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GUIDELINE INTERPRETATION

# Influence of SCENIC recommendations on terminology used for histopathologic diagnosis of inflammatory bowel disease-associated dysplasia

Yuan Li, Hanlin L Wang

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## Abstract

### *BACKGROUND*

Published in 2015, the International Consensus Recommendations on Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients (SCENIC) recommended abandoning the use of diagnostic term "dysplasia-associated lesion or mass (DALM)" for polypoid dysplastic lesions detected in patients with inflammatory bowel disease (IBD). The aim of this study was to investigate whether this recommendation had any influence on diagnostic terminologies used by pathologists in their practice.

### *METHODS*

We retrospectively reviewed all pathology reports for surveillance colonoscopic biopsies from ulcerative colitis (UC) patients in our institution during 1/2012-12/2014 (pre-SCENIC) and 1/2016-12/2018 (post-SCENIC). These included 1203 biopsies from 901 UC patients during the pre-SCENIC period and 1273 biopsies from 977 UC patients during the post-SCENIC period. Their corresponding endoscopic findings and histopathologic diagnoses were recorded. Clinical indications for total colectomy for UC patients and corresponding histopathologic findings in colectomy specimens were also recorded and compared.

### *RESULTS*

A total of 347 and 419 polyps/polypoid lesions were identified during the pre-SCENIC and post-SCENIC periods, among which 60 and 104 were dysplastic/adenomatous, respectively. More polypoid dysplastic lesions were simply diagnosed as "adenoma" during the post-SCENIC period in comparison with the

pre-SCENIC period (97.1% *vs* 65.0%; $P < 0.001$). The number of cases with a comment in pathology reports regarding the distinction between DALM and sporadic adenoma was also significantly decreased during the post-SCENIC period (5.8% *vs* 38.3%; $P < 0.001$). In addition, the term "dysplasia" was more consistently used for random biopsies during the post-SCENIC period. Furthermore, the terms "sessile serrated adenoma/polyp" (SSA/P) and "serrated epithelial change" (SEC) were more consistently used for polypoid lesions and random biopsies, respectively, during the post-SCENIC period, although these were not specifically addressed in the SCENIC recommendations. The indications for colectomy remained unchanged, however, despite the standardization of diagnostic terminologies.

***CONCLUSION***

The SCENIC recommendations relieve pathologists from the burden of distinguishing DALM from sporadic adenoma in IBD patients, which helps the standardization of diagnostic terminologies used by pathologists. The consistent use of the diagnostic terminologies may help reduce potential confusions to clinicians and patients.

**Key Words:** Inflammatory bowel disease; Ulcerative colitis; Dysplasia; Terminology; SCENIC



**Core Tip:** The Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients recommendations help relieve pathologists from the burden of histologically distinguishing dysplasia-associated lesion or mass from sporadic adenoma in inflammatory bowel disease patients, which is an extremely challenging and stressful differential. This has a significant influence on diagnostic terminologies used by pathologists in their practice.

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## INTRODUCTION

Patients with inflammatory bowel disease (IBD), either ulcerative colitis (UC) or Crohn disease (CD), have an increased risk of developing colorectal cancer (CRC). IBD-associated CRC constitutes 10%-15% of deaths in IBD patients[1]. The risk of developing CRC in UC is similar to that in CD[2,3]. Dysplasia, which is stratified by histopathologic features into low-grade dysplasia (LGD) and high-grade dysplasia (HGD), is currently considered the best marker of CRC risk in IBD. Surveillance colonoscopy therefore is recommended to detect dysplasia for early CRC prevention. Dysplastic foci may be visible under colonoscopy as raised lesions or invisible found on random (non-targeted) biopsies of the colonic mucosa. Invisible dysplasia, especially HGD, is usually an indication of total colectomy.

Raised or polypoid dysplasia in the setting of IBD has been termed "dysplasia-associated lesion or mass (DALM)" in the past, which was believed to be associated with a high risk for CRC development [4,5]. Therefore, a diagnosis of DALM usually meant total colectomy for cancer prevention[5]. However, it is extremely difficult or even impossible for endoscopists and pathologists to distinguish a DALM lesion from a sporadic adenoma, another polypoid precancerous lesion of CRC that is not associated with IBD. Similar to that in the general population, the occurrence of sporadic adenoma in IBD patients also increases with age, but its progression to CRC appears to take much longer. Complete removal of a sporadic adenoma by endoscopic polypectomy is considered an adequate treatment for cancer prevention[6]. In addition, with the advancement in endoscopic technology and increasing use of high-resolution endoscopy, chromoendoscopy, image-enhanced endoscopy and magnifying endoscopy, many invisible dysplastic foci under routine endoscopy in IBD patients now become visible[7], and many of them can be completely removed under endoscopy without the necessity of colectomy. Furthermore, recent studies have shown that cancer risk is not increased if DALM lesions can be completely removed by endoscopy, and thus total colectomy is also unnecessary[8,9]. These changes in practice have greatly reduced the necessity of total colectomy for CRC prevention in IBD patients.

With these new developments, the International Consensus Recommendations on Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients (SCENIC) were published in 2015[10], which addressed two important issues: How surveillance colonoscopy should be performed for dysplasia detection, and how dysplasia should be managed.

According to SCENIC recommendations, endoscopically visible dysplasia can be categorized into polypoid (≥ 2.5 mm, pedunculated or sessile) and non-polypoid (superficially elevated and < 2.5 mm, flat or depressed) lesions. It is also recommended that the terms "DALM", "adenoma-like" and "non-adenoma-like" be abandoned. The aim of this study was to investigate whether the SCENIC recommendations had any influence on the terminologies used by pathologists to diagnose dysplasia detected during surveillance colonoscopies in IBD patients.

## MATERIALS AND METHODS

### Study groups

We retrospectively reviewed all pathology reports from patients who had clinically established diagnosis of UC and underwent surveillance colonoscopy with biopsy at Ronald Reagan UCLA Medical Center during two periods of time: January 2012 through December 2014 (pre-SCENIC) and January 2016 through December 2018 (post-SCENIC). Patients with CD, indeterminate colitis and first-time diagnosis of UC were excluded. Endoscopic biopsies from UC patients who had already undergone total colectomy were also excluded from the study. A total of 1203 colonoscopic biopsies from 901 UC patients during the pre-SCENIC period (2012-2014) and a total of 1273 biopsies from 977 UC patients during the post-SCENIC period (2016-2018) were reviewed. Their corresponding endoscopic findings (*e.g.*, polyp or other elevated lesions) and histopathologic diagnoses (*e.g.*, adenoma, hyperplastic polyp or others) were recorded. Clinical indications for total colectomy for UC patients and corresponding histopathologic findings in colectomy specimens were also recorded and compared between the pre- and post-SCENIC periods. The study was approved by the Institutional Review Board at UCLA.

### Statistical analysis

Clinicopathologic and outcome findings were compared between the pre- and post-SCENIC groups using the $\chi^2$ or Fisher exact test (for categorical features). All statistical analyses were performed using the SPSS software. $P < 0.05$ was considered statistically significant.

## RESULTS

### Polyps and polypoid lesions removed by targeted biopsies/polypectomies

A total of 347 polyps/polypoid lesions were detected and removed among 1203 endoscopic biopsies (28.8%) during 2012-2014 (Table 1). Among these polyps/polypoid lesions, 60 (17.3%) were found to be dysplastic/adenomatous. In pathology reports, 39 of 60 (65.0%) cases were directly diagnosed as "adenoma" ($n$ = 36) or "adenomatous change" ($n$ = 3). These included 17 cases with multiple adenomatous lesions. Nineteen (31.7%) cases were diagnosed as "dysplasia" (LGD, $n$ = 14; polypoid LGD, $n$ = 1; LGD with tubulovillous features, $n$ = 2; HGD, $n$ = 2), of which 9 had multiple dysplastic lesions. One (1.7%) polypoid dysplastic lesion was diagnosed as "DALM", and another polyp was diagnosed as "combined serrated and low-grade adenomatous features". A comment on the distinction between sporadic adenoma and DALM was included in pathology reports for 23 (38.3%) of these cases (Table 2). These included 9 of 39 (23.1%) cases diagnosed as "adenoma" or "adenomatous change" and 13 of 19 (68.4%) cases diagnosed as "dysplasia". Of the cases diagnosed as "adenoma", sporadic adenoma was favored for 5 (55.6%) cases in the comment, DALM for 2 (22.2%), and indistinguishable for 2 (22.2%). Of the cases diagnosed as "dysplasia", sporadic adenoma was favored for 4 (30.8%) cases, DALM for 2 (15.4%), and indistinguishable for 7 (53.8%). The single case diagnosed as "DALM" also had a comment to further favor the diagnosis.

As shown in Table 1, a total of 419 polyps/polypoid lesions were identified and removed among 1273 endoscopic biopsies (32.9%) during 2016-2018. Of the 104 (24.8%) polyps/polypoid lesions that were found to be dysplastic/adenomatous, 101 (97.1%) were directly diagnosed as "adenoma". These included 21 cases with multiple adenomatous lesions. Only 3 (2.9%) polyps was diagnosed as "dysplasia", which were all diagnosed in 2016. As shown in Table 2, only 6 (5.8%) of these cases had a comment in pathology reports on the distinction between sporadic adenoma and DALM, including 4 cases diagnosed as "adenoma" (all favored sporadic adenoma) and 2 diagnosed as "dysplasia" (both stated as indistinguishable). Except for one case diagnosed in early 2017, all cases with comments were diagnosed in 2016.

Compared to the pre-SCENIC period, more cases were simply diagnosed as adenoma (97.1% *vs* 65.0%; $P < 0.001$) and much fewer cases had a comment in pathology reports (5.8% *vs* 38.3%; $P < 0.001$) during the post-SCENIC period. In fact, all polyps or polypoid lesions that showed dysplastic/adenomatous features were simply diagnosed as adenoma since 2017, and none of these cases had a comment in pathology report regarding the distinction between sporadic adenoma and DALM after early 2017.

**Table 1 Histopathologic diagnoses of polyps and polypoid lesions detected during surveillance colonoscopies in ulcerative colitis patients during pre-SCENIC (2012-2014) and post-SCENIC (2016-2018) periods**

| Histopathologic diagnosis | Pre-SCENIC (*n* = 347) | Post-SCENIC (*n* = 419) |
|---|---|---|
| Adenocarcinoma | 3 | 3 |
| TA | 32 | 80 |
| TVA | 2 | 17 |
| VA | 0 | 2 |
| Adenoma with HGD | 2 | 2 |
| LGD | 14 | 1 |
| LGD with focal HGD | 0 | 1 |
| HGD | 2 | 1 |
| Polypoid LGD | 1 | 0 |
| LGD with tubulovillous features | 2 | 0 |
| Adenomatous change/LGD | 2 | 0 |
| Adenomatous change with focal HGD | 1 | 0 |
| DALM | 1 | 0 |
| Combined serrated and low-grade adenomatous features | 1 | 0 |
| IND | 14 | 6 |
| HP | 55 | 63 |
| SSA/P | 9 | 22 |
| TSA | 0 | 1 |
| Hyperplastic change | 51 | 81 |
| Serrated epithelial change | 1 | 0 |
| Inflammatory polyp/pseudopolyp | 143 | 96 |
| Benign lymphoid aggregate | 5 | 17 |
| Well-differentiated NET | 0 | 1 |
| Pneumatosis intestinalis | 0 | 1 |
| Mucosal prolapse | 0 | 2 |
| Collagenous colitis | 0 | 1 |
| Submucosal giant cells | 1 | 0 |
| Atypical epithelial proliferation | 0 | 1 |
| Polypoid normal mucosa | 4 | 20 |
| Branching crypts | 1 | 0 |

SCENIC: Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients; UC: Ulcerative colitis; TA: Tubular adenoma; TVA: Tubulovillous adenoma; VA: Villous adenoma; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; DALM: Dysplasia-associated lesion or mass; IND: Indefinite for dysplasia; HP: Hyperplastic polyp; SSA/P: Sessile serrated adenoma/polyp; TSA: Traditional serrated adenoma; NET: Neuroendocrine tumor.

### *Invisible dysplasia on random biopsies*

During 2012-2014, 17 of 1203 (1.4%) cases were diagnosed to have dysplasia on random biopsies (Table 3). Among them, 8 (47.1%) showed multiple foci of or extensive dysplasia. Five (29.4%) cases had a comment in pathology reports. For 2 cases, IBD-associated dysplasia was favored considering the background of chronic colitis and the random nature of the biopsies. One case was diagnosed as "low-grade adenomatous change" and sporadic adenoma was favored in the comment despite the random nature of the biopsy. For the remaining 2 cases, the comment stated that a definitive distinction between IBD-associated dysplasia and sporadic adenoma could not be made based on histologic assessment alone and recommended clinical and endoscopic correlation.

**Table 2 Comparison of comments on histopathologic diagnoses of polypoid adenomatous/dysplastic lesions detected in ulcerative colitis patients during surveillance colonoscopies between pre-SCENIC (2012-2014) and post-SCENIC (2016-2018) periods**

| Histopathologic diagnosis | No. of cases (%) | Comment | | | |
|---|---|---|---|---|---|
| | | No. of cases | Favor sporadic adenoma | Favor DALM | Cannot distinguish |
| Pre-SCENIC | | | | | |
| TA | 32 (53.3) | 6 | 5 | | 1 |
| TVA | 2 (3.3) | 0 | | | |
| TA with focal HGD | 2 (3.3) | 0 | | | |
| Adenomatous change/LGD | 2 (3.3) | 2 | | 1 | 1 |
| Adenomatous change with focal HGD | 1 (1.7) | 1 | | 1 | |
| LGD | 14 (23.3) | 10 | 4 | 1 | 5 |
| Polypoid LGD | 1 (1.7) | 1 | | | 1 |
| LGD with tubulovillous features | 2 (3.3) | 2 | | 1 | 1 |
| HGD | 2 (3.3) | 0 | | | |
| DALM | 1 (1.7) | 1 | | 1 | |
| Combined serrated and low-grade adenomatous features | 1 (1.7) | 0 | | | |
| Total | 60 (100) | 23 | 9 | 5 | 9 |
| Post-SCENIC | | | | | |
| TA | 80 (76.9) | 4 | 4 | | |
| TVA | 17 (16.3) | 0 | | | |
| TVA with focal HGD | 2 (1.9) | 0 | | | |
| VA | 2 (1.9) | 0 | | | |
| LGD | 1 (1.0) | 1 | | | 1 |
| LGD with focal HGD | 1 (1.0) | 1 | | | 1 |
| HGD | 1 (1.0) | 0 | | | |
| Total | 104 (100) | 6 | 4 | 0 | 2 |

SCENIC: Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients; UC: Ulcerative colitis; DALM: Dysplasia-associated lesion or mass; TA: Tubular adenoma; TVA: Tubulovillous adenoma; VA: Villous adenoma; LGD: Low-grade dysplasia; HGD: High-grade dysplasia.

During 2016-2018, 16 of 1273 (1.3%) cases were diagnosed to have dysplasia on random biopsies, including 9 (56.3%) that showed multiple foci of or extensive dysplasia. Three (18.8%) cases had a comment in pathology report on the nature of dysplasia, including 2 diagnosed in 2016 and one in 2017. IBD-associated dysplasia was considered in the comment for 2 cases. For the other case, diagnosed in 2016, the comment stated that the distinction between IBD-associated dysplasia and sporadic adenoma could not be made reliably on histologic grounds.

There was no significant difference in the frequency of dysplasia diagnosed on random biopsies between the pre- and post-SCENIC periods (1.4% *vs* 1.3%; $P > 0.05$). However, the terminologies used for the diagnosis appeared to be more consistent during the post-SCENIC period in comparison to the pre-SCENIC period.

### *SSA/P on targeted biopsies/polypectomies*

During 2012-2014, 9 of 1203 (0.7%) biopsies from UC patients had a diagnosis of SSA/P with variable terms used by pathologists (Table 4). These included "SSA" ($n = 5$), "SSP" ($n = 1$), "SSA/P" ($n = 2$), and "SSA/P with low-grade cytologic dysplasia" ($n = 1$). Three (33.3%) cases, biopsied from "thickened fold", had a comment in pathology reports on the significance of serrated polyps in the setting of IBD. Seven (77.8%) cases had follow-up colonoscopic biopsies ($n = 5$) or surgical resections ($n = 2$). Of the 2 resection cases, one case that showed SSA/P with cytologic dysplasia and multiple synchronous tubular adenomas (TA) on surveillance biopsies still showed adenomas in resection specimen. No HGD or

**Table 3 Histopathologic diagnoses of dysplastic lesions on random endoscopic biopsies from ulcerative colitis patients during pre-SCENIC (2012-2014) and post-SCENIC (2016-2018) periods**

| Histopathologic diagnosis | No. of cases | Multiple or extensive | Comment | | | |
|---|---|---|---|---|---|---|
| | | | No. of cases | Favor sporadic adenoma | Favor IBD dysplasia | Cannot distinguish |
| Pre-SCENIC | | | | | | |
| LGD | 13 | 5 | 3 | | 2 | 1 |
| LGD, villous type | 1 | 1 | 1 | | | 1 |
| Low-grade adenomatous change | 1 | 0 | 1 | 1 | | |
| Low-grade villous dysplasia | 1 | 1 | 0 | | | |
| HGD | 1 | 1 | 0 | | | |
| Total | 17 | 8 | 5 | | | |
| Post-SCENIC | | | | | | |
| LGD | 12 | 7 | 3 | | 2[1] | 1[2] |
| LGD/TA | 2 | 1 | 0 | | | |
| HGD | 2 | 1 | 0 | | | |
| Total | 16 | 9 | 3 | | | |

[1]Diagnosed in 2016 and 2017.
[2]Diagnosed in 2016.
SCENIC: Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; TA: Tubular adenoma.

invasive carcinoma was identified. The other case had resection for prior diagnosed dysplasia and sigmoid stricture. No dysplasia, adenoma or invasive carcinoma was identified in resection specimen for this case. The remaining 5 cases had no dysplasia or adenoma identified in the follow-up biopsies.

During 2016-2018, 22 of 1273 cases (1.7%) from 19 patients were diagnosed as "SSA/P", including one case diagnosed as "SSA" in 2018 and one case diagnosed as "SSA/P with low-grade cytologic dysplasia". None of the cases had a comment in pathology report on the significance of the lesion. Six (27.3%) patients had follow-up data from subsequent colonoscopic biopsies. Adenoma was found in one and SSA/P in 2 patients. No dysplastic lesions were detected in the remaining 3 patients. None of the patients underwent surgical resection.

### *SEC on random biopsies*

During 2012-2014, a total of 49 (4.1%) cases showed serrated colonic mucosa on random biopsies (Table 5), which was termed "hyperplastic change" ($n$ = 47) or "SEC" ($n$ = 2). The most common locations were the left colon and rectum (71.4%). Synchronous adenoma/dysplasia was found in 9 (18.4%) cases. Five of them had synchronous TA including 2 with multiple TAs. The other 4 cases had synchronous LGD on random biopsies including 2 with multiple foci of LGD. Thirty-five (71.4%) cases had follow-up biopsies. Metachronous adenoma/LGD was found in 7 (20.0%) of these cases including one case with multiple tubulovillous adenomas (TVA) and 3 cases with LGD on random biopsies.

During 2016-2018, a total of 66 (5.2%) cases showed serrated colonic mucosa on random biopsies, which was termed "hyperplastic change" ($n$ = 61), "SEC" ($n$ = 3), and "SSA/P" ($n$ = 2). Similar to that seen during pre-SCENIC period, the left colon and rectum were the most common locations (72.7%). Synchronous adenoma/dysplasia was found in 9 (13.6%) cases. Five of them had synchronous TA, 2 had LGD on random biopsies, and 2 had SSA/P. Thirty-five (53.0%) cases had follow-up biopsies. Metachronous adenoma/dysplasia was found in 10 (28.6%) cases including 2 cases showing LGD on random biopsies.

### *Indications for total colectomies*

Table 6 shows that during 2012-2014, a total of 54 UC patients underwent total colectomies, 40 (74.1%) of which were done for medically refractory colitis ($n$ = 34) or nonneoplastic complications ($n$ = 6). These patients had no prior history of dysplasia or neoplasia. Histopathologic examination of colectomy specimens showed no dysplasia or neoplasia in 38 (95%) cases. One of the two remaining cases was incidentally found to have multiple foci of well-differentiated (low-grade) neuroendocrine tumor in the

**Table 4 Comparison of Sessile serrated adenoma/polyp diagnosed in ulcerative colitis patients between pre-SCENIC (2012-2014) and post-SCENIC (2016-2018) periods**

| | | Pre-SCENIC | | Post-SCENIC | |
|---|---|---|---|---|---|
| | | No. | % | No. | % |
| No. of biopsies/patients | | 9/9 | | 22/17 | |
| Frequency | | 9/1203 | 0.7 | 22/1273 | 1.7 |
| Diagnosis | SSA/P | 3[1] | 33.3 | 21[1] | 95.5 |
| | SSA | 5 | 55.6 | 1 | 4.5 |
| | SSP | 1 | 11.1 | 0 | |
| Single | | 9 | 100 | 17 | 77.3 |
| Multiple | | 0 | | 5 | 22.7 |
| | Right | 4 | 44.4 | 9 | 41 |
| | Transverse | 1 | 11.1 | 5 | 22.7 |
| Location | Left | 3 | 33.3 | 4 | 18.2 |
| | Rectum | 1 | 11.1 | 2 | 9.1 |
| | Multiple sites | 0 | | 2 | 9.1 |
| Synchronous adenoma | | 4[2] | 44.4 | 4[3] | 21.1 |
| No. of patients with follow-up | | | | | |
| | Biopsy | 5 | | 6 | |
| | Resection | 2[4] | | 0 | |
| Metachronous adenoma | | 4[5] | 44.4 | 3[6] | 15.8 |

[1]One case with low-grade cytologic dysplasia.
[2]Tubular adenoma (TA) = 2, TA with focal high-grade dysplasia = 1, indefinite for dysplasia = 1.
[3]TA = 4.
[4]One case had history of pseudopolyps, dysplasia, and sigmoid stricture. The other case had synchronous multiple TAs/low-grade dysplasia (LGD).
[5]TA/LGD = 3, dysplasia (grade not provided) = 1.
[6]TA = 2, TA and sessile serrated adenoma/polyp = 1.
SCENIC: Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients; SSA: Sessile serrated adenoma; SSP: Sessile serrated polyp; SSA/P: Sessile serrated adenoma/polyp.

rectum that ranged in size from 0.3 cm to 1.0 cm and invaded the lamina propria, muscularis mucosae and focally the superficial submucosa. No lymph node metastasis was identified. The other case was found to have a small TA. Fourteen (25.9%) patients underwent surgeries for adenocarcinoma or dysplasia detected on surveillance colonoscopies. Six (42.9%) cases were found to have invasive adenocarcinoma in resection specimens.

During 2016-2018, 40 patients underwent total colectomies, 28 (70.0%) of which were done for refractory colitis ($n$ = 26) or nonneoplastic complications ($n$ = 2). These patients did not have a prior history of dysplasia or neoplasia. On resection specimens, focal LGD was incidentally found in one case. There was another case where surveillance biopsy showed a focus indefinite for dysplasia but the resection specimen showed extensive HGD. Twelve (30.0%) patients had colectomies for carcinoma, dysplasia or large adenomas detected on surveillance colonoscopies. Invasive carcinoma was found in resection specimens in 7 (58.3%) cases, among which 2 were poorly differentiated neuroendocrine carcinomas. Two cases with preoperative diagnosis of adenocarcinoma showed no residual carcinoma or dysplasia in resection specimens. One case had a 1.5 cm polyp that was completely removed by endoscopic polypectomy prior to surgery. The other case was treated with neoadjuvant chemotherapy prior to surgery with complete response.

## DISCUSSION

Dysplasia in IBD can be either flat (endoscopically invisible) or elevated (endoscopically visible). Elevated lesions were used to be called "DALMs", which were believed to have a high association with cancer and thus regarded as a strong indication for colectomy[11]. DALMs are a group of heterogeneous

**Table 5 Comparison of serrated epithelial change diagnosed on random endoscopic biopsies from ulcerative colitis patients between pre-SCENIC (2012-2014) and post-SCENIC (2016-2018) periods**

| | | Pre-SCENIC | | Post-SCENIC | | |
|---|---|---|---|---|---|---|
| | | Hyperplastic change (%) | SEC | Hyperplastic change (%) | SEC | SSA/P |
| Total | | 47 | 2 | 61 | 3 | 2 |
| Location | Right | 2 (4.3) | 0 | 5 (8.2) | 0 | 1 |
| | Transverse | 2 (4.3) | 0 | 3 (4.9) | 0 | 1 |
| | Left | 17 (36.2) | 2 | 14 (23.0) | 1 | 0 |
| | Rectum | 16 (34.0) | 0 | 31 (50.8) | 0 | 0 |
| | Multiple sites | 10 (21.3) | 0 | 8 (13.1) | 2 | 0 |
| Synchronous adenoma/dysplasia | | | | | | |
| | TA | 4[1] (8.5) | 1[1] | 5 (8.2) | 0 | 0 |
| | LGD | 4[2] (8.5) | 0 | 2 (3.3) | 0 | 0 |
| | SSA/P | 0 | 0 | 1 (1.6) | 1 | 0 |
| No. of cases with follow-up biopsies | | 33 | 2 | 31 | 3 | 1 |
| Metachronous dysplasia | | | | | | |
| | TVA | 1[1] (3.0) | 0 | 1 (3.2) | 0 | 0 |
| | TA | 1 (3.0) | 1 | 6[1] (19.4) | 0 | 0 |
| | LGD | 3 (9.1) | 0 | 2 (6.5) | 0 | 0 |
| | SSA/P | 1 (3.0) | 0 | 1 (3.2) | 0 | 0 |
| | IND | 0 | 0 | 1 (3.2) | 0 | 0 |

[1]One case had multiple adenomas.
[2]Two cases had multiple foci of LGD.
SCENIC: Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients; SEC: Serrated epithelial change; SSA/P: Sessile serrated adenoma/polyp; TA: Tubular adenoma; TVA: Tubulovillous adenoma; LGD: Low-grade dysplasia; IND: Indefinite for dysplasia.

lesions which can be further divided into adenoma-like and non-adenoma-like based on their endoscopic appearance. Non-adenoma-like DALMs refer to velvety patches, plaques, irregular bumps and nodules, wart-like thickenings, stricturing lesions, and broad-based masses. These lesions are believed to carry a high risk of concurrent malignancy, often representing the surface of an invasive adenocarcinoma and therefore often requiring colectomy[8]. On the other hand, adenoma-like DALMs are well-circumscribed lesions similar to sporadic adenomas endoscopically and pathologically. It has been suggested that adenoma-like DALMs that occur outside or proximal to the areas of mucosa involved by inflammation are considered sporadic in origin and can be managed conservatively by polypectomy. On the contrary, adenoma-like DALMs detected within the area of inflammation may be IBD-associated and thus colectomy and close surveillance may need to be considered. Other features favoring IBD-associated adenoma-like DALMs include young age at diagnosis, long duration of disease, prominent villous architecture, a mixture of normal and dysplastic epithelia at the surface of polyp, "bottom-up" dysplasia, increased inflammation in polyp, presence of stalk dysplasia, and a high frequency of *p53* and a low frequency of *KRAS* mutations[12-15]. However, none of these features has proven to be specific despite the great efforts made by pathologists in the distinction between IBD-associated adenoma-like DALMs and sporadic adenomas.

In 2015, the SCENIC recommendations were published, which incorporated the latest understanding on surveillance and management of dysplasia in IBD[10]. According to this consensus, dysplastic lesions can be simply classified as endoscopically visible and invisible. Visible dysplasia, by definition, is histopathologically proven dysplasia on a targeted biopsy of a concerning area recognized on colonoscopic examination. Invisible dysplasia is histopathologically proven dysplasia on a random biopsy from a visually unremarkable colonic mucosa[10]. For endoscopically visible lesions, the determination of endoscopic resectability, rather than the distinction between adenoma-like and non-adenoma-like or between IBD-associated dysplasia and sporadic adenoma, becomes important according to the consensus recommendations. Therefore, the term "DALM" becomes no longer useful and should be abandoned. For endoscopically visible and resectable lesions, either polypoid or non-polypoid,

**Table 6 Indications for total colectomies and postoperative findings for ulcerative colitis patients during pre-SCENIC (2012-2014) and post-SCENIC (2016-2018) periods**

| | No. of cases (%) | Preoperative biopsy/polypectomy (No. of cases) | Postoperative findings (No. of cases) |
|---|---|---|---|
| Pre-SCENIC | | | |
| Total | 54 | | |
| Refractory UC | 34 (63.0) | No dysplasia/adenoma or malignancy (34) | Incidental well-differentiated (low-grade) NET (1); TA (1); no dysplasia/adenoma or malignancy (32) |
| Complications | 6 (11.1) | Perforation (2); fistula (1); stricture (1); volvulus (1); obstruction (1)[1] | No dysplasia/adenoma or malignancy (6) |
| Dysplasia/malignancy | 14 (25.9) | Invasive adenocarcinoma (4.5 cm mass lesion), a separate focus of LGD | pT4a pN2b adenocarcinoma, a separate focus of HGD |
| | | Invasive adenocarcinoma (6.3 cm mass), a separate focus of LGD | pT3 pN0 adenocarcinoma |
| | | At least HGD (2.6 cm polypoid lesion) | pT1 pN1a adenocarcinoma (2 foci) |
| | | At least HGD (2.0 cm polypoid lesion) | pT1 pN1a adenocarcinoma (2 foci), separate foci of HGD |
| | | Dysplasia (3.6 cm mass) | pT2 pN1b mucinous adenocarcinoma |
| | | HGD (3.0 cm polypoid lesion), also separate foci of LGD | HGD |
| | | Multiple TAs and foci of LGD, one TA with HGD, one SSA/P with low-grade cytologic dysplasia (0.2-1.5 cm sessile polyps) | Multiple TAs and foci of LGD |
| | | LGD (1.0 cm lesion) | pT1 pN0 mucinous adenocarcinoma arising from extensive LGD |
| | | LGD with tubulovillous features (6.5 cm mucosal plaque) | LGD |
| | | LGD (4.6 cm polypoid lesion) | LGD |
| | | Dysplasia (outside diagnosis) | LGD |
| | | LGD (focal on a random biopsy) | No residual dysplasia or carcinoma |
| | | Extensive LGD | Extensive LGD |
| | | Multiple foci of LGD | Focal LGD |
| Post-SCENIC | | | |
| Total | 40 | | |
| Refractory UC | 26 (65.0) | No dysplasia/adenoma or malignancy (26) | Focal LGD (1); no dysplasia/adenoma or malignancy (25) |
| Complications | 2 (5.0) | Perforation (1); GI bleeding (1)[2] | Extensive HGD (1)[2]; no dysplasia/adenoma or malignancy (1) |
| Dysplasia/malignancy | 12 (30.0) | Invasive adenocarcinoma (4.1 cm mass) | pT4a pN1a poorly differentiated NEC |
| | | Invasive adenocarcinoma (1.5 cm mass) | pT3 pN1a adenocarcinoma, separate foci of LGD |
| | | Invasive adenocarcinoma (5.5 polypoid mass), a separate focus of LGD | pT3 pN1a poorly differentiated NEC |
| | | Invasive adenocarcinoma with mucinous features arising in a polypoid lesion with serrated/villiform dysplasia (1.5 cm polyp) | No residual carcinoma or dysplasia |
| | | Invasive adenocarcinoma (2.8 cm mass) | No residual carcinoma or dysplasia (s/p neoadjuvant chemotherapy) |
| | | Atypical cells concerning for adenocarcinoma (13.0 cm mass) | pT4b pN0 mucinous adenocarcinoma |
| | | At least HGD (2.5 cm mass) | pT1 pN0 adenocarcinoma with signet-ring cell features (3 foci) |

| | | |
|---|---|---|
| | Extensive HGD (3.5 cm flat induration) | pT2 pN0 adenocarcinoma (3 foci) |
| | TVA with focal HGD (6.1 cm mass) | pT2 pN0 adenocarcinoma |
| | Villous adenoma (2.0 cm sessile polyp) | No residual adenoma or carcinoma |
| | Multifocal LGD, one TA | Focal LGD and HGD |
| | LGD with tubulovillous architecture (12 cm polypoid lesion), a separate focus of LGD, multiple TVAs | Villous adenoma |

[1]Surveillance colonoscopy found a small polypoid area in the sigmoid colon that caused partial obstruction. Biopsy showed features of sessile serrated adenoma/polyp without cytologic dysplasia. No dysplasia or carcinoma was found on resection specimen.
[2]Surveillance biopsy showed indefinite for dysplasia, resection specimen identified extensive high-grade dysplasia.
SCENIC: Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients; UC: Ulcerative colitis; SSA/P: Sessile serrated adenoma/polyp; NET: Neuroendocrine tumor; TA: Tubular adenoma; TVA: Tubulovillous adenoma; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; IND: Indefinite for dysplasia; NEC: Neuroendocrine carcinoma.

complete endoscopic polypectomy or excision followed by continued surveillance is a sufficient treatment, though the borders of non-polypoid lesions may be difficult to delineate and complete excision can be technically challenging. For patients with endoscopically invisible dysplasia, referral to an experienced IBD specialist with further examination using chromoendoscopy with high-definition colonoscopy is suggested[10,16,17]. If dysplasia is still invisible, management will depend on the grade of dysplasia. While the SCENIC consensus provides recommendations for surveillance and management of dysplasia in IBD patients, no specific suggestions are made on diagnostic terminologies that pathologists should use when reporting dysplastic lesions in IBD patients.

We were curious about whether the SCENIC recommendations had any influence on the terminologies used by pathologists in their reports for the diagnosis of dysplastic/adenomatous lesions detected in IBD patients. According to our single institutional experience, the diagnostic terms used by pathologists were more uniform and consistent in the post-SCENIC period. Specifically, more polypoid dysplastic lesions were directly diagnosed as adenomas (with or without HGD) in the post-SCENIC period (97.1%) in comparison to the pre-SCENIC period (65.0%). In the pre-SCENIC period, approximately one-third of polypoid dysplastic lesions were diagnosed as "LGD" or "HGD", which could potentially be confused with invisible dysplasia diagnosed on random biopsies. These diagnostic terms have never been used again by pathologists in our institution for targeted biopsies or polypectomies on visible lesions after 2016. It is interesting to note that our pathologists made much less efforts to attempt to distinguish adenoma-like DALM from sporadic adenoma in their practice in the post-SCENIC period. This is evidenced by a dramatic reduction in the number of pathology reports that included a diagnostic comment on the distinction between sporadic adenoma and DALM. In fact, the few cases that had a comment in the post-SCENIC period were all diagnosed in 2016, with only one in early 2017. None of the cases diagnosed after early 2017 carried a diagnostic comment. These changes in practice indicate that pathologists were much less struggling once the stress of distinguishing IBD-associated dysplasia from sporadic adenoma was relieved. It is also interesting to note that in the pre-SCENIC period, only one of 60 (1.7%) polypoid dysplastic lesions was directly diagnosed as "DALM" and only 5 of 23 (21.7%) cases with a comment were favored to be "DALM", further indicating how cautious the pathologists were in making such a diagnosis given its potential clinical consequence.

For endoscopically invisible dysplasia, histopathologic interpretation of random surveillance biopsies plays an essential role in clinical management. Current recommendation for invisible HGD is colectomy given the high risk of synchronous and metachronous carcinoma[18]. For endoscopically invisible LGD, the management is controversial. The American Society for Gastrointestinal Endoscopy recommended colectomy for multifocal LGD but an individualized approach for unifocal LGD[19]. We had a total of 94 colectomies for UC patients during the pre- and post-SCENIC periods. The majority (72.3%) of the surgeries were performed for medically refractory disease and nonneoplastic complications. The rest of patients (27.7%) had resections for carcinoma, HGD, multifocal LGD, and unifocal LGD diagnosed on surveillance biopsies. There were 4 patients who underwent colectomies for unifocal LGD, all of which occurred during the pre-SCENIC period. Three cases had a polyp/mass detected during surveillance endoscopy, which ranged in size from 1.0 cm to 6.5 cm. Only one case had resection based on histopathologic diagnosis of unifocal LGD on a random surveillance biopsy.

Colorectal serrated polyps, which include hyperplastic polyp, SSA/P and traditional serrated adenoma, have been implicated in the pathogenesis in a subset of CRC. SSA/P in general population has been widely studied[20] and the serrated neoplasia pathway has been thought to be responsible for at least 20% of sporadic CRC[21,22]. These polyps are distinct from conventional adenomas as they frequently harbor *BRAF* mutations and show CpG island methylation. There is evidence, though limited, to support the notion that the clinicopathologic and molecular characteristics of SSA/P found in IBD patients are similar to those in general population[23-25]. Similar to conventional adenomas, these serrated lesions are endoscopically detectable as polyps and thus can be easily removed by

polypectomy. No correlation of occurrence of these lesions with the background inflammation has been reported[25,26]. The changes in our pathology reports also reflected this recognition. During 2012-2014, before SCENIC consensus, variable diagnostic terms had been used including SSA, SSP and SSA/P. A diagnosis comment was included in 1/3 of pathology reports on the nature of the lesion. Since 2016, however, the term SSA/P was consistently used, with no further comment.

SEC, previously called hyperplastic change, is the currently preferred term to describe mucosal changes similar to SSA/P or hyperplastic polyp on biopsies from non-polypoid colonic mucosa from IBD patients. Histologically, it is recognized by distorted architecture but lacks typical features of cytologic dysplasia. It is typically found on random biopsies during surveillance colonoscopy and characterized by serrated crypt architecture, usually involving the upper half of the crypt, and without cytologic features of dysplasia[27]. When endoscopically visible, SEC is typically flat or shows nodular mucosa without a discrete polypoid configuration[28]. Whether SEC carries a risk of progression to dysplasia and CRC is currently unknown, but several studies have suggested that the finding of SEC in IBD patients may be associated with higher rates of colonic synchronous and metachronous neoplasia [27,29,30]. Our limited data also showed a high association of SEC with synchronous and metachronous neoplasia in UC patients. Specifically, of the 81 patients who had a SEC diagnosis, 38 (46.9%) had synchronous or metachronous adenomas. There was no significant difference between the pre-SCENIC and post-SCENIC periods. Further controlled studies are needed to determine whether SEC is indeed a preneoplastic marker in IBD patients.

There are a couple of limitations in this retrospective study. First, this is a single institutional study. Our experience might not be the same as that in other institutions. Second, all data were collected from previous pathology reports signed by different pathologists. It is understandable that different pathologists might have used different diagnostic criteria for various entities and might have different thresholds for the diagnosis of dysplasia even though they were practicing in the same institution. Nonetheless, these limitations did not appear to affect the conclusions of the study.

## CONCLUSION

Although the SCENIC recommendations were aimed to address management issues, they had a significant impact on the terminologies pathologists used in their practice based on our institutional experience. Specifically, the recommendations relieved pathologists from the burden of distinguishing "DALM" from sporadic adenoma in IBD patients, which is an extremely challenging and stressful differential. Currently, all polypoid or visible dysplastic lesions are simply diagnosed as "adenoma" in our institution, irrespective of whether or not they are IBD-associated because of the same management approaches. The term "dysplasia" is reserved only for invisible lesions found in random biopsies. The consistent use of the diagnostic terminologies may help reduce potential confusions to clinicians and patients.

## FOOTNOTES

**Author contributions:** Wang HL designed the research, supervised data analysis and manuscript preparation; Li Y collected and analyzed the data, and wrote the manuscript.

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REVIEW

# *KAI1/CD82* gene and autotaxin-lysophosphatidic acid axis in gastrointestinal cancers

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## Abstract

The *KAI1/CD82* gene inhibits the metastasis of most tumors and is remarkably correlated with tumor invasion and prognosis. Cell metabolism dysregulation is an important cause of tumor occurrence, development, and metastasis. As one of the important characteristics of tumors, cell metabolism dysregulation is attracting increasing research attention. Phospholipids are an indispensable substance in the metabolism in various tumor cells. Phospholipid metabolites have become important cell signaling molecules. The pathological role of lysophosphatidic acid (LPA) in tumors was identified in the early 1990s. Currently, LPA inhibitors have entered clinical trials but are not yet used in clinical treatment. Autotaxin (ATX) has lysophospholipase D (lysoPLD) activity and can regulate LPA levels *in vivo*. The LPA receptor family and ATX/lysoPLD are abnormally expressed in various gastrointestinal tumors. According to our recent pre-experimental results, KAI1/CD82 might inhibit the migration and metastasis of cancer cells by regulating the ATX-LPA axis. However, no relevant research has been reported. Clarifying the mechanism of ATX-LPA in the inhibition of cancer metastasis by KAI1/CD82 will provide an important theoretical basis for targeted cancer therapy. In this paper, the molecular compositions of the *KAI1/CD82* gene and the ATX-LPA axis, their physiological functions in tumors, and their roles in gastrointestinal cancers and target therapy are reviewed.

**Key Words:** KAI1/CD82; Autotaxin; Lysophosphatidic acid; Pancreatic cancer; Liver cancer

**Core Tip:** The *KAI1/CD82* gene inhibits the metastasis of most tumors and is significantly correlated with their invasion and prognosis. According to our recent pre-experimental results, we speculated that KAI1/CD82 might inhibit the migration and metastasis of cancer cells by regulating autotaxin (ATX)-lysophosphatidic acid (LPA) axis. However, no relevant research has been reported. To clarify the mechanism of ATX-LPA in KAI1/CD82 inhibition of cancer metastasis will provide an important theoretical basis for targeted cancer therapy, and further research is necessary. In this paper, the molecular composition of the *KAI1/CD82* gene and ATX-LPA axis, their physiological functions in tumors, and their roles in gastrointestinal cancers and target therapy are reviewed.

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## INTRODUCTION

The *KAI1/CD82* gene is an important tumor suppressor gene. As a metastasis-related suppressor gene of prostate cancer discovered by Dong *et al*[1] in 1995, *KAI1/CD82* is located on human chromosome 11p11.2 and consists of l0 exons and 9 introns with a length of about 80 kb. The protein encoded by this gene is composed of 267 amino acids residues and has a relative molecular weight of 29600 Da. KAI1/CD82 is a member of the transmembrane 4 superfamily (TM4SF). TM4SF proteins promote the interactions between cells and the extracellular matrix, enhance the cohesion between tumor cells, reduce phagocytosis and invasion, and inhibit tumor cell metastasis. Cell dysmetabolism is an important cause of tumor occurrence, development, and metastasis. As one of the hallmarks of cancer, cell dysmetabolism has increasingly attracted the attention of researchers in recent years. Phospholipid is an indispensable substance in cell metabolism and participates in the metabolism of various tumor cells. Phospholipid metabolites have become important cell signaling molecules. Lysophosphatidic acid (LPA) is secreted by platelets, fibroblasts, cancer cells, and fat cells and is a multifunctional "phospholipid messenger". In tumor tissues, LPA induces intracellular signal transduction by binding G protein-coupled LPA receptors (LPARs) on the cell surface and regulates tumor cell proliferation, adhesion, migration, and invasion. Autotaxin (ATX) is a key enzyme catalyzing LPA synthesis. Clarifying the role and molecular mechanism of ATX-LPA and LPARs in cancer invasion and metastasis is necessary. According to our previous experimental results and recent pre-experimental results, as well as current reports on ATX-LPA, KAI1/CD82 might inhibit the cancer cell migration and metastasis by regulating the ATX-LPA axis. The abnormal metabolism of the ATX-LPA axis may be associated with the high metastasis characteristics of cancer. The ATX-LPA axis and their receptors may serve as molecular markers for cancer metastasis and prognosis. Clarifying the mechanism of the ATX-LPA axis in the inhibition of cancer metastasis by KAI1/CD82 will provide an important theoretical basis for targeted cancer therapy and further research.

## MOLECULAR COMPOSITION OF THE *KAI1/CD82* GENE AND THE ATX-LPA AXIS

### *Molecular composition of KAI1/CD82*

*KAI1* (named after Anticancer Kang Ai) is a tumor-suppressor gene first discovered by Dong *et al*[1] in 1995 on chromosome 11 of rabbit AT6.1 metastatic prostate cancer cells. Later, researchers confirmed that *KAI1* has the same structure as the *CD82* gene; therefore, it was named *KAI1/CD82*. The 5′-end promoter region of the *KAI1/CD82* gene is 735 bp long and rich in CpG island with nine transcription factor-specific protein SPI binding sites, five AP2 binding sites, and tcF-1, Myb, and MEP.1 binding sites, which suggests that the gene is regulated by multiple mechanisms[2,3]. KAI1/CD82 is located on the cell membrane and is a member of TM4SF, which comprises four conservative hydrophobic transmembrane domains (TM1–TM4) and one extracellular glycosyl-based binding site. This structure indicates that KAI1/CD82, like other TM4SF members, can affect plasma membrane molecular rearrangement, cell aggregation, adhesion, and migration, and other physiological and pathological activities through various mechanisms, as well as inhibit the migration and metastasis of various malignant tumors[4].

### *Molecular composition of the ATX-LPA axis*

ATX is a secretory glycoprotein called autocrine motility factor. ATX was first identified in A2058 melanoma cells and induces cell migration through the pertussis toxin G protein[5]. ATX has phosphod-

iesterase activity[6], and LPA is catalyzed by lysophosphatidylcholine (LPC)[7]. LPA is a multifunctional "phospholipid messenger" secreted by platelets, fibroblasts, adipocytes, and cancer cells. Although LPA is the simplest phospholipid, it is not a simple biomolecule. LPA has six G-protein-coupled receptors that mediate several physiological and pathological processes, including embryogenesis, wound healing, chronic inflammation, cancer progression, and treatment tolerance[8]. In tumor tissues, LPA binds to LPARs on the cell surface to induce intracellular signal transduction, which in turn regulates tumor cell proliferation, adhesion, migration, and invasion[7]. At present, ATX-LPA target inhibitors are not yet used as a therapeutic measure clinically, and the therapeutic effects of LPA monoclonal antibodies, LPAR antagonists, and ATX inhibitors are still being explored.

ATX is also called extracellular pyrophosphatase/phosphodiesterase $(ENPP)_2$ because of its 47%-55% homology with pc-1/NPP1 and B-10/NPP3 amino acid sequences in the ENPP family. ATX is a multidomain protein[9], and lysophospholipase D (lysoPLD) catalyzes LPA formation[10]. ATX has a slightly U-shaped hydrophobic pocket in the catalytic region, which tends to contain unsaturated substrates, such as unsaturated fatty acids[11], and all five selective splicing isomers have catalytic activity[12,13]. Therefore, its affinity with LPC is strong. Although LPA can be produced by other processes, such as phospholipase A2, $Ca^{2+}$-independent phospholipase A2, and phosphatidate[14-16], ATX is still the main pathway of extracellular LPA generation.

Serum contains 2-20 μm LPA, and its metabolites extensively affect biological activities inside and outside cells[17]. LPA is one of the smallest glycerophosphatides and comprises three domains: Phosphate head, linker, and lipophilic terminal. The function of the phosphoric head is to activate the receptor; the lipophilic terminal sequence determines its biological activity; and the head and tail are linked by acyl, alkyl, or alkenyl groups[18]. Its free hydroxyl and phosphate groups make LPA more soluble in water than long-chain phospholipids, which likely contributes to its biological activities. The family of lipid phosphate phosphohydrolases (LPPs) dephosphorylates LPA[19,20].

LPARs are divided into two subfamilies: $LPA_{1-3}$ receptors belonging to the endothelial cell differentiation gene (Edg) family, and $LPA_{4-6}$ receptors belonging to the purine (P2Y) receptor family[9,21]. $LPA_1$ ($Edg_2$) has 50%-60% amino acid homology with $LPA_2$ (Edg4) and $LPA_3$ ($Edg_7$). $LPA_1$ and $LPA_2$ need to pass through the $G_{i/O}$, $G_{q/11}$, and $G_{12/13}$ signaling pathways, whereas $LPA_3$ passes only through the $G_{i/O}$ and $G_{q/11}$ signaling pathways[22]. The function of $G_{i/O}$ is to stimulate mitotic division through the Ras-Raf-MAPK signaling pathway and promote tumor cell survival through the PI3K-Akt signaling pathway[23,24]. $LPA_4$ (P2Y9/GPR23), $LPA_5$ (GPR92), and $LPA_6$ (P2Y5) have 35%-55% amino acid homology. $LPA_4$ acts through the $G_s$, $G_{i/O}$, $G_{q/11}$, and $G_{12/13}$ signaling pathways and is the only LPAR that activates adenosine cyclase and leads to cyclic adenosine monophosphate elevation. $LPA_5$ plays a role through the $G_{q/11}$ and $G_{12/13}$ signaling pathways, whereas $LPA_6$ plays a role through the $G_{12/13}$ activation of the Rho signaling pathways[22]. The effect of LPARs on tumors depends on the G protein signaling pathway that it activates[25].

## PHYSIOLOGICAL FUNCTIONS OF THE *KAI1/CD82* GENE AND THE ATX-LPA-LPP AXIS IN CANCERS

### *Inhibition of the KAI1/CD82 gene in cancers*

Low KAI1 expression accelerates tumor invasion and metastasis[26]. In 2017, a meta-analysis involving 31 studies showed that high KAI1 expression is significantly associated with overall survival (OS) [hazard ratio (HR) = 0.56, 95% confidence interval (CI): 0.47-0.67] and disease-free/relapse-free/progression-free survival (PFS) (HR = 0.42, 95%CI: 0.30-0.59) in patients with cancer. In addition, they performed a subgroup analysis showing that KAI1/CD82 is associated with a good prognosis in patients with cancer. KAI1/CD82 may be a promising biomarker for predicting the prognosis of patients with malignant tumors, and its biological function has important research value for this topic [27]. The Human Protein Atlas is an outstanding initiative associated to the Human Proteome Project, which has made available valuable information about the functional and pathological aspects of about 17000 proteins. In particular, they are able to propose scores that suggest the prognostic value of proteins in diseases based on the expression levels of these proteins in healthy and diseased tissues. Considering that only 31 studies were included in the meta-analysis, more studies may be needed in the future to verify whether KAI1 can be used as a prognostic factor. KAI1/CD82 may inhibit cell metastasis and migration through two pathways. The first is that KAI1/CD82 inhibits cell migration as an initiating signal. However, the possibility of this pathway is low because of the simple structure of KAI1/CD82 and the lack of corresponding enzymes in the cytoplasm. However, evidence also indicates that KAI1/CD82 may be an initiating signal[28,29]. KAI1/CD82 is crosslinked with monoclonal antibody to induce morphological changes and signal transduction[30]. Integrins are also essential for cell adhesion and migration, and KAI1/CD82 is associated with several integrins, including α3β1, α4β1, α5β1, α6β1, and αLβ2[31-35], which may also be one of the pathways through which KAI1/CD82 inhibits tumor. Epidermal growth factor receptor (EGFR) is a member of the ErbB family. In tumor tissues, the receptors and ligand of the ErbB pathway are overproduced and overactivated. Odintsova *et al*[36] found that KAI1/CD82 is correlated with EGFR, ErbB2, and ErbB3 and inhibits the endocytosis of the

EGF signaling pathway and EGFR. KAI1/CD82 redistributes molecules on the cell membrane surface; KAI1/CD82 overexpression results in the redistribution and aggregation of urokinase-type plasminogen activator receptor (uPAR) into a stable α5β1 complex. Moreover, KAI1/CD82 overexpression also results in the redistribution of EGFR and gangliosides in the plasma membrane. However, whether the redistribution of these substances is related to KAI1/CD82 tumor inhibition remains unknown[37].

### Physiological function of the ATX-LPA-LPP axis in cancers

LPA signals can be roughly divided into three parts, namely, ATX, LPARs, and LPP of extracellular LPA [38,39]. ATX has lysoPLD activity and promotes LPA generation in blood[40,41]. Many tumor cells secrete ATX[42], LPAR expression is higher on tumor cell surfaces than on normal cells, and LPP expression is lower in tumor cells than in normal cells. Understanding the metabolic pathway of the ATX-LPA-LPP axis in the tumor microenvironment (TME) is important to study its target therapy (Figure 1).

The TME is produced by tumor cells, such as neuroblastoma[43], glioblastoma[44], liver cancer[45], B-cell lymphoma[46], melanoma[47], kidney cancer[48], thyroid cancer[49], breast cancer, and non-small cell lung cancer[50], as well as stromal cells such as fibroblasts and adipocytes[51-53]. How to regulate ATX expression remains unclear. ENPP overexpression may be one of the reasons for ATX upregulation in cancer tissues[54]. The Cancer Genome Atlas shows that ENPP overexpression is present in serous ovarian cystadenocarcinoma (about 33%) and invasive breast carcinoma (about 20%). The $ENPP_2$ gene is overexpressed in hepatocellular carcinoma (HCC; about 20%), lung adenocarcinoma (about 11%), bladder transitional cell carcinoma (about 10%), and head and neck squamous cell carcinoma (about 10%)[13]. Moreover, ATX is involved in the physiological wound-healing response, and ATX levels are increased in some inflammatory diseases[55]. Park *et al*[56] found that the levels of interleukin (IL)-4, IL-5, and ATX increase in patients with asthma who received bronchoalveolar lavage fluid when stimulated by allergens. ATX induces pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-1β[57,58], NOD receptor family (NLRP3), ATM kinase, ATR protein kinase, and nuclear transcription factor-kappa B (NF-κB)[59]. At present, although ATX research has made some progress, the overall understanding remains limited.

LPA is present in intracellular and extracellular fluids (blood, ascites, follicular fluid, saliva, *etc.*)[56]. In 1989, van Corven *et al*[60] found that LPA may be involved in cell diffusion and migration. Two years later, Merchant *et al*[61] found increased LPA levels in malignant colon tumor tissues. LPA may be a simple lipid, but it is involved in all aspects of tumor development; it stimulates proliferative signals [62], prevents growth inhibition and resists apoptosis[63,64], regulates telomerase[64], promotes vascular endothelial growth factor (VEGF)-A and VEGF-C, and induces angiogenesis[65-67]. LPA induces the gene instability caused by reactive oxygen species and stimulates the production of inflammatory factors, such as COX-2, IL, and TNF-α[68,69]. LPA activates at least three signaling pathways: (1) Promotes phosphoinositol hydrolysis and therefore activates protein kinase C (PKC) and $Ca^{2+}$ mobilization; (2) Promotes the release of guanosine triphosphate (GTP); and (3) Inhibits adenylate cyclase activity. In recent years, the activation of the downstream signaling Ras pathway may promote LPA fibrogenesis[70]. Moreover, MAK-related kinase, as an effector of RhoC, regulates LPA-induced cell invasion through myosin, extracellular signal-regulated kinase (ERK), and P38[71], whereas LPA induces the G12/13-Rhoa-Rock signaling pathway to mediate focal adhesion kinase autophosphorylation and promote tumor cell migration[72]. Furthermore, Lee *et al*[73] found that LPA interacts with T lymphocytes, B lymphocytes, acidic granulocytes, neutrophils, macrophages, mast cells, dendritic cells, and natural killer cells in the immune system and blood. Currently, no clinical treatment for LPA target is available, and the study of TME's molecular mechanism is helpful to guide clinical treatment.

LPA is hydrolyzed and inactivated by LPPs. Studies have found that LPP1 and LPP3 are reduced in various tumor tissues[74]. LPPs activate ERK signaling by thrombin; induce LPP1 and LPP2 overexpression; and attenuate cell migration, cell differentiation, and angiogenesis[75]. Pilquil *et al*[76] found that increased $LPP_1$ expression weakens PLD activation, which is an intermediate substance necessary for LPA to stimulate cell migration. $LPP_1$ also weakens fibroblast migration. Tanyi *et al*[77] found that $LPP_3$ reduces cell apoptosis, decreases the migration ability of transfected $LPP_3$ cells, and slows down tumor growth *in vivo* and *in vitro*.

### Comparative analysis of LPAR-mediated signals in tumors

**$LPA_1$:** $LPA_1$ is the most widely expressed Edg LPAR in tissues[69]. LPA signaling through $LPA_1$ regulates a variety of malignant properties in cancer cells[78]. Murph *et al*[79] found that $LPA_1$ downregulates the tumor suppressor gene *p53* and weakens its inhibitory effect. Marshall *et al*[80] found that the tumor-suppressor gene *Nm23* could inhibit $LPA_1$ expression. Additionally, Stadler *et al*[81] found that $LPA_1$ is a signaling receptor downstream of fibroblast growth factor receptor 4 (FGFR4) that promotes cell transformation of cells into fibroblasts, which are one of the main components of TME matrix. $LPA_1$ preferentially binds to Gα Q proteins in tumors to activate PKC. PKC is involved in many cellular processes, including proliferation and metastasis. Valdés-Rives *et al*[82] found that when the $LPA_1$/PKC α signaling pathway is blocked, the number of cells is reduced; this finding suggests a correlation

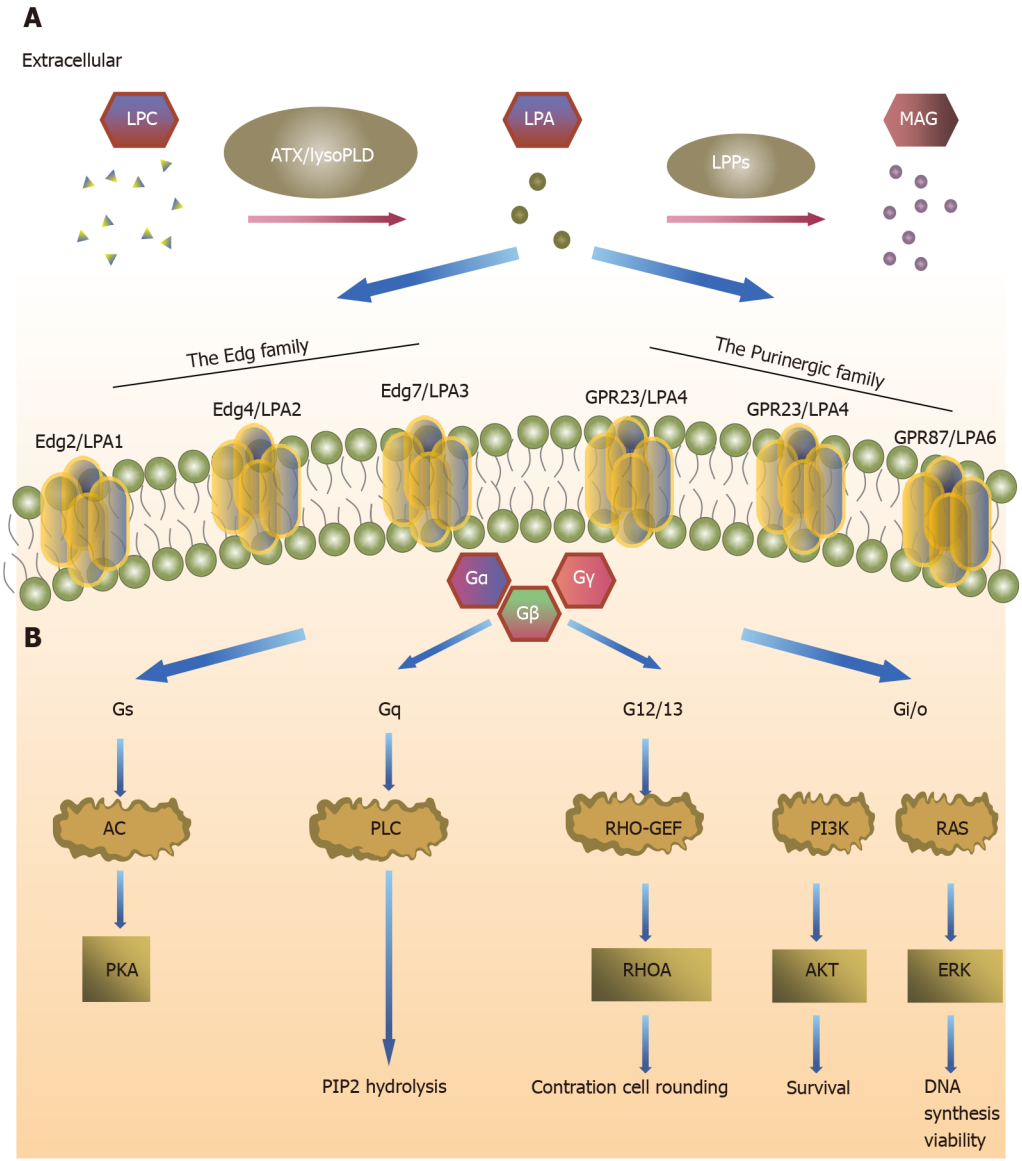


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**Figure 1 Autotaxin-lysophosphatidic acid axis plays a key role in the pathophysiology of tumor cells.** A: The anabolism and catabolism of tumor extracellular lysophosphatidic acid (LPA). Autotaxin/lysophospholipase D catalyzes the generation of LPA from lysophosphatidylcholine (LPC), and lipid phosphate phosphohydrolases promotes LPC hydrolysis; B: LPA activates multiple pathological processes in tumor cells by binding GPRs (lysophosphatidic acid receptors) to promote tumor occurrence and development. LPC: Lysophosphatidylcholine; LPA: Lysophosphatidic acid; ATX: Autotaxin; Edg: Endothelial cell differentiation gene; LPPs: Lipid phosphate phosphohydrolases; LysoPLD: Lysophospholipase D.

between $LPA_1$ and PKCα in glioblastoma multiforme growth. Stadler *et al*[81] found that patients with high expression of the $LPA_1$ receptor for R388 FGFR4 phenotype are more likely to develop cancer. Lin *et al*[83] found that $LPA_1$ signaling mediates tumor lymphangiogenesis by promoting calreticulin expression in prostate cancer. Elevated $LPA_1$ receptors also contribute to cancer development.

**$LPA_2$:** $LPA_2$ is elevated in tumor tissue[84]. Studies showed that $LPA_2$ is associated with many human tumors, and the binding of $LPA_2$ with its ligand, LPA, can activate the LPA signaling pathway and promote cell proliferation and malignant transformation. For example, the high expression level of the $LPA_2$ receptor in breast cancer suggests a poor prognosis[85]. The high expression of *$LPA_2$* mRNA in HCC is related to the low differentiation of cancer cells[86], and the high expression of $LPA_2$ receptor in colon cancer cells promotes the acquisition of drug resistance and the failure of anticancer drugs[87]. $LPA_2$-mediated signaling plays an important role in the enhancement of the chemoresistance of A375 cells treated with anticancer drugs[78]. Ren *et al*[88] transfected SGC-7901 gastric cancer (GC) cells with $LPA_2$ expression vector and found that the expression of E-cadherin gradually decreases and the expression of vimentin gradually increases with the increase in $LPA_2$ level. These findings suggest that $LPA_2$ is involved in the epithelial-mesenchymal transition (EMT) process of GC cells. GC cells with increased $LPA_2$ level are likely to metastasize. Dong *et al*[89] believed that an effective drug that can inhibit *$LPA_2$* gene expression, inhibit GC cell proliferation, and promote apoptosis might be a potential

new target for GC treatment. Xu *et al*[90] found that thyroid receptor interacting protein 6 activates $LPA_2$ and its downstream signal and therefore promotes cell adhesion and migration. The carcinogenic mechanism of $LPA_2$ is still unknown, and most studies have focused on the LPA stimulation of the expression of cytokines, such as IL-6, VEGF, hypoxia-inducible factor 1α, C-MyC, cyclin D1, Kruppel-like factor 5, and COX-2. Moreover, $Na^+/H^+$ regulatory factor 2 (NHERF-2) may enhance *$LPA_2$* gene expression and other LPA-induced cellular processes[91].

**$LPA_3$:** Research found that $LPA_3$ promotes cancer cell proliferation and metastasis. Zhao *et al*[92] found that the high expression of the $LPA_3$ protein is considerably correlated with the occurrence and recurrence of epithelial ovarian cancer. Hayashi *et al*[93] and Kitayoshi *et al*[94] found that $LPA_3$ inhibits tumor cell migration. Sun *et al*[95] found that $LPA_3$ overexpression is associated with lymph node metastasis and the loss of the expression of estrogen receptor, progesterone receptor, and human EGFR2. Studies found that $LPA_3$ may be related to the activation of the YAP protein in breast cancer and that $LPA_3$ overexpression may promote the activation of YAP protein and the proliferation and metastasis of breast cancer cells. Fang *et al*[96] found that $LPA_3$ affects B cell lymphoma (Bcl)-2 and Bax expression; therefore, it affects the Bcl-2/Bax ratio, inhibits the apoptosis of ovarian cancer cells, and promotes the development of ovarian cancer. The vasodilator-stimulated phospho-protein phosphorylation induced by LPA receptor is a key mediator of migration initiation. $LPA_3$ plays a role in cellular motility and may contribute to cell invasion and metastasis[97].

**$LPA_{4\text{-}6}$:** $LPA_4$ may be involved in the invasion and metastasis of breast cancer cells, and the migration and invasion ability may involve the regulation of MMP2 and MMP9 protein expression. Takara *et al*[98] found that $LPA_4$ is involved in the formation of vascular networks. $LPA_4$ activation induces the subcellular binding of circumferential actin and enhances the linear adhesion of vascular-endothelial cadherin in endothelial cells. Studies found that $LPA_5$ knockout cells show high motor activity. The gelatinase spectrum shows that $LPA_5$ inhibits the activation of MMP2. $LPA_5$ also inhibits the cellular motility of endothelial cells, which is correlated to the expression level of the *VEGF* gene[99]. However, Tsujino *et al*[100] found no mutation in the *$LPA_5$* gene in colon cancer cells DLD1, SW480, HCT116, CACO-2, SW48, and LoVo. $LPA_6$-mediated tube formation, which reflects the stabilization of barrier integrity, was confirmed by *in vitro* angiogenesis assay. By contrast, $LPA_6$-mediated protective actions are associated with the activation of Src and Rap1 and attenuated by the abrogation of their activities [101]. A considerable correlation between LPA6 and PIM-3 expression levels is also observed in patients with HCC. Furthermore, the biological roles of $LPA_{4\text{-}6}$ remain unknown[102,103].

## THE *KAI1/CD82* GENE AND ATX–LPA AXIS IN GASTROINTESTINAL CANCERS

### *KAI1/CD82 in pancreatic cancer*

Pancreatic cancer (PC) is the seventh most common cancer worldwide and causes more than 300000 deaths a year[104]. The 5-year survival rate of PC is only 3%-5%. In the early stages of PC, it directly invades peripancreatic tissues or metastasizes to organs near and far *via* lymphatic and/or blood vessels. More than 80% of patients with PC are initially diagnosed at advanced stages, lose the chance of surgical treatment, and have poor radiotherapy and chemotherapy effects. In 1996, Guo *et al*[105] found that the expression of *KAI1/CD82* mRNA in early pancreatic tumors (I and II) is significantly higher than that in advanced tumors (III and IV) with lymph node metastasis or distant metastasis ($P < 0.01$), and the *KAI1* mRNA level in poorly differentiated tumors is significantly higher than that in moderately differentiated or well-differentiated tumors ($P < 0.05$). Friess *et al*[106] and Xu *et al*[107] also found similar results. Subsequent studies have shown that low KAI1/CD82 level is associated with the inhibition of PC cell invasion and metastasis, and the *KAIl/CD82* gene may control PC cell metastasis by inhibiting cancer cell invasion and motor function[108-111].

KAIl/CD82 protein, a member of TM4SF, has been accepted for its inhibitory effect on tumor metastasis; the mechanism of this effect has not yet been clearly explained, but it may be related to its localization on the cell membrane, extensive glycosylation, and cell-cell and cell-extracellular matrix interactions. Mashimo *et al*[112] found that the loss of p53 leads to the downregulation of the *KAI1/CD82* gene and promotes cancer metastasis. KAI1 may inhibit the metastasis of the PC cells PANC-1 and Miapaca-2, caused by hepatocyte growth factor (HGF) by downregulating sphingosine kinase (SphK) expression. After they were infected with the *KAI1* gene, the PANC-1 and Miapaca-2 cells induced by HGF had decreased invasive ability in the Boyden chamber assay. KAI1 overexpression in cells leads to the deactivation of SphK and a decreased level of intracellular sphingosine-1-phosphate[108]. Liu *et al* [108] found that KAI1/CD82 induces the downregulation of VEGF-C expression through the Src/STAT3 signaling pathway, which may also inhibit the lymph node metastasis of PC. Wu *et al*[111] found that KAI1 induces the expression of the autophagy proteins LC3 and Beclin1, and further confirmed that KAI1 could induce autophagy in the human PC cell line MiAPACA-2 and therefore promote cell apoptosis and inhibit proliferation. EMT plays an important role in the pathogenesis of PC. KAI1 reverses the expression of EMT-related factors, such as Snail, Vimentin, MMP2, and MMP9 ($P <$

0.05), and inhibits PC cell metastasis and invasion. In conclusion, KAI1 may be a new potential therapeutic target for PC in the future.

### KAI1/CD82 in HCC

HCC is a common malignant tumor with the second highest mortality rate in China. Rapid intrahepatic and extrahepatic metastases lead to poor prognosis[113]. Zhang *et al*[114] found that the combined detection of KAI1 and VEGF can greatly improve the diagnostic efficiency for HCC. Mu *et al*[115] found that KAI1/CD82 suppresses the HGF-induced migration of hepatoma cells *via* SphK1 downregulation. HGF induces hepatoma cell migration through cellular SphK1 activation. The adenovirus-mediated gene transfer of KAI1 downregulates SphK1 expression and suppresses the HGF-induced migration of SMMC-7721 human HCC cells. Guo *et al*[116] found that the *wTP53* fusion gene and JunB inhibit tumor cell invasiveness and promote tumor cell apoptosis by regulating KAI1/CD82 expression. Si *et al*[117] and Yang *et al*[118] found that changing KAI1 expression could alter the migration and invasion ability of MHCC97-H in HCC cells. Xu *et al*[119] found that KAI1 is negatively correlated with tumor grade, venous invasion, lymph node metastasis, intrahepatic metastasis, and TNM stage and positively correlated with patients' OS. KAI1/CD82 may also play an important role in HCC metastasis and prognosis.

### KAI1/CD82 in GC

GC is one of the most common malignant tumors. Although GC-related morbidity has shown a downward trend in recent years, the mortality rate remains high[120,121]. KAI1 has been studied to identify novel therapeutic targets[122-126]. Ilhan *et al*[122] and Knoener *et al*[123] found that KAI1/CD82 is negative in all tissues with distant metastasis or tissues in stage IV GC with statistical significance ($P < 0.05$). KAI1 inhibits tumor growth and metastasis and is a prognostic factor for patients with GC. Hinoda *et al*[124] found that the positive rate of KAI1/CD82 in patients with stages Ia-IIIa GC is 16.6% (8/48), and all patients with stages IIIb-IVb GC are negative for KAI1/CD82 (0%, 0/25; $P = 0.05$). KAI1/CD82 is highly expressed in normal gastric epithelial cells. In GC, KAI1/CD82 expression decreases with increased tumor differentiation, tumor invasion depth, and lymph node metastasis[127, 128]. Guan *et al*[129] found that reduced KAI1/CD82 expression promotes lymph node metastasis and liver metastasis in patients with GC. The detection of *KAI1/CD82* mRNA expression level can be used as a prognostic index for patients with GC.

### KAI1/CD82 in colorectal cancer

Colorectal cancer (CRC) is a common malignant tumor, and metastasis is the main cause of its poor prognosis. KAI1 may affect cellular connectivity and may be related to its metastasis. KAI1 may be a new therapeutic target for CRC[130,131]. KAI1 mRNA and protein are increased in early CRC tumors, decreased in late CRC tumors, and no longer expressed in distant metastasis[132]. Integrin-α3 and TAp73 regulate CRC invasion and metastasis by regulating KAI1 transcription[133,134].

### ATX-LPA in PC

The expression of ATX in PC remains unclear, and its molecular biological mechanism has not yet been reported. Ryder *et al*[135] and Nakai *et al*[136] found that ATX expression is increased in PC tissues, but it is more increased in chronic pancreatitis or pancreatic cysts than in PC. Quan *et al*[137] found that TNF-α, NF-κB, Wnt/β-catenin pathway, V-Jun, EGF, and B-FGF are all activated or abnormally expressed in PC tissues, which may provide a direction for future research on mechanisms. LPA activates downstream signaling pathways, such as PI3K/AKT, RAS/ERK, Rho, and Hippo, and promotes PC cell proliferation, migration, and invasion[138,139]. Additionally, LPA is remarkably increased in the serum and ascites[140,141], which suggests that ATX activity is elevated in patients with PC.

ATX catalyzes LPA synthesis from LPC and exerts biological effects through the receptors $LPA_{1\text{-}6}$. Fukushima *et al*[142] found that the invasion ability of PANC-R9 cells is 15 times that of PANC-1 cells, $LPA_1$ expression in PANC-R9 cells is remarkably higher than that in PANC-1 cells, and $LPA_3$ is decreased. Kato *et al*[143] also found that $LPA_1$ and $LPA_3$ play opposite roles in PC cell migration. Tsujiuchi *et al*[144], Komachi *et al*[145], and Yamada *et al*[146] found that $LPA_1$ induces PC cell migration. Liao *et al*[141] and Yoshikawa *et al*[147] found that $LPA_2$ may induce PC cell migration by enhancing the proto-oncogene K-RAS pathway. However, Komachi *et al*[145] found that $LPA_2$ may inhibit PC cell migration through the conjugated G12/13/Rho signaling pathway. Ishii *et al*[148] conducted a cell activity assay after LPARs were knocked out from PANC-1 cells (PANC-SH4, PANC-SH5, and PANC-SH6 cells). They found that PANC-SH4 and PANC-SH5 enhance cell migration ability, whereas PANC-SH6 inhibits cell migration. Currently, few studies have been conducted on the molecular biology of LPAR and PC, and further research is needed.

### ATX-LPA axis in HCC

The main risk factors for HCC are hepatitis virus infection; alcohol consumption; and metabolic disorders, such as obesity, diabetes, and non-alcoholic fatty liver disease[149]. The abnormal expression

of the ATX-LPA axis may cause liver metabolism disorder and induce steatohepatitis and liver cancer [150,151]. The ATX-LPA axis is currently considered one of the most promising signaling pathways in liver cancer[152]. Watanabe *et al*[153] found elevated ATX and LPA levels in hepatic fibrosis tissues. Memet *et al*[149] found that high expression of ATX in HCC is an independent prognostic factor (HR = 13.70, 95%CI: 3.26-57.62, $P$ = 0.0004), and high expression of ATX (+3) also increases the risk of death by eight-fold. Wu *et al*[154] found that ATX is significantly elevated in Hep3B and Huh7 cells. Park *et al* [155] found that $LPA_1$ is significantly elevated in liver cancer. $LPA_3$ may be highly expressed in HCC tissues through the lPA3-GI-ERK signaling pathway[156]. Enooku *et al*[86] found that increased $LPA_2$ mRNA level may be associated with the low differentiation degree of HCC. Okabe *et al*[157] found that $LPA_3$ induces the invasion of rabbit RH7777 hepatoma cells. $LPA_6$ is not expressed in normal tissues but is expressed in liver cancer tissues. Zheng *et al*[158] found that nuclear receptor coactivator 3 induces the acetylation of histone 3-LYS-27 at the $LPA_6$ site after HGF treatment and inhibits $LPA_6$ transcription. High $LPA_6$ expression promotes HCC proliferation. Lippolis *et al*[159] found that high $LPA_6$ expression promotes the development of HCC with poor prognosis. Gnocchi *et al*[160] and Mazzocca *et al*[161] found that $LPA_6$ may be an important therapeutic target for HCC, although $LPA_6$ overexpression promotes HCC cell growth.

### ATX-LPA axis in GC

The role of ATX-LPA axis in GC invasion and metastasis remains to be explored. Zeng *et al*[162] found that LPA is increased in GC tissue samples with peritoneal metastasis ($P$ = 0.046) and is significantly increased in ascites ($P$ < 0.001). Serum LPA decreases after chemotherapy ($P$ = 0.028). PFS and OS are significantly decreased in an ascites LPA > 24000 ng/mL group ($P$ < 0.001). Ramachandran *et al*[163] and Shida *et al*[164] found that LPA upregulates SphK1 through the ERK1 signaling pathway. Kim *et al* [165] found that LPA can induce uPAR to stimulate the downstream signaling pathways, rho-family GTPase, JNK, AP-1, and NF-κB. Budnik[18] found that LPA upregulates human epidermal growth factor receptor 2 expression in GC cells and promotes GC cell invasion. LPA promotes cell proliferation, but the molecular biological mechanism between LPA and GC still needs further exploration, and LPA may become a new target for GC treatment.

### ATX-LPA in CRC

CRC is the fourth leading cause of cancer deaths in the world[166]. Kazama *et al*[167] found that ATX overexpression is associated with tumor angiogenesis in the early stage of colon cancer. LPA may stimulate the proliferation and migration of CRC cells through the EGFR pathway. It may also promote hcT-116 colon cancer cell migration by regulating the cell cycle through the rho-Rock and STAT3 pathways. Whether $LPA_1$ stimulates colon cancer cell proliferation remains controversial. A study found that HCT116 and LS174T cells with $LPA_1$ knockout do not affect the spread of cancer cells[168], and DLD cancer cells are affected when they spread[169,170]. $LPA_2$ promotes the spread and migration of colon cancer by regulating the NHERF-2 pathway[171,172]; therefore, $LPA_2$ may be one of the therapeutic targets for CRC in the future[173]. Shida *et al*[174] found that $LPA_3$ mRNA is micro-expressed in normal and tumor tissues. Fukui *et al*[175] found that the expression levels of VEGF-A and VEGF-C are increased in HCT-SH3-3 cells with $LPA_3$ knockout, and $LPA_3$ inhibits the metastasis of HCT116 colon cancer cells. Takahashi *et al*[176] found that $LPA_4$ and $LPA_6$ inhibit the activities of DLD1 and HCT116 colon cancer cells. Studies on ATX-LPA axis target inhibitors and colon malignancies are still few and require further exploration.

## KAI1/CD82 AND ATX-LPA AXIS TARGET THERAPY

### KAI1/CD82 target therapy

Most studies have shown that KAI1/CD82 inhibits tumor metastasis and migration, but knowledge about KAI1/CD82 antibody reagents is still lacking. Custer *et al*[177] found that the KAI1 polyclonal antibody produced by rabbits is expressed similarly in normal tissues of mice and humans and could specifically detect mouse KAI1/CD82 protein. KAI1/CD82 is a novel tumor therapeutic target, and more KAI1/CD82 antibodies are expected to be developed in the future[178,179].

### ATX inhibitors

ATX inhibitors decrease serum LPA levels by more than 95%[180]. Oral ATX inhibitors have better bioavailability owing to their low hydrophobicity and slow degradation *in vivo*[181]. PF-8380 is the first ATX inhibitor to permanently reduce LPA levels *in vivo*. Bhave *et al*[182] and Schleicher *et al*[183] found that PF-8380 reduces lPA-induced inflammation and delays tumor growth for more than 20 d in a mouse model of glioblastoma multiforme. Tang *et al*[184] found that the inhibition of GLPG1690 on ATX enhances the efficacy of chemoradiotherapy in mouse breast cancer models. ONO-8430506 is also a highly effective ATX inhibitor, and the oral administration of 30 mg/kg ONO-8430506 effectively reduces serum ATX and LPA levels in rats[185]. ONO-8430506 in combination with adriamycin delays

the growth time of orthotopic 4T1 breast tumors in 60% Balb/C mice by about 10 d and reduces the growth time of 70% tumors by about 17 d[186,187]. Cholera toxin treatment increases the expression of the anti-inflammatory cytokines IL-4 and IL-10 and inhibits *ATX* mRNA[188], and the knockdown of *ATX* mRNA inhibits the growth of Hep3B and Huh7 hepatoma cells[189]. Gupte *et al*[190] found that ATX inhibitors, such as 4-pentadecylbenzylphosphonic acid, reduce plasma LPA levels by 50%. Plasma LPA in ATX-KO mice lacking dominant heterozygosity is reduced by 50%. ATX inhibitors have not shown remarkable side effects to date.

### *LPA monoclonal antibody and LPA receptor antagonist*

Antibody interventional therapy is superior to traditional therapy, and its antibody bioavailability and receptor binding are longer than other therapies[191]. Goldshmit *et al*[192] found that monoclonal antibody B3 can reduce inflammation and glial cell death and improve neuronal function. Monoclonal antibody B3, also known as lpathomab, reduces IL-6 expression and the lesion area and has improved function in a mouse model of traumatic brain injury[193].

Many LPA receptor antagonists have been found, but few work *in vivo*. LPA receptor antagonists are divided into lipid and small-molecule inhibitors, which are derived from fibrosis model studies[194]. BrP-LPA, a pan-LPAR antagonist, was used to treat breast MDA-MB-231 cancer cells[195]. Through LPAR2, BrP-LPA may also sensitize vascular endothelial cells in mouse GL-261 glioma cells to improve malignant glioma response to radiation therapy[183]. LPA accelerates pulmonary fibrosis through $LPA_1$, and the $LPA_1$ antagonist AM966 can inhibit bleomycin-induced idiopathic pulmonary fibrosis. Zhao *et al*[196] found that Ki16425 ($LPA_1$ and $LPA_3$ antagonist) and ono7300243 ($LPA_1$ antagonist) completely block LPA-induced actions. Recently, lysophospholipid *GPCR* genes have been used to develop receptor subtype-selective agonists and antagonists. The discovery of FTY720, a novel immune modulator, along with other chemical tools, has provided a means of elucidating the functions of each lysophospholipid GPCR on an organ and the whole body level[197]. In some cancers, targeting $LPAR_5$ is considered a good option against cancer development[87,198]. $LPAR_5$ antagonist TCLPA5 attenuates the proliferation and migration of thyroid carcinoma cells[199]. In addition, the loss of $LPA_5$ in mouse B16-F10 melanoma results in fewer lung metastases[200], which suggests that the drug inhibition of $LPA_5$ can also control melanoma-mediated metastasis. MP-LPA analogs exhibit an unanticipated pattern of partial agonist/antagonist activity for the LPA G protein-coupled receptor family and the intracellular LPA receptor peroxisome proliferator-activated receptors-γ[201]. Currently, all are based on $LPA_1$, $LPA_2$, or $LPA_{1/3}$ dual antagonists[194]. However, the development of PAN-LPA receptor antagonists may be a more effective approach owing to the complexity of LPAR signals[202].

## CONCLUSION

This paper systematically reviews the physiological functions of the *KAI1/CD82* gene and the ATX-LPA axis in tumors, as well as their roles in digestive system tumors and targeted therapies. The results demonstrate that KAI1/CD82 is indeed an important inhibitor of tumor metastasis. Further elucidation of the molecular mechanism and regulatory network of KAI1/CD82 and the inhibition of tumor metastasis is needed to discover the molecular markers of pancreatic tumor metastasis, adopt effective strategies to treat PC and prevent PC metastasis, and provide a new approach for the diagnosis and treatment of patients with refractory PC. Although the ATX-LPA axis is considered an important target of cancer, its clinical application is still faced with obstacles. LPA is degraded quickly in the body, and many other factors, such as diet, smoking, and alcohol consumption, can affect the detection results. Other lipids may also generate LPA during extraction, storage, and detection. Therefore, many technical problems need to be overcome in LPA detection. In recent years, clinical trials on the ATX-LPA axis have begun. LPA monoclonal antibodies, LPA receptor antagonists, and ATX inhibitors may become feasible treatment measures. Moreover, ATX-LPA axis-targeted therapy may affect the efficacy of existing chemical drugs. Therefore, an in-depth exploration of specific biomarkers related to LPA activity should be conducted to track disease progression during LPA treatment and ensure the rational application of drugs.

## FOOTNOTES

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*REVIEW*

# Poorly cohesive cells gastric carcinoma including signet-ring cell cancer: Updated review of definition, classification and therapeutic management

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## Abstract

While the incidence of gastric cancer (GC) in general has decreased worldwide in recent decades, the incidence of diffuse cancer historically comprising poorly cohesive cells-GC (PCC-GC) and including signet ring cell cancer is rising. Literature concerning PCC-GC is scarce and unclear, mostly due to a large variety of historically used definitions and classifications. Compared to other histological subtypes of GC, PCC-GC is nevertheless characterized by a distinct set of epidemiological, histological and clinical features which require a specific diagnostic and therapeutic approach. The aim of this review was to provide an update on the definition, classification and therapeutic strategies of PCC-GC. We

focus on the updated histological definition of PCC-GC, along with its implications on future treatment strategies and study design. Also, specific considerations in the diagnostic management are discussed. Finally, the impact of some recent developments in the therapeutic management of GC in general such as the recently validated taxane-based regimens (5-Fluorouracil, leucovorin, oxaliplatin and docetaxel), the use of hyperthermic intraperitoneal chemotherapy as well as pressurized intraperitoneal aerosol chemotherapy and targeted therapy have been reviewed in depth for their relative importance for PCC-GC in particular.

**Key Words:** Poorly cohesive cells gastric carcinoma; Review; Definition; Classification; Therapeutic management



**Core Tip:** Although the worldwide incidence of gastric cancer (GC) has decreased in recent decades, the incidence of diffuse cancer historically comprising poorly cohesive cells-GC (PCC-GC) and including signet ring cell cancer is rising. While the existing literature concerning PCC-GC is scarce, this narrative review aims to provide an update on the classification and management of PCC-GC in light of several recent developments: (1) The updated definition according to World Health Organization classification and Verona consensus; (2) An update in curative approaches following the recent validation of 5-Fluorouracil, leucovorin, oxaliplatin and docetaxel regimen and development of hyperthermic intraperitoneal chemotherapy; and (3) Role of chemotherapy and targeted therapies in the treatment of PCC-GC.

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## INTRODUCTION

Worldwide, gastric cancer (GC) is ranked as the 5$^{th}$ most frequently diagnosed cancer. Because of its poor prognosis, it is responsible for the 3$^{rd}$ highest cancer-related death rate[1]. Despite a global decline in the overall incidence of GC, the relative incidence of diffuse-type GC historically comprising poorly cohesive cells-GC (PCC-GC) and including signet ring cell (SRC) cancer has shown a steady increase in the past few decades, especially in the United States and Europe[2-4]. Based on data from the Surveillance, Epidemiology and End Results (SEER) database, collected between 1973 and 2000, an increase of 400% of the diffuse type GC has been noted[4]. In contrast to other histological types of GC, SRC-GC is known to be associated with a younger age at the time of diagnosis along with a more female sex distribution[5-8]. Since the publication of the first edition of the World Health Organization (WHO) classification of GC in 1977, the definition of SRC-GC has changed several times until the 5$^{th}$ edition in 2019[9-13]. Before 2010, SRC-GC was classified as a separate specific subtype of GC[9,10,13]. In the edition of 2010, the SRC-GC category was redefined entirely as a subtype of PCC-GC[10]. Previously, alternative classification systems such as the Lauren and the Ming classification, categorized SRC-GC as 'diffuse/mixed' and 'infiltrative' type carcinoma, respectively[14,15]. As such, these multiple definitions and classifications render correct assessment and comparison of this histological subtype in the current literature challenging to make. In this context, an updated review on PCC-GC was needed to address the following topics: (1) Recent definition according to WHO classification[12] and Verona consensus [16]; (2) Update in curative approaches following validation of the new perioperative chemotherapy (CT) regimen 5-Fluorouracil, leucovorin, oxaliplatin and docetaxel (FLOT)[17,18] and the increasing role of hyperthermic intraperitoneal chemotherapy (HIPEC) in the prevention of, or as a curative treatment for, peritoneal metastases; and (3) Recent developments in future-based therapeutic strategies including CT, pressurized intraperitoneal aerosol chemotherapy (PIPAC) and targeted therapies including immunotherapy.

## LITERATURE SEARCH

A literature search in the MEDLINE/PubMed and *Reference Citation Analysis* (https://www.referencitationanalysis.com/) database was conducted with the use of the following search terms: 'Signet

ring cell carcinoma' ($n$ = 3345), 'PCC' ($n$ = 136), 'Lauren and diffuse type' ($n$ = 257), 'linitis plastica' ($n$ = 423) and 'Bormann type IV' ($n$ = 178) up to 2021. Only studies in the English language published after January 1980 were eligible for inclusion. Studies were screened based on the abstract. Additional studies were retrieved by screening the references of each article. Case reports and studies including patients < 18-years-old were excluded as well as studies reporting on non-gastric PCC-GC. Studies reporting on < 30 cases were also excluded. Abstracts and meeting reports were only included if the information was found to be relevant enough in the context of the subject. Studies were only included after the agreement of both VD and GP.

## OVERVIEW AND UPDATE ON HISTOLOGICAL AND MOLECULAR CLASSIFICATIONS

Overview and update on histological and molecular classifications of SRC- and PCC-GC.

The most commonly used classifications in GC are the WHO and the Laurén classifications[10,11,14].

### *WHO and Verona classification*

The WHO definition of SRC-GC and-more recently-PCC-GC has evolved in function of the different published editions of the WHO classification. In the very first edition, published in 1977, SRC-GC was considered as a separate subtype of GC and was defined as 'a tumor which contained more than 50% of isolated or small groups of malignant cells containing intracytoplasmic mucin'. As such, four morphological SRC types were defined[9]. By the time the 3rd edition of the WHO classification was published in 2000, this was extended to 5 morphological SRC types[11]. In the 4th edition in 2010, the SRC-GC category was completely redefined as a subtype of PCC-GC[10]. PCC-GC is composed of neoplastic cells that are isolated or arranged in small aggregates without well-formed glands. The definition of the extent of SRC to qualify as SRC-GC evolved to "predominantly" or "exclusively" in the 4th and 5th editions of the WHO[10,12]. SRCs are characterized by a central optically clear, globoid droplet of cytoplasmic mucin with an eccentrically placed nucleus[10]. Other cellular subtypes not fulfilling the requirements of this definition should be defined as PCC not otherwise specified (PCC-NOS). PCC-NOS include tumors composed of neoplastic cells resembling histiocytes or lymphocytes; others have deeply eosinophilic cytoplasm; some PCC are pleomorphic with bizarre nuclei. A mixture of the different cell types can be seen, including a mixture of PCC-NOS and SRC. Historically, mucinous adenocarcinoma has frequently been misclassified as SRCC due to the frequent observation of SRC in this subtype[19,20]. Overall, this added a lot of confusion in analyzing data from the literature.

Invited by the European chapter of the International Gastric Cancer Association (IGCA), a multidisciplinary expert panel convened in 2017 with the intent to clarify the pathological definition of PCC-GC[16]. In a consented conclusion, it was proposed that only PCC-GC with more than 90% of cells representing an SRC morphology should be classified as SRC-type. The two other categories were PCC with SRC component (< 90% but > 10% of SRC) and PCC-NOS: < 10% of SRC[16]. An overview of the proposed definition and classification is shown in Table 1 and Figure 1. On another level, this newly defined classification also incorporates the theory that the extent of SRC in the tumor may be an expression of the differentiation grade of PCC[16]. The importance of this consensus definition cannot be underestimated since it will enable future studies to standardize results and facilitate comparison between studies in order to avoid the major heterogeneity that has characterized studies concerning SRC-GC for the past few decades.

### *Laurén and other classifications*

The Laurén classification, which is the oldest and most general classification, categorizes tumors into two major categories: Intestinal-type tumors, characterized by cohesive neoplastic cells organized in well-differentiated glandular structures and diffuse tumors, diffusely infiltrating the gastric wall, with little to no gland formation. The latter type consists of PCCs, with or without SRC morphology and thus corresponds most with the PCC category of the WHO classification[14]. Comparative studies are shown in Table 2. Tumors exhibiting features of both the intestinal and diffuse types (> 25% of either component) are designated as mixed-type adenocarcinoma and account for approximately 10% of all gastric adenocarcinomas[21,22]. Some tumors may be unclassified. Although widely implemented, the Laurén classification does not allow for any clinical or pathological evaluation according to the proportion of the SRC component, which is an additional justification for the implementation of the recently proposed renewed definition of PCC by the WHO[12] and the European chapter of IGCA[16].

The original Japanese classification system categorized GC into differentiated and undifferentiated tumors, with undifferentiated type corresponding to diffuse type[23]. A more recent version of the classification proposed by the Japanese Gastric Cancer Association (JCGA) is however mainly based on the WHO classification and distinguishes between papillary, tubular, poorly differentiated and mucinous adenocarcinoma as well as SRC tumors[24]. Finally, the Ming classification describes an expanding and infiltrative type, the latter being strongly correlated to diffuse type[25,26].

**Table 1 Subcategories of poorly cohesive cell carcinoma as proposed by the Verona consensus[16] in two recent clinical studies[47,48]**

| Ref. | Category of PCC | | |
|---|---|---|---|
| | SRC type: > 90% of Signet Ring cells | PCC with SRC component: < 90% but > 10% of SRC | PCC-NOS: < 10% of SRC |
| Bencivenga *et al*[47] | 32 (18.5) | 98 (56.6) | 43 (24.9) |
| Roviello *et al*[48] | 0 (0) | 87 (60.8) | 56 (39.2) |

PCC: Poorly cohesive cell; SRC: Signet ring cells; NOS: Not otherwise specified.

**Table 2 Concordance rates between World Health Organization and Laurén classification systems**

| Ref. | Reclassification of SRC and PCC-GC according to Laurén classification | | | |
|---|---|---|---|---|
| | *n* | % Intestinal | % Diffuse | % Mixed |
| Pyo *et al*[7], 2016 | 3170 | 0.6 | 96.3 | 3.1 |
| Pyo *et al*[173], 2017 | 5309 | 0.0 | 96.1 | 3.9 |
| Wanebo *et al*[174],1993 | 187 | 2 | 87 | 11 |
| Hass *et al*[175], 2011 | 160 | 7.6 | 66.2 | 26.2 |
| Lee *et al*[176], 2012 | 320 | 0.0 | 90.6 | 9.4 |
| Heger *et al*[58], 2014 | 235 | 0.0 | 75.3 | 20.0 |
| Chon *et al*[53], 2017 | 1646 | 1.2 | 96.4 | 2.4 |

SRC: Signet ring cells; PCC: Poorly cohesive cells; GC: Gastric cancer.

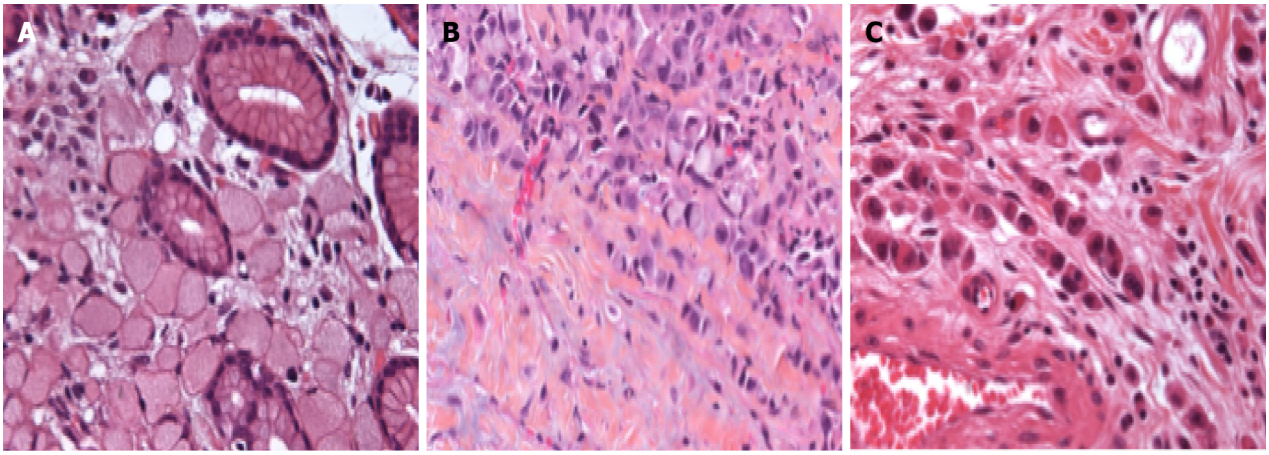


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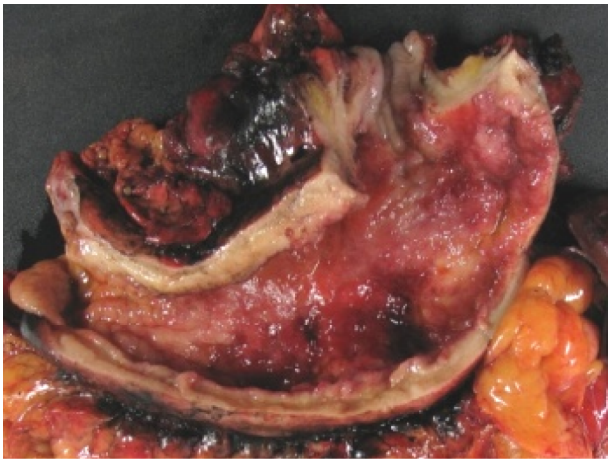
**Figure 1 Subcategories of poorly cohesive cell gastric carcinoma as proposed by the European consensus meeting of the European Chapter of International Gastric Cancer Association (Verona, 2017).** A: Signet ring cells type (SRC): > 90% of SRC; B: Poorly cohesive cell (PCC) with SRC component: < 90% but > 10% of SRC; C: PCC-non other specified: < 10% of SRC.

### *Linitis plastica*

Linitis plastica (LP) is macroscopically described as an increased thickening and rigidity of the gastric wall with an aspect of linen. From a histological point of view, it corresponds to involvement of the entire stomach wall by carcinoma cells, mostly SRC, with a very abundant sclerous stroma. LP is an uncommon variant of gastric adenocarcinoma occurring in 7%–17.4% of cases[27-31]. LP is rarely individualized in studies for two main reasons; (1) Some authors confuse the histological and macroscopical definition[32-34] assimilating SRC-GC with LP, thus adding to the confusion; and (2) LP is also referred to as Borrmann type IV or scirrhous gastric carcinoma in the Eastern literature. An illustration of gastric LP is presented in Figure 2. In one study at our center, among 159 patients with SRC-GC and non-SRC_GC, LP occurred in 35.6% in the SRC group *vs* 6% in the non-SRCC group ($P$ < 0.001)[35]. Most LP in the non-SRC-group had a minor component of SRC. In other words, LP and SRCC



**DOI:** 10.4251/wjgo.v14.i8.1406 

**Figure 2 A macroscopic view of a gastric linitis plastica.**

are not synonyms[36] but are closely associated. However, we believe that the current definition of SCR-GC should be used systematically. The term 'linitis plastica' can be additionally used when applicable.

### *Molecular characteristics*

From a molecular point of view, GC has been classified into four genomic subtypes in a landmark project by The Cancer Genome Atlas[37]. These four subtypes comprise: (1) The Epstein-Barr virus (EBV) subtype (9%), characterized by extreme DNA hypermethylation, recurrent PIK3CA mutations and amplification of JAK2, programmed death-ligand 1 (PD-L1) and PD-L2; (2) The microsatellite instability (MSI) subtype (21%), containing mutations in genes encoding for targetable oncogenic signaling proteins and associated with a more favorable oncological outcome; (3) A genomically stable (GS) subtype (20%), in which most but not all PCC-GC are categorized; and (4) The chromosomal instability (CIN) subtype (50%), associated with aneuploidy and amplification of genes involved in receptor tyrosine kinase/RAS/MAPK signaling[38]. More recently, another molecular analysis for GC identified four subgroups of tumors associated with distinct clinical outcomes: (1) A mesenchymal-type, including diffuse-subtype tumors and most PCC-GC tumors; (2) An MSI subtype, characterized by numerous mutations and a better prognosis; (3) A tumor protein 53 (TP53)-active subtype, associated with higher rates of EBV infection; and (4) A TP53-inactive subtype, similar to the CIN subgroup[39]. The importance of these molecular classifications cannot be underestimated as they provide a roadmap for patient stratification. In addition to the prognostic impact, it has been proven that these genomic subtypes are associated with distinct features regarding tumor response. As such, this subtype classification is primordial in the implementation of current and future clinical trials that evaluate the role of targeted therapies, among others[40,41]. However, we have to bear in mind that GC consists of heterogeneous tumors and that several histological and molecular components can be present in the same tumor and may be modified by the treatment applied[42]. In addition, there is no strict correlation between the histological types and molecular subtypes. PCC-GC are mostly GS but can also be MSI or EBV type with potential therapeutic implications since both molecular subtypes are associated with response to immune checkpoint inhibitors[43].

### *Prognostic features of PCC-GC*

**All stages studies:** Although most studies agree about the poor prognosis of diffuse GC according to the Laurén classification, more discrepancies exist about the specific prognosis of PCC-GC[22,35,44,45]. An overview of studies reporting on the prognosis of all stages of SRC- and PCC-GC, is shown in Table 3. The reported prognosis of PCC-GC in Western studies is in general worse compared to that of most Eastern studies with, however, significant differences in terms of tumor stages; the majority of studies in early gastric cancer (EGC) (*i.e.* GC pT1a or pT1b regardless of lymph node status)[46] originate from Eastern series.

Among PCC tumors, the prognostic impact of the relative percentages of an SRC component within the tumors remains controversial[40]. Two studies evaluated the prognostic role of the Verona consensus with marked differences between the distribution of the three categories questioning the reproducibility of the classification (Table 1)[40,47,48]. Bencivenga *et al*[47] showed that the percentage of SRC was associated with tumor stage and survival in PCC-GC: The percentage of SRC was inversely related to tumor aggressiveness, pT stage ($P < 0.001$) and the number of positive nodes coded as a continuous variable ($P = 0.009$). Long-term survival was significantly higher in SRC-type (> 90% SRC) compared with PCC with SRC component (< 90% but > 10% of SRC) and PCC-NOS (< 10% of SRC) tumors[47]. In the other study, on pathological revision no patients with SRC-type (> 90% SRC) were

**Table 3 Summary of studies reporting the all-stage prognostic value of signet ring cell- and poorly cohesive cell-gastric cancer**

| Ref. | *n* | *n* SRC-CG (%) | % LNM | 5-yr survival rate %, SR-CGC *vs* other | Univariate | Multivariate | Compared to |
|---|---|---|---|---|---|---|---|
| **Eastern studies** | | | | | | | |
| Maehara *et al*[49], 1992 | 1500 | 51 (3.4) | 33.3 | 74.5 *vs* 52.4 | $P < 0.01$ | - | Non SRC-GC |
| Kim *et al*[177], 1994 | 3702 | 450 (12.2) | 50.6 | 59.7 *vs* 57.7/48.6/43.1 | NS | - | WD/MD/PD |
| Otsuji *et al*[178], 1998 | 1498 | 154 (10.3) | 27.9 | 68.2 *vs* 43.9 (10-yr survival rate) | $P < 0.05$ | - | Non SRC-GC |
| Yokota *et al*[179], 1998 | 923 | 93 (10.1) | 43 | Worse | NS | - | Non SRC-GC |
| Kim *et al*[180], 2004 | 2358 | 204 (8.7) | 26.5 | 60.2 *vs* 48.9 | $P < 0.01$ | NS | Non SRC-GC |
| Park *et al*[181], 2008 | 2275 | 251 (11.0) | 46.2 | 66.2 *vs* 66.7/54.5/51.0 | WD: NS; PD/MC: $P < 0.001$ | $P = 0.002$[a] | WD/PD/MC |
| Zhang *et al*[45], 2010 | 1439 | 218 (15.1) | 76.1 | 44.9 *vs* 36 | $P = 0.013$ | NS | Non SRC-GC |
| Chiu *et al*[182], 2011 | 2439 | 505 (20.7) | 53.7 | 57.6 *vs* 56 | NS | - | Non SRC-GC |
| Jiang *et al*[55], 2011 | 1439 | 211 (14.7) | 52,0 | 49.8 *vs* 41.4 | $P = 0.001$ | - | Non SRC-GC |
| Lee *et al*[176], 2012 | 1002 | 320 (31.9) | 37.2 | 84.8 *vs* 71.9/57.8 | $P < 0.001$ | NS | PD/MC |
| Kwon *et al*[50], 2014 | 769 | 108 (14.0) | 43.5 | 55.4 *vs* 64.5/46.2 (10-yr survival rate) | $P < 0.001$ | NS | WD-MD/PD-MC |
| Liu *et al*[162], 2015 | 1464 | 138 (9.4) | 30.4 | 36.2 *vs* 49.5 | $P < 0.001$ | $P < 0.001$ | Non SRC-GC |
| Chon *et al*[53], 2017 | 7667 | 1646 (21.5) | 25.8 | 80.0 *vs* 70.0 (10-y survival rate) | $P < 0.001$ | NS | WMD/PD |
| Lu *et al*[183], 2016 | 2199 | 354 (16.1) | - | 15.9 mo *vs* 22.1 mo | $P = 0.002$ | < 0.001 | Non SRC-GC |
| **Western studies** | | | | | | | |
| Theuer *et al*[184], 1999 | 3020 | 453 (15.0) | NR | Similar | NS | NS | Non SRC-GC |
| Piessen *et al*[35], 2009 | 180 | 59 (32.8) | 83.1 | 28 *vs* 46 | $P = 0.004$ | $P = 0.004$ | Non SRC-GC |
| Taghavi *et al*[44], 2012 | 10246 | 2666 (26) | 59.7 | Similar (Disease-specific survival) | NS | $P = 0.15$ | Non SRC-GC |
| Bamboat *et al*[51], 2014 | 569 | 210 (36.9) | 61.0 | 49 *vs* 24/43 (5-y cumulative-mortality) | $P < 0.0001$ | - | WMD/PD |
| Postlewait *et al* [6], 2015 | 768 | 312 (40.6) | 66.3 | 33.7 mo *vs* 46.6 mo (OS) | $P = 0.011$ | NS | Non SRC-GC |
| Voron *et al*[8], 2016 | 1799 | 899 (50) | 73.2 | 26 mo *vs* 51 mo (median survival) | $P < 0.001$ | $P < 0.041$ | Non SRC-GC |

[a]The survival rate of patients with stage IV signet ring cells-gastric cancer was poorer than those with the other three types.

LNM: Lymph node metastasis; SRC-GC: Signet ring cells-gastric cancer; non SRC-GC: gastric cancer other types than SRC-GC; WMD: Well-and moderately-differentiated gastric cancer; PD: Poorly differentiated; MC: Mucinous cancer; NS: Non-significant.

identified[48]. The 5-year overall survival (OS) was significantly higher in PCC with an SRC component (< 90% but > 10% of SRC) compared with PCC-NOS (< 10% of SRC) (63.3% *vs* 12.7%)[48].

**EGC:** An overview of studies reporting on the prognostic outcomes of SRC- or PCC-EGC is shown in Table 4. Most studies demonstrated that the prognosis of SRC- or PCC-EGC is similar to or even better than that of other EGC[49-52]. The largest of these studies, including data on 3272 patients, concluded that the prognosis of SRC-EGC was better than that of well-and moderately-differentiated EGC [hazard ratio (HR) for OS = 0.66, 95%CI: 0.44-0.98][53]. In one of the few Western studies, Gronnier *et al*[54] showed that SRC-EGC was associated with a 5 year-OS benefit (85% *vs* 76%, $P = 0.035$) compared to

**Table 4 Summary of studies reporting prognostic value of signet ring cell- and poorly cohesive cell-early gastric cancer**

| Ref. | *n* | *n* SRC-GC (%) | % LNM | 5-yr survival rate %, SRC-GC *vs* other | Univariate | Multivariate | Compared to |
|---|---|---|---|---|---|---|---|
| **Eastern studies** | | | | | | | |
| Maehara *et al*[49], 1992 | 384 | 28 (7.3) | 10.7 | 100 *vs* 94.8 | NS | - | Non SRC-GC |
| Kim *et al*[177], 1994 | 785 | 185 (23.6) | 7.6 | 92.9 *vs* 83.9/87.3/93.6 | NS | - | WD/MD/PD |
| Otsuji *et al*[178], 1998 | 568 | 94 (16.5) | 5.3 | 93 *vs* 76.3 | $P < 0.05$ | - | Non SRC-GC |
| Yokota *et al*[179], 1998 | 253 | 41 (16.2) | - | Similar | NS | - | Non SRC-GC |
| Hyung *et al*[80], 2002 | 933 | 263 (28.2) | 5.7 | 94.2 *vs* 91.6 | $P = 0.01$ | - | Non SRC-GC |
| Kim *et al*[180], 2004 | 561 | 94 (16.8) | 2.1 | 96.3 *vs* 90.8 | NS | NS | Non SRC-GC |
| Kunisaki *et al* [185], 2004 | 513 | 120 (23.4) | 9.2 | Better | $P = 0.033$ | $P = 0.036$ | Non SRC-GC |
| Ha *et al*[186], 2008 | 1520 | 388 (25.5) | 9.5 | 99.7 *vs* 99.1/97.2 | NS/$P = 0.019$ | - | WMD-PA/PD-MC |
| Zhang *et al*[45], 2010 | 138 | 49 (35.5) | - | Similar | NS | - | Non SRC-GC |
| Chiu *et al*[182], 2011 | 579 | 149 (25.7) | 10.7 | 96.1 *vs* 89.6 | $P = 0.01$ | - | Non SRC-GC |
| Jiang *et al*[55], 2011 | 269 | 54 (20.1) | 16.7 | 94.3 *vs* 90.6 | $P = 0.007$ | $P = 0.011$ | Non SRC-GC |
| Kwon *et al*[50], 2014 | 326 | 51 (15.6) | 9.8 | 84.0 *vs* 76.0/65.7 (10-yr survival rate) | NS | - | WD-MD/PD-MC |
| Kim *et al*[52], 2014 | 2085 | 345 (16.5) | 9.0% | Similar (disease-related survival) | NS | - | WD/MD/PD |
| Wang *et al*[187], 2015 | 334 | 115 (34.4) | 8.5 | 93.9 *vs* 85.8 | $P = 0.027$ | 0.001 | UD |
| Chon *et al*[53], 2017 | 3272 | 1091 (33.3) | - | 95 *vs* 85 (10-yr survival rate) | $P < 0.001$ | $P = 0.041$ (WMD) | WMD-PD |
| Imamura *et al* [188], 2016 | 746 | 152 (20.4) | 2.0 | 97.4 *vs* 89.9 | $P = 0.012$ | $P = 0.038$ | Non SRC-GC |
| **Western studies** | | | | | | | |
| Gronnier *et al*[54], 2013 | 421 | 104 (24.7) | 24,0 | 85 *vs* 76 | $P = 0.035$ | NS | Non SRC-GC |
| Bamboat *et al*[51], 2014 | 437 | 174 (39.8) | - | 0 *vs* 8/24 (5-disease-specific mortality) | $P = 0.001$ | - | WMD/PD |

EGC: Early gastric cancer; LNM: Lymph node metastasis; SRC-GC: Signet ring cells-gastric cancer; non SRC-GC: Gastric cancer other types than SRC-GC; WMD: Well-and moderately-differentiated gastric cancer; PD: Poorly differentiated; MC: Mucinous cancer; NS: Non-significant.

non-SRCEGC, although SRC-EGC was more frequently associated with submucosal invasion[54]. However, the survival benefit in this study was no longer objectivated after multivariable analysis, possibly because of the lower rate of non-cancer-related deaths in the younger SRC group. More studies in Western populations are required to validate further the superior prognostic results of PCC- or SRC-EGC as reported by the Eastern series and should include an analysis according to the new WHO classification and Verona consensus[12,16].

### *Advanced GC (GC invading beyond the submucosa)*

Table 5 presents an overview of studies reporting on the prognostic characteristics of SRC- or PCC-advanced GC (AGC). At an advanced stage, SRC-AGC is associated with deeper tumor invasion, a higher rate of lymph node involvement, an increased potential for diffuse infiltration of the gastric wall (LP), a greater risk of metastatic peritoneal disease, lower rates of R0 resection and higher rates of early disease recurrence[44,55-57]. Whether the dismal prognosis of PCC-GC is related to a more advanced stage of the disease at the time of diagnosis or to inherently more aggressive tumor biology is much debated[35,44]. Results from a large population-based study in the United States demonstrated that

**Table 5 Summary of studies reporting prognostic value of signet ring cell- and poorly cohesive cell-gastric cancer**

| Studies | *n* | *n* SRC (%) | % LNM | 5-yr survival rate % (PCC-GC *vs* other) | Univariate | Multivariate | Compared to |
|---|---|---|---|---|---|---|---|
| **Eastern studies** | | | | | | | |
| Maehara *et al* [49], 1992 | 1116 | 23 (2.1) | 60.8 | 42.5 *vs* 37.6 | NS | - | Non SRC-GC |
| Kim *et al*[177], 1994 | 2917 | 265 (9.1) | 80.8 | 33 *vs* 45.4/38.8/35.3 | $P < 0.05$ | - | WD/MD/PD |
| Otsuji *et al*[178], 1998 | 930 | 60 (6.4) | 63.3 | 44.4 *vs* 27.5 (10-yr survival rate) | NS | - | Non SRC-GC |
| Yokota *et al*[179], 1998 | 430 | 52 (12.1) | - | Worse | NS | - | Non SRC-GC |
| Kunisaki *et al* [185], 2004 | 600 | 54 (9.0) | 57.4 | Similar | NS | - | Non SRC-GC |
| Kim *et al*[180], 2004 | 1797 | 110 (6.1) | 47.3 | 35.1 *vs* 39.5 | NS | - | Non SRC-GC |
| Li *et al*[56], 2007 | 4759 | 662 (13.9) | 75.7 | 42.4 *vs* 50.1 | 0.009 | NS | Non SRC-GC |
| Chiu *et al*[182], 2011 | 1860 | 356 (19.1) | 71.6 | 41.5 *vs* 46.3 | $P = 0.018$ | - | Non SRC-GC |
| Jiang *et al*[55], 2011 | 2046 | 157 (7.7) | 64.3 | 31.5 *vs* 35.7 | NS | NS | Non SRC-GC |
| Zu *et al*[57], 2014 | 741 | 44 (5.9) | 56.8 | 43.4 *vs* 87.1/57.1/50.6/62.7 | $P = 0.012$ | 0.028 | WD/MD/PD/MC |
| Kwon *et al*[50], 2014 | 443 | 57 (12.9) | 73.7 | 26.0 *vs* 50.5/38.4 (10-yr survival rate) | $P = 0.044$ | NS | WD-MD/PD-MC |
| Chon *et al*[53], 2017 | 1777 | 555 (31.2) | - | 53 *vs* 58/52 (10-yr survival rate) | $P < 0.001$ | $P < 0.001$ | WMD/PD |
| **Western studies** | | | | | | | |
| Heger *et al*[58], 2014 | 723 | 235 (32.5) | 63.0 | 26.3 *vs* 46.6 mo (median survival) | $P < 0.001$ | $P = 0.02$ (backward analysis) | Non SRC-GC |

EGC: Early gastric cancer; LNM: Lymph node metastasis; SRC-GC: Signet ring cell-gastric cancer; PCC-GC: Poorly cohesive cells-gastric cancer; WMD: Well-and moderately-differentiated gastric cancer; PD: Poorly differentiated; MC: Mucinous cancer; NS: Non-significant.

after adjustment for stage, SRC histology was not independently associated with a worse prognosis[44]. These findings seem to be confirmed by several other studies that reported a worse prognosis in univariable analysis, but not in multivariable analysis after adjustment for tumor stage[6,56-58]. Critics, however, state that a posteriori adjustment by multivariable analysis results in an oversimplification of the issue. In the absence of any possibility for prospective randomization, some authors noted that a matched case-control analysis should be the methodological tool of choice to clarify this debate[59]. Piessen *et al*[35] confirmed that SRC histology entailed a worse stage-independent prognosis in patients with GC than other histological subtypes[35].

The underlying factors that may cause the discrepancy between the prognostic characteristics of early and advanced PCC-GC remain uncertain. This topic is even more complicated by the geographical differences and potential variability in the molecular tumor characteristics between Western and Eastern populations[60]. Within the group of GCs, early and advanced PCC-GC may represent two distinct entities, each with its own prognostic features[61].

### *Pre-therapeutic evaluation in PCC-GC*

A thorough anamnestic evaluation with emphasis on family history should be performed to detect clinical criteria for hereditary diffuse GC[62]. Because the tumoral spread in PCC-GC mainly occurs within the deeper tissue layers, mostly in the absence of any mucosal alterations, conventional endoscopy and superficial biopsies may miss the diagnosis. Repeated endoscopies should consequently be performed with deep biopsies guided by endoscopic ultrasonography. A CT scan can give useful additional information by identifying areas of the stomach characterized by an increased wall thickness in the case of LP.

In light of the WHO criteria from 2000 for SRC-GC (*i.e.* more than 50% SRC), the overall reliability of pretherapeutic biopsies to predict specimen histology has been evaluated. Among 254 patients, the presence of SRC in routine pre-therapeutic endoscopic biopsies could accurately predict SRC histology

and its associated poor prognosis (Sensitivity: 88.1%, Specificity: 95.4%, Positive predictive value: 92.7%, Negative predictive value: 92.4%)[5]. Future studies evaluating the concordance between pretherapeutic biopsies and specimens in PCC-GC will have to be performed using the new WHO definition and the Verona consensus[12,16].

Positron emission tomography (PET) imaging using fluoro-2-deoxy-D-glucose (FDG) may be helpful to eliminate distant metastases in the case of advanced disease[63,64]. However, PCC-GC has proven to be associated with a lower PET sensitivity and a lower standard uptake value (SUV) than no PCC-GC, with a potential risk of false-negative results[65-67]. In addition, two studies suggested that a higher SUVmax was a predictive factor of poor prognosis in SRC histology[68,69].

Staging laparoscopy is currently recommended by the European Society for Medical Oncology (ESMO) for tumors ≥ stage Ib[70] and by the National Comprehensive Cancer Network (NCCN) for tumors ≥ T1b[71]. Several studies reported high rates of peritoneal carcinomatosis (5%-21%) discovered during surgical exploration after a standard workup, including CT scan in advanced PCC-GC or diffuse tumors[35,72-74]. In the PlASTIC-study, comparing staging laparoscopy and FDG-PET/CT in preoperative workup of locally AGC, treatment intent changed from curative to palliative in 73 patients (19%) after staging laparoscopy (detecting peritoneal or locally non-resectable disease) *vs* in 12 patients (3%) after FDG-PET/CT (detecting distant metastases)[74]. This risk was 1.5 to 3 times higher than in other tumors[35,74]. Staging laparoscopy has been consequently proposed as an essential tool for pretherapeutic evaluation of PCC-GC[75]. In addition to a complete and systematic exploration of the abdominal cavity, staging laparoscopy provides the possibility to perform a peritoneal lavage with cytology. A positive cytology classifies the disease as stage IV, necessitating a change in therapeutic strategy[30,76,77]. Alternative procedures such as laparo-endoscopic single-site surgery are currently being evaluated to optimize the detection of peritoneal disease. Even with standard staging laparoscopy, lesions on the mesenteric side of the small bowel are still frequently missed[78,79]. A small periumbilical incision to explore the small bowel by means of palpation may be helpful in advanced PCC-GC.

### *Curative treatment*

**Endoscopic resection:** An increasing amount of evidence has been gathered that endoscopic treatment using an endoscopic submucosal dissection could represent a valid option for non-ulcerated undifferentiated lesions, ≤ 2 cm in diameter, limited to the mucosa and without LVI[50,80-82]. Lesions in this category are currently excluded from the absolute indication by the JGCA recommendations due to the lack of sufficient evidence for long-term outcome. Still, they may in the future be included pending the results of the JCOG1009/1010 study[83]. For Western countries, the European Organisation for Research and Treatment of Cancer has defined the indications for endoscopic resection for EGC during the St. Gallen international consensus meeting. For diffuse EGC, gastrectomy is considered mandatory[84]. In the NCCN and ESMO guidelines, undifferentiated tumors (including PC-GC) are contra-indicated for endoscopic treatment[71].

**Surgery:** Multiple studies have demonstrated a higher risk of positive resection margins due to the specific infiltrative characteristics of PCC-GC and a higher risk of lymph node involvement[6,8,35]. Consequently, some surgical specificities should be proposed.

According to the JCGA, a proximal margin of 5 cm is recommended in cases of AGC with an infiltrative growth pattern (*i.e.* PCC-GC). A frozen section is advisable in case of doubt. For EGC, a gross resection margin of 2 cm should be respected[83]. A margin of 4 cm is recommended by the NCCN regardless of histological type[71]. According to the ESMO guidelines, a subtotal gastrectomy is indicated if a macroscopic proximal margin of 5 cm can be achieved. For diffuse GC and consequently PCC-GC, a margin of 8 cm should be respected. If not, a total gastrectomy is advised[70]. In the case of an antropyloric location of PCC-GC, a frozen section of the distal margin should be proposed since there is a significant risk of duodenal invasion due to submucosal and subserosal spreading of the tumor[40].

Neither JCGA nor ESMO, nor NCCN guidelines advocate a modification of the D2 Lymphadenectomy without systematic splenectomy for AGC in PCC-GC[70,71,83]. Only the guidelines of the Italian Research Group for Gastric Cancer recommend a D2+ lymphadenectomy (D2 + stations 8p, 12p/b, 13, station 14 v along the mesenteric vein and para-aortic lymph node station 16a2/16b1) for tumors classified as diffuse-type according to the Laurén classification and located in the distal two-thirds of the stomach[85]. Whether or not the extent of lymphadenectomy should be adapted to the higher potential of lymph node metastasis in PCC-GC is questionable and has so far not been investigated by any randomised controlled trial (RCT).

### *Impact of PCC-GC in peri-operative CT*

**In Western countries, before the FLOT era:** The added value of perioperative CT for GC has been demonstrated in two randomized trials[17,86,87]. Perioperative CT allows for an increased R0-resection rate, tumor- and lymph node downstaging and significant improvement in OS. In a post hoc analysis of the MAGIC trial, no statistically significant difference in pathological response rate could be identified between the different histological types according to the Lauren classification. Of note, only 18 % of included patients presented with diffuse-type GC and SRC-presence was not specifically evaluated[88].

Other studies, mainly retrospective, have suggested that Laurén diffuse-type GC and SRC-GC specifically were less chemosensitive than other histological subtypes[8,89-92]. In a large multicentric retrospective cohort study among 1050 patients with SRC-GC defined as tumors with > 50% SRC, Messager *et al*[92] found that perioperative CT (ECF or 5FU/Cisplatin) did not result in tumor- or lymph node downstaging, nor did it entail any benefit in terms of R0 resection[92]. Perioperative administration of CT was even identified as an independent factor of poor prognosis in the SRC-GC group (HR = 1.4, 95%CI: 1.1-1.9). Several hypotheses could account for these findings: (1) Innate chemoresistance of SRC-GC; (2) Disease progression during neoadjuvant CT; or (3) Toxicity resulting in relative immunodepression with subsequent facilitation of disease progression[93]. The results found by Messager *et al*[92] highlighted the urgent need for a randomized controlled trial dedicated to identifying optimal therapeutic strategies in the management of SRC-GC. In this context, the phase II/III PRODIGE 19 randomized controlled trial was designed to evaluate whether upfront surgery with adjuvant CT (6 cycles of ECF regimen) would provide a survival benefit compared to perioperative CT (perioperative ECF regimen) in patients with stage Ib-III SRC-GC[94]. The phase II study met its primary endpoint of > 26 mo of 2-year OS in the upfront surgery + adjuvant CT arm. However, 2-year OS rates were 60% in the perioperative arm *vs* 53.5% in the upfront surgery arm, with a median survival of 39 mo *vs* 28 mo respectively (exploratory HR = 0.71, 95%CI: 0.40-2.64). Subsequently, phase III was not launched[18].

Another retrospective study, including 235 patients with SRC-GC, defined as tumors with any percentage of SRC, suggested that SRC-GC had a lower clinical (21.1% *vs* 33.7%, $P = 0.001$) and histopathological (16.3% *vs* 28.9%, $P < 0.001$) response rate to neoadjuvant CT than non-SRC-GC[58]. However, within the cohort of SRC-GC patients that displayed a clinical or histopathological response, the outcome was favorable which led to the conclusion that perioperative CT should not be abandoned for SRC-GC. In the same study, the addition of a taxane-based CT regimen did not have any positive influence on prognosis in SRC-GC patients.

**In Western countries in the FLOT era:** Taxane-based CT regimens and more specifically the FLOT regimen, have in recent years proven their added value in the peri-operative treatment of GC[17,95,96]. Results concerning the benefit of the FLOT regimen in the treatment of PCC-GC remain, however, controversial: Homan *et al*[97] found that the pathological complete response rate to FLOT-therapy in intestinal-type GC was higher as compared to diffuse/mixed type GC (30.8% *vs* 0%, $P < 0.05$)[97]. Likewise, in the phase II NeoFLOT study, it was demonstrated that when considering near-complete responders (< 10% residual tumor), 85% had an intestinal-type GC in contrast to only 10% and 5% of these patients that exhibited a diffuse and mixed type tumor, respectively[98]. However, the results from the FLOT4 trial demonstrated a beneficiary treatment effect of the FLOT regimen *vs* ECF regardless of histological type and presence of an SRC component[17]. The definition of SRC in the FLOT trial, was the presence of any SRC in the pathological report, which does not correlate with the recent definition of PCC-GC[12]. The beneficial effect on OS was more pronounced in the SRC-GC than in diffuse GC. These findings are difficult to analyze in the absence of pathological reassessment of the pathological specimen. However, this was an additional argument not to launch the phase III of PRODIGE 19 trial.

**In Eastern countries:** In Eastern countries where primary surgery followed by adjuvant CT is the standard treatment, three trials evaluating preoperative CT dedicated to LP have been identified[99-102]. The first study with S1 (JCOG02) did not reach its expected survival rate and consequently, no phase III study was performed; the second study with S1+ cisplatin showed interesting tumor response (JOG0210) but did not show any superiority of the neoadjuvant arm in the long term in the phase III (JCOG0501).

**Impact of PCC-GC on adjuvant CT:** In Eastern countries, adjuvant CT is the preferred therapeutic strategy in GC based on two major trials: The ACTS-GC (Adjuvant CT Trial of TS-1 for GC) trial and the CLASSIC study with CAPOX[103,104]. There was no subgroup analysis based on diffuse or SRC-GC type in both trials. However, in the ACTS-GC trial, the S-1 setting had a significant favorable HR for death in the undifferentiated group (that includes PCC-GC) compared to surgery alone, contrary to the differentiated group, where the effect was not significant[103]. After 5 years, the results were maintained in both subgroups[105]. A retrospective study suggested no tumor response of SRC-GC to either oxaliplatin or docetaxel adjuvant-based CT. In contrast, the mixed SRC-GC group responded to both regimens with even more improved survival with the docetaxel-based regimen[90]. Although the exact definition of SRC-GC and mixed SRC-GC was not mentioned in this study, it supports the fact that PCC-GC could behave differently according to the percentage of SRC and underlines the potential benefit of taxane-based CT in PCC-GC.

**Impact of PCC-GC on adjuvant radiotherapy:** Several RCT's evaluated the potential benefit of adjuvant CRT in GC (Intergroup 0116, ARTIST, ARTIST2, CRITICS)[106-110]. They failed to show a favorable outcome in PCC or diffuse GC subgroups. An analysis of the SEER database using a propensity score however showed favorable outcome of adjuvant RT in patients with diffuse-type GC (median survival time: 30 mo with adjuvant RT *vs* 18 mo without adjuvant RT, $P < 0.001$, HR: 0.75, $P < 0.001$). A major bias was the absence of data regarding the use of CT[111].

**Impact of PCC-GC on neo adjuvant chemoradiotherapy:** Phase III trials evaluating RT or preoperative CRT in GC, excluding the gastroesophageal junction (GEJ), are scarce and small[112-114]. Several phase II trials showed encouraging results in tumor response and survival but this type of strategy has so far been limited by the related toxicity[115-119]. At least two trials are ongoing: TOPGEAR[120] and CRITICS-II[121] with a planned subgroup analysis according to histological type in the CRITICS-II study.

A study analyzing 107 localized GA ($n$ = 45 non-SRC-GC and $n$ = 62 SRC-GC) treated with preoperative CRT showed that the presence of SRC was associated with a lower rate of pCR (11% *vs* 36%, $P$ = 0.004) which remained significant even with a low percentage of SRC (1%–10%; $P$ = 0.014). The higher the fraction of SRC, the lower the probability of pCR ($P$ = 0.03). Poorly differentiated and SRCC led to shorter OS ($P$ = 0.046 and $P$ = 0.038, respectively)[89].

### *Impact of PCC-GC in intraperitoneal chemotherapy combined with surgery*

**Preventive setting:** The high failure rate of surgical curative therapy for GC and PCC-GC in particular, is mainly due to a high rate of peritoneal recurrence. In this context, a strategy of preventive intraperitoneal chemotherapy (IPC) during the surgical intervention has been hypothesized. Two meta-analyses (including mostly Asian studies) showed a clear benefit of preventive IPC in terms of survival[122,123]. However, no subgroup analysis for PCC-GC was performed. The phase III GASTRICHIP trial (NCT01882933) is currently evaluating the role of oxaliplatin-based HIPEC in addition to curative gastrectomy in patients with GC or Siewert II/III cardia adenocarcinoma with either serosal infiltration, LN positivity, positive peritoneal cytology or perforated tumor. Stratification according to the presence of SRC on pretherapeutic biopsies, has been anticipated[124]. The ongoing PREVENT trial (FLOT-9) (NCT04447352) is a multicenter, randomized, controlled, open-label study including a total of 200 patients with localized and locally advanced non-metastatic diffuse or mixed type (Laurens's classification) adenocarcinoma of the stomach and Type II/III esogastric junction tumors. Patients undergo perioperative FLOT and are randomized between curative gastrectomy alone and curative gastrectomy + intra operative cisplatin-based HIPEC[125]. In Japan, the PHOENIX-GC2 Trial will evaluate the impact of IPC as adjuvant or perioperative CT for patients with type 4 scirrhous GC in addition to S1 CT [126].

**Curative setting:** In a curative setting, cytoreductive surgery (CRS) plus HIPEC has been strongly recommended for AGC by a panel of international experts[127,128]. However, controversy concerning this topic remains, with further high-quality evidence being expected to confirm the value of this treatment strategy, which could be of particular interest for PCC-GC.

At present, no published RCT has compared CRS + HIPEC *vs* CT alone. Two ongoing randomized phase III trials evaluate the role of surgery in limited- metastatic adenocarcinoma of the stomach or esophagogastric junction in patients responding to CT and will include patients with peritoneal carcinomatosis[129,130]. In the RENAISSANCE trial no stratification based on histological type has been anticipated and HIPEC is not described in the protocol (NCT02578368)[129].In the SURGIGAST trial, stratification based on histological type (PCC-GC on biopsy) has been anticipated (NCT03042169)[130].

In the multicenter, open-label, phase III PERISCOPE II trial, patients with peritoneal metastasis are currently randomized between CT alone *vs* CRS + HIPEC with CT. Study completion is expected by October 2022[131]. Stratification based on the main histological subtype (diffuse *vs* intestinal) has been anticipated.

Based upon the available evidence, it is presumed that for GC in general, only patients with a peritoneal cancer index (PCI) < 12, who display a clinical response after neoadjuvant CT and in whom no diffuse bowel involvement is found, may benefit from the added value of CRS + HIPEC[132,133]. For PCC-GC, little to no specific selection criteria have been proposed so far. In a retrospective study on 89 patients, Chia *et al*[134] demonstrated that after treatment with CRS + HIPEC, non-PCC-GC patients had a better OS (21.8 mo *vs* 13.2 mo, $P$ = 0.0214) compared to PCC-GC patients. The authors suggested that if complete CRS was achievable in patients with a PCI < 7, the presence of an SRC component should not be considered as a contra-indication for CRS + HIPEC[134].

In 2018, Bonnot *et al*[135] published the results from the large multicenter retrospective CYTO-CHIP study, which evaluated the survival results of CRS compared to CRS + HIPEC in patients with AGC with peritoneal involvement[135]. Only patients with a complete CRS (CC-0 or CC-1) were included in the study. After propensity scored weighting, this study showed that CRS + HIPEC was associated with an increased OS and the potential of disease eradication compared to CRS alone. Subgroup analysis confirmed the superiority of CRS + HIPEC in patients with PCC-GC defined according to WHO classification[11]. An ancillary study recently published showed that PCC-GC was associated with poorer OS (HR: 0.43, $P$ = 0.003), as were pN3, PCI, and resection with a completeness of cytoreduction score of 1, whereas HIPEC was associated with improved OS (HR: 0.52; $P$ < 0.001). The benefit of CRS-HIPEC over CRS alone was consistent, irrespective of histology, with a median OS of 16.7 mo *vs* 11.3 mo (HR: 0.60, $P$ = 0.018) in the PCC-GC group, and 34.5 mo *vs* 14.3 mo (HR: 0.43, $P$ = 0.003) in the non-PCC-GC group. Non PCC-GC and HIPEC were independently associated with improved recurrence-free survival and fewer peritoneal recurrences. In patients who underwent HIPEC, PCI values < 7 and < 13 were

predictive of OS in PCC-GC and non PCC-GC populations, respectively[136]. Consequently, those patients should be well-selected to avoid the excess morbidity rate associated with an unnecessary exploratory laparotomy[137].

### *Role of PCC-GC on non-curative treatments*

**CT:** Several studies demonstrated that SRC-GC had different infiltrative and metastatic mechanisms than non-SRC-CG. It lacked free ribosomes but were rich in lysosomes and mucus impeding anticancer drugs from getting to the cell[20,138]. In a metastatic setting, there are few data concerning the chemosensitivity of PCC-GC. Rougier *et al*[139] reported among 87 patients with metastatic or recurrent tumor ($n$ = 57) or with locally AGC ($n$ = 30) a significantly poorer response rate of CT using infusional 5-FU and cisplatinum for linitis plastic or SRC histology ($P$ = 0.003 and $P$ = 0.16, respectively)[139].

A retrospective analysis of the FLAGS trial suggested that survival was improved among patients with advanced diffuse GC treated with S-1 and cisplatin compared to 5-FU and cisplatin[140]. A dedicated phase III trial compared both regimens in patients with metastatic diffuse gastric and GEJ adenocarcinoma previously untreated[141]. However, both regimens were similar in efficacy and safety and the primary endpoint was not met. A study of the AGEO evaluated the place of docetaxel added to 5-FU, leucovorin and oxaliplatin (TEFOX) as first-line treatment in 65 patients with metastatic or locally advanced non-resectable gastric or GEJ SRC-GC including 17 LP. This regimen gave an interesting response rate of 66% with an OS of 14.3 mo. Interestingly, 26 patients (40%) initially unresectable had secondary resection ($n$ = 24) or radiotherapy ($n$ = 2) with curative intent[142].

**PIPAC:** PIPAC is a recently developed promising technique that allows for homogeneous loco-regional application of intraperitoneal CT at lower doses than achievable in conventional HIPEC[143]. This technique could offer a valuable alternative for patients with unresectable peritoneal disease from GC and with PCI-scores that are considered as too high for CRS + HIPEC (PCI > 7 or 12 depending on histological type). Several retrospective studies have evaluated the feasibility of this technique on patients with unresectable peritoneal metastasis from GC. The majority of patients included in these studies were affected by an SRC histology and the results show that PIPAC treatment (with low-dose cisplatin + doxorubicin) is associated with improved survival, without compromising the quality of life [143-145]. Further results from the randomized controlled multicenter phase II PIPAC EstoK 01 trial evaluated the interest of PIPAC in addition to intravenous CT and are awaited[143].

**Targeted drugs in gastric SRCC:** Due to some specific oncogenic pathways in GC, the efficacy of several targeted agents has been tested in recent trials, in which SRC histology has only rarely been the subject of subanalysis. On the other hand, diffuse type GC has been evaluated frequently within these trials.

**Human epidermal growth factor receptor 2 targeting agents:** The incidence of human epidermal growth factor receptor 2 (HER2) amplification in GC ranges from 12% to 22.1%. It is more often noted in intestinal GC than diffuse-type GC and characterized by a more frequent location in the proximal stomach and gastroesophageal junction[146-150]. Although still controversial, HER2 positive status is, in general, associated with a poor outcome and more aggressive disease[147,149,150]. Some authors found that the unfavorable prognostic value of HER2 positivity was present in intestinal-type GC, but not in diffuse-type GC[151,152]. In PCC-GC, the diagnosis of HER2 status can be somewhat troublesome due to the presence of a marginalized cytoplasm and nucleus, entailing a frequent misinterpretation of intense, non-specific staining[153-155]. The phase III ToGa trial demonstrated the added value of the humanized monoclonal antibody against HER2 (Trastuzumab) in combination with CT (capecitabine or 5-FU and cisplatin) compared to CT alone in HER2-positive AGC[156]. Of note, a sub-group analysis among patients with a diffuse-type tumor showed no benefit of trastuzumab, although the number of patients in this sub-analysis was quite low. A Korean study found resistance to trastuzumab of more than 50% among 13 patients with SRC-GC who were HER2 positive, with a low HER2 amplification index being identified as an independent molecular predictor for trastuzumab resistance in a multivariate analysis[157]. Despite these findings, it remains recommended to routinely test all patients with GC for HER2 amplification, regardless of the histological type[146,156,158]. Future studies are required to investigate more profoundly a potential benefit of trastuzumab in PCC-GC.

**Anti-angiogenic agents:** The randomized phase III AVAGAST trial evaluated the effect of bevacizumab [a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody] in combination with CT (fluoropyrimidine-cisplatin) as first-line therapy in AGC. Although AVAGAST did not reach its primary objective (OS of 10.1 mo in the placebo arm *vs* 12.1 mo in the bevacizumab arm, $P$ = 0.1002), the addition of bevacizumab to CT was found to be associated with a significant increase in progression-free survival (PFS) and overall response rate[159]. An additional analysis according to disease subtype, suggested a benefit of bevacizumab in a subset of non-Asian patients with the diffuse histologic type (HR = 0.68; 95%CI: 0.48-0.97)[159]. The phase III REGARDS trial compared ramucirumab (an anti-VEGF-R2 antibody) *vs* best supportive care after first-line platinum-containing or fluoropyrimidine-containing CT in AGC or gastro-esophageal junction adenocarcinoma. Ramucirumab provided a significant benefit in terms of OS (5.2 mo *vs* 3.8 mo, HR = 0.78, 95%CI: 0.603-0.998)[160]. In subgroup analysis, a significant benefit was found for diffuse-type GC (HR = 0.56; 95%CI: 0.36-0.85), but not for

the intestinal-type (HR = 1.009, 95%CI: 0.583-1.745), suggesting a higher sensitivity to anti-angiogenics. Conversely, the RAINBOW trial showed that for ramucirumab in combination with paclitaxel in a second-line treatment, the OS benefit concerned only the intestinal histological subtype [HR: 0.705 (0.534–0.932)][161]. Supplemental data are needed to establish the role of anti-angiogenic targeted therapies in patients with diffuse-type GC. Currently, no data concerning the role of anti-angiogenic therapies in the therapy of PCC-GC are available.

**Anti-epidermal growth factor receptor:** Epidermal growth factor receptor (EGFR) expression has been identified as an independent predictor of poor prognosis in patients with PCC-GC compared to non-PCC-GC patients[162]. Data from the EXPAND and REAL3 trials have suggested no additional benefit of anti-EGFR treatment in combination with CT for AGC[163,164]. In a subgroup analysis of the EXPAND trial in function of the histological subtype, it was even found that anti-EGFR could be harmful in diffuse-type tumors (HR for OS: 1.44, 95%CI: 1.01-2.03)[163].

### *Mammalian target of rapamycin inhibitors*

Since phospho-mammalian target of rapamycin (mTOR) is expressed in 60% of intestinal and 64% of diffuse-type GC, mTOR inhibitors were considered an interesting therapeutic option from a biological point of view[165]. However, results from the phase III GRANITE-1 trial showed no benefit of everolimus (an oral mTOR-inhibitor) on OS compared to best supportive care for previously treated AGC[166]. In a subgroup analysis, no benefit in diffuse-type GC was found either.

### *CLDN18.2 antibody (zoltemuximab)*

In advanced gastric/gastro-esophageal junction and esophageal adenocarcinoma patients expressing CLDN18.2, adding zolbetuximab to first-line EOX provided longer PFS and OS *vs* EOX alone in a phase 2 trial[167]. Interestingly, the vast majority of these populations had diffuse- or mixed type GC. Zolbetuximab is being evaluated in phase III studies based on clinical benefits observed in the overall population and in patients with moderate-to-strong CLDN18.2 expression in > 70% of tumor cells.

### *Immunotherapy*

Among new treatment strategies for GC, immunotherapy, and more specifically, PD-L1 inhibitors have proven to be the most promising. PD-L1 is expressed in 30% to 63% of GC[168,169]. The results of the CheckMate 649 study demonstrated the superiority of nivolumab in combination with CT compared to CT alone. In a study population of patients with HER2 negative, previously untreated, unresectable advanced or metastatic GC or gastro-esophageal junction cancer, nivolumab in combination with CT (XELOX or FOLFOX) resulted in significantly improved OS and PFS *vs* CT in patients whose tumors expressed a PD-L1 combined positive score (CPS) ≥ 5 (HR for OS = 0.71, 98.4%CI: 0.59–0.86 and HR for PFS = 0.68, 98%CI: 0.56–0.81). This survival benefit was also observed in patients with a PD-L1 CPS ≥ 1 and in the all-randomized population[170]. The rate of patients with SRC-GC or diffuse tumors was close between patients with a CPS ≥ 5 and the overall population[170]. However, other studies found that in SRC histology, PD-L1 CPS > 1 was significantly less observed[171]. The question remains how the recent findings of the CheckMate 649 trial could be applied to PCC-GC. A group of specifically selected PCC-GC patients with S-I may benefit from immunotherapy. However, Hirotsu *et al*[172] reported that PCC-GC exhibits high MSI at low frequencies[172].

## CONCLUSION

In contrast to GC in general, the relative incidence of PCC-GC has risen over the past few decades. PCC-GC represents a distinct pathological entity within the GC spectrum, characterized by specific epidemiological and clinical features, including younger age at presentation and a significantly worse prognosis, primarily due to peritoneal dissemination early in the disease. In light of these distinct features, the recently redefined pathological definition of PCC-GC by the WHO and the European chapter of IGCA will facilitate methodological standardization in future studies which in turn will help to identify which therapeutic strategies for GC in general apply to PCC-GC. We believe that the updated definition will help standardize future research concerning the prognostic results of SRC-ECG in Western populations and evaluate the correlation between pre-therapeutic biopsies and the final pathology result. Concerning the pre-therapeutic evaluation, the infiltrative growth pattern of PCC-GC along with early peritoneal dissemination justifies the use of repeat endoscopies with deep biopsies, CT-graphic imaging as well as systematic staging laparoscopy with peritoneal lavage. Since correct PCI determination is essential for therapeutic management, a small incision with palpation of the entire small bowel should be considered. Surgery is considered the mainstay of curative treatment for PCC-AGC. The role of the extent of the lymphadenectomy however in PCC-AGC should be evaluated in future studies. For PCC-EGC, no endoscopic treatment is currently advocated. The added value of peri-operative CT for PCC-GC with FLOT regimen is probable but should be further confirmed using histological reassessment. No

role of adjuvant radiotherapy has been demonstrated in PCC-GC. In the case of peritoneal disease, IPC using HIPEC or PIPAC offer a valuable treatment option on the condition that patients are well selected. To what extent the promising results of immunotherapy could apply to PCC-GC needs to be confirmed in future studies. PCC-GC in general requires a highly individualized diagnostic and therapeutic approach to optimize the inherent poor prognosis of this disease in the future. Molecular and genetic differentiation will be important in offering a patient-tailored therapeutic strategy.

## FOOTNOTES

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*REVIEW*

# Lymph node regression grading of locally advanced rectal cancer treated with neoadjuvant chemoradiotherapy

Lei He, Juan Xiao, Ping Zheng, Lei Zhong, Qian Peng

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## Abstract

Neoadjuvant chemoradiotherapy (nCRT) and total rectal mesenteric excision are the main standards of treatment for locally advanced rectal cancer (LARC). Lymph node regression grade (LRG) is an indicator of prognosis and response to preoperative nCRT based on postsurgical metastatic lymph node pathology. Common histopathological findings in metastatic lymph nodes after nCRT include necrosis, hemorrhage, nodular fibrosis, foamy histiocytes, cystic cell reactions, areas of hyalinosis, residual cancer cells, and pools of mucin. A number of LRG systems designed to classify the amount of lymph node regression after nCRT is mainly concerned with the relationship between residual cancer cells and regressive fibrosis and with estimating the number of lymph nodes existing with residual cancer cells. LRG offers significant prognostic information, and in most cases, LRG after nCRT correlates with patient outcomes. In this review, we describe the systematic classification of LRG after nCRT, patient prognosis, the correlation with tumor regression grade, and the typical histopathological findings of lymph nodes. This work may serve as a reference to help predict the clinical complete response and determine lymph node regression in patients based on preservation strategies, allowing for the formulation of more accurate

treatment strategies for LARC patients, which has important clinical significance and scientific value.

**Key Words:** Lymph node regression grade; Histopathological; Rectal cancer; Chemoradiotherapy; Treatment response; Neoadjuvant therapy



**Core Tip:** Studies on lymph node regression grading after neoadjuvant chemoradiotherapy (nCRT) for rectal cancer are limited but serve clinicians for assessing the lymph node response to treatment based on the efficacy of the primary tumor after preoperative nCRT, providing guidance in formulating more accurate surgical or therapeutic strategies for the next stage of patient management and in determining patient prognosis. We discuss its histopathology, prognosis, correlation with tumor regression grading, and clinical applications and prospects.

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## INTRODUCTION

Neoadjuvant chemoradiotherapy (nCRT) and total rectal mesenteric excision (TME) are the main standards of treatment for locally advanced rectal cancer (LARC)[1-5]. The response of lymph nodes (LNs) to neoadjuvant therapy is reflective of the possibility of regression, similar to the main tumor body. LN regression grade (LRG) is based on postsurgical metastatic LN pathology and is an indicator of the response to preoperative nCRT and patient prognosis[6,7]. The status of tumor-draining LNs (TDLN) has been considered the most significant indicator of prognosis in patients with LARC, and the number of LN metastases is currently the only measure of ypN staging[8-12]. Several studies have demonstrated that nCRT decreases the detection of positive LNs and the total number of positive LNs, thereby affecting the accuracy of the patient's ypN stage[13-16]. In addition, the majority of studies and applications focused on tumor regression have centered on the primary tumor, while the impact of LRG on tumor regression and prognosis has not been fully explored. nCRT treatment based on well-predicted and assessed regression is beneficial for individualized clinical decision making and multidisciplinary diagnosis and treatment.

In the following study, we present the characteristics and histopathological findings of LNs observed as a result of nCRT, summarize the concepts for LRG, introduce some LRG staging systems for rectal cancer, describe the patient prognosis and the relationship with tumor regression grade (TRG), explore the limitations and critical issues, and discuss the clinical impact of LRG on rectal cancer.

## LITERATURE SEARCH

The main purpose of the present review is to identify the latest studies relating to LRG after neoadjuvant radiotherapy in patients with LARC and to compare their main elements. We performed a database search on PubMed and selected papers published in English between January 2000 and January 2022. PubMed was last accessed on 2 February 2022. The following keywords and terms were used. ("rectal OR rectum") AND ("carcinoma OR neoplasm OR malignant OR malignancy OR cancer") AND ("lymph node grade OR LRG OR lymph node grading") AND ("chemoradiotherapy OR therapy OR chemotherapy OR radiotherapy") AND ((2000/1/1[PDAT]: 2022/1/31[PDAT])), to retrieve relevant articles. All articles are in English. Meta-analyses, reviews, and other articles containing nonoriginal data were excluded from our review. All articles retrieved were selected and screened by three independent authors. Related data on the articles were retrieved by a standardized data collection method. A flow chart of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses is shown in Figure 1.

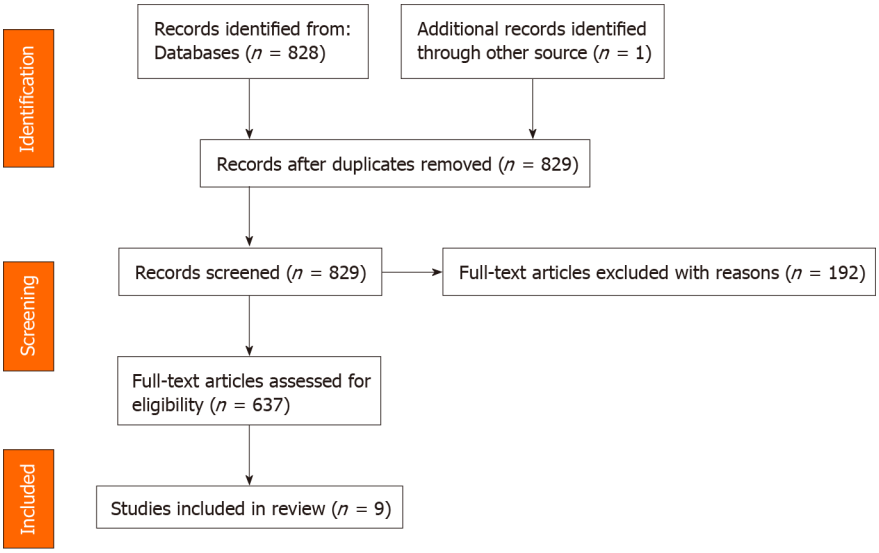


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**Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (2020) flow diagram.**

## HISTOPATHOLOGICAL DISCOVERIES FOLLOWING NCRT

The primary purpose of the pathologic procedure was the macrosurvey of the resected tumor and LN specimens[17]. Operative specimens were detached from the anterior wall with a fixation for 24 h in 40 g/L formaldehyde. External surfaces of the specimen were stained with black ink for the easy identification of surgical margins. Serial sections of the entire tumor and attached mesentery were performed at 3- to 4-mm intervals vertically along the longitudinal axis of the rectum. To assess the LNs around the rectum, the interrectal fat was removed after tumor sampling. All LNs were identified by palpation and removed using scissors and a scalpel, followed by histological examination[18].

Based on the histology, tumor regression after nCRT essentially constitutes subacute to subchronic inflammation that follows the cytotoxic effects occurring weeks before. In the majority of cases, the tumor was removed sometime after completing the final cycle of preoperative chemotherapy[17].

At the cellular level, in the case of complete LN regression, the malignant cells were eradicated through cytotoxic therapy and/or subsequently by the inflammatory response, and the LNs were displaced by fibrous tissue. In contrast, there was a high probability of an abundance of residual tumor cells in the LNs, such as small single cells or tumor cell clusters. Microscopic analysis of metastatic disease was performed on all dissected LNs[19]. The following modes of tumor regression could be observed: Necrosis, hemorrhage, nodular fibrosis, foamy histiocytes, cystic cell reaction, areas of hyalinosis, residual cancer cells, and pools of mucin (Figure 2)[20,21]. Fernández-Aceñero *et al*[21] analyzed the potential prognostic effects of those response modes, such as cystic cell reaction and mucus pool, on disease-free survival (DFS) and disease-specific survival (DSS) and found no significant correlation between survival and response. In addition, several other LN markers have prognostic significance. For instance, mounting evidence suggests that extracapsular LN involvement is one prognostic contributor to recurrence and poor prognosis in malignancies of the gastrointestinal tract[22,23]. The presence or absence of fibrosis is usually used to differentiate nonmetastatic LNs from metastatic LNs that have completely regressed[19].

However, histopathological assessments have several limitations. First, the number of patients with stage ypN0 disease downgraded to only microscopic LN involvement is difficult to assess. Second, patients receiving nCRT had fewer LNs retrieved than those who underwent only radical surgery. After nCRT, fibrosis in the metastatic LNs is not as pronounced as in the primary tumor. Normal lymphocytes still occupied most LNs, and only fibrosis occurred around metastatic tumor cells. However, the changes in normal lymphocytes after radiotherapy were uncertain, with most showing no response and some fibrosis, making it much more difficult for pathologists to distinguish normal LNs from completely regressed LNs, especially when only a small number of metastatic tumor cells were present. Therefore, only some LRG1 patients were in complete remission after nCRT, while others had normal LNs, so pathologists could not assess whether the small fibrotic tissue lesion was normal LN or a metastatic LN before treatment. Finally, pathologists cannot distinguish patients with fibrosis-free LNs from those with residual ypN0 tumors as complete responders and non-responders. Nevertheless, we ought to recognize that a complete response is not a safe assumption among patients with clinical LN+ on magnetic resonance imaging with no pathological abnormalities. Does the absence of fibrosis among the LN imply that no tumor cells were present before nCRT was performed, or does the presence of fibrosis among the LN imply that tumor cells were once present? These questions should be invest-

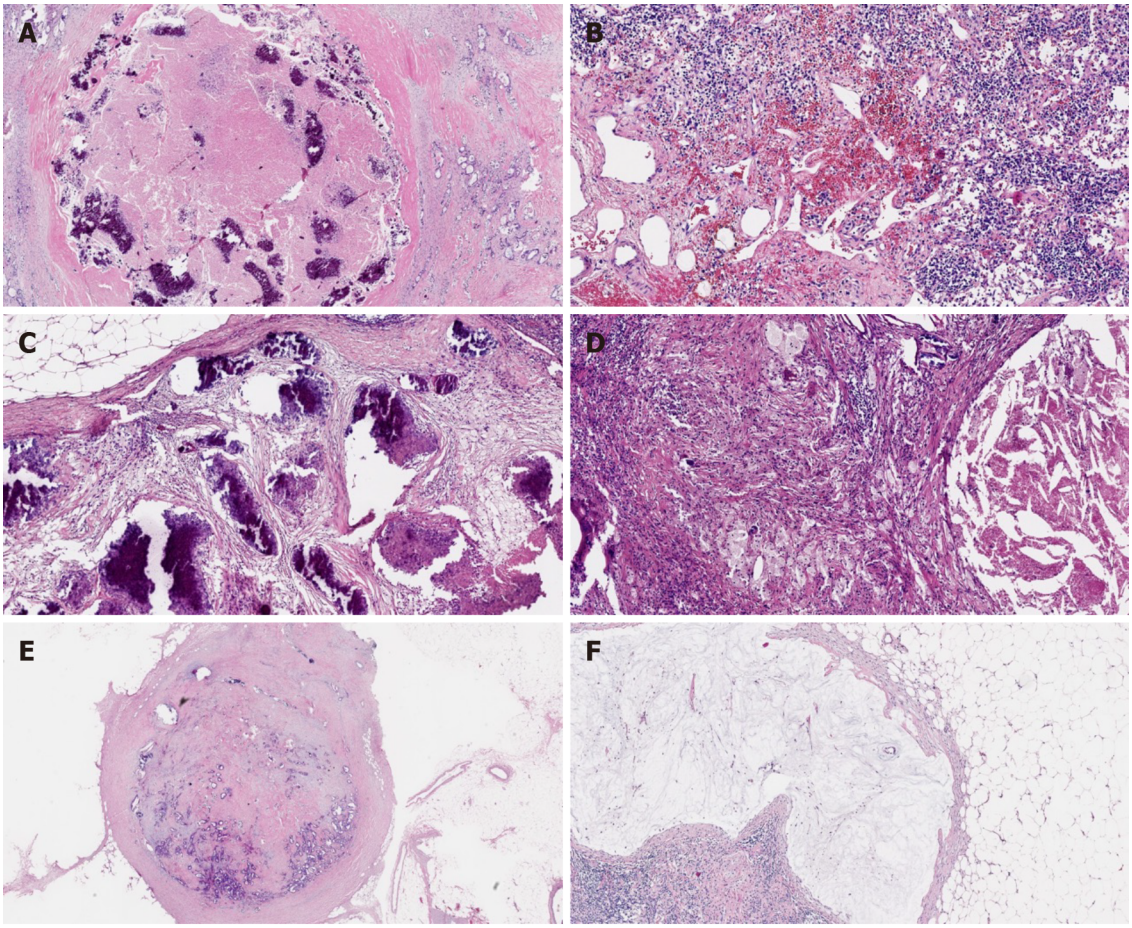


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**Figure 2 Example of modes of lymph node tumor regression.** A: Necrosis; B: Hemorrhage, nodular; C: Fibrosis; D: Foamy histiocytes; E: Residual cancer cells; F: Pools of mucin.

igated in future studies.

## CLASSIFICATION OF LRG

Numerous publications have shown that TRG is significantly relevant to the assessment of patient outcomes[13,24] and is an essential prognostic indicator for patients with LARC[25-27]. LRG, like TRG, is an assessment of local metastatic LN treatment response indicators for nCRT based on postoperative patient histopathology[9,28,29]. When classifying the degree of LN regression, the following two aspects should be assessed: the relationship between residual cancer cells and regressive fibrosis, the basis of which is usually described, and the number of LNs with residual cancer cells, which is usually expressed as a percentage (%) (Figure 3).

Relevant studies have documented that residual tumor cells may still be present in local LNs despite complete regression of the primary disease[30]. In some studies[31], this occurred in up to 17% of cases, especially when a watch-and-wait strategy after nCRT was chosen, likely leading to recurrence and treatment failure. Therefore, pathologic evaluation of LNs in patients undergoing surgery after nCRT can contribute to an accurate determination of the clinical stage of the tumor and the metastatic LN response to nCRT (Table 1).

### *Caricato et al[18]*

In 2007, Caricato *et al*[18] retrospectively analyzed colorectal LNs in 35 patients undergoing preoperative CRT with LARC and reported, for the first time, the tissue effects of preoperative CRT on colorectal LNs and defined the grade of LN regression as follows: LRG1 for the absence of histologically identifiable residual cancer and fibrosis extending through the different areas of the LN; LRG2 for near-complete pathologic response (pCR); LRG3 for the presence of residual cancer cells with evident fibrosis; LRG4 for poor response; and LRG5 for nodal metastasis with the absence of regressive changes. It was also concluded that LRG was significantly correlated with TRG in primary tumors. However, this study had a small sample size, and no follow-up was performed clinically, so the prognosis of patients

**Table 1 Examples for lymph node regression grading systems**

| Descriptive | Caricato *et al*[18] | Mirbagheri *et al*[28] | Beppu *et al*[32] | Lee *et al*[34] | Sun *et al*[35] | Cui *et al*[38] |
|---|---|---|---|---|---|---|
| Negative/normal | LRG1 | LRG0 | - | pLRG0 | LRG0 | LRG0 |
| | Absence of histologically identifiable residual cancer and fibrosis extending through the different areas of the lymph node | Normal lymph nodes | - | LN-preserving normal nodal architecture without evidence of cancer cells or fibrosis was scored | Normal lymph node architecture without evidence of regression or cancer cells | Negative lymph node |
| Complete | LRG2 | LRG1 | LRG3 | pLRG1 | LRG1 | LRG1 |
| | Near complete pathologic response (pCR) | 100% fibrosis, no residual cancer | Total regression. No cancer cells, single cells or small groups of cancer | LN with 100% fibrosis | 100% fibrosis | Complete regression with no residual tumor cells |
| Subtotal | LRG3 | LRG2 | LRG2 | pLRG2 | LRG2 | LRG2 |
| | Presence of residual cancer cells with evident fibrosis | 75%-100% fibrosis, 0-25% cancer | Good regression. Residual cancer outgrown by fibrosis | LN with < 25% cancer cells | < 25% remaining cancer cells | Rare residual tumor cells |
| Partial | LRG4 | LRG3 | LRG1 | pLRG3 | LRG3 | LRG3 |
| | Poor response | 50%-75% fibrosis, 25%-50% cancer | Minor regression. Fibrosis outgrown by cancer or no fibrosis with extensive residual cancer | Scattered glandular elements with fibrosis | 25%-50% scattered glandular elements with fibrosis | Fibrosis outgrown by residual tumor cells |
| No regression | LRG5 | LRG4 | - | pLRG4 | LRG4 | LRG4 |
| | Nodal metastasis with absence of regressive changes | 25%-50% fibrosis, 50%-75% cancer | - | LN with > 50% cancer cells | > 50% viable cancer cells | Residual tumor cell outgrown by fibrosis |
| | | LRG5 | | pLRG5 | LRG5 | LRG5 |
| | | 0-25% fibrosis, 75%-100% cancer | - | Complete replacement with cancer cells | Complete replacement with cancer cells | Absence of regression with no fibrosis |

LRG: Lymph node regression grade; pCR: Complete pathologic response; LN: Lymph node.

with LRG was not investigated further.

### *Mirbagheri et al[28]*

In 2014, Mirbagheri *et al*[28] retrospectively analyzed clinical data from 190 patients who had LARC and received nCRT and found that LRG, similar to the TRG standard, could be used as an influencing factor for tumor recurrence. They also proposed a TRG-like LRG scoring system as follows for LRG0 for normal LNs; LRG1 for 100% fibrosis, no residual cancer; LRG2 for 75%-100% fibrosis, 0-25% cancer; LRG3 for 50%-75% fibrosis, 25%-50% cancer; LRG4 for 25%-50% fibrosis, 50%-75% cancer; and LRG5 for 0-25% fibrosis, 75%-100% cancer (Figure 4). Their study results indicated that: (1) LVI ($P$ = 0.029), tumors in the middle of the rectum and higher TRG scores were correlated with higher LRG scores; and (2) LN regression was a major factor in the prediction of tumor recurrence, and lower LN regression scores were associated with an enhanced survival curve. Mirbagheri *et al*[28] also proposed not only the LRG score but, for the first time, LRG maximum (LRG-max) and LRG-sum (LRG-sum). Subsequent analysis of these parameters indicated significant associations with tumor prognosis. Further research has provided additional evidence supporting a significant association between these parameters and tumor prognosis.

**LRG-max:** Since the number of LNs varies in each specimen and different regression scores may be calculated for different LNs depending on their treatment response, total scores were determined according to the worst score for each patient (specimen). For example, if one specimen contains two LNs whose scores were 2 and 3, the LRG-max would be 3.

**LRG-sum:** This reflects the overall tumor burden of the specimen for all LNs. For example, if one specimen contains two LNs whose scores were 2 and 3, the LRG-sum would be 5.

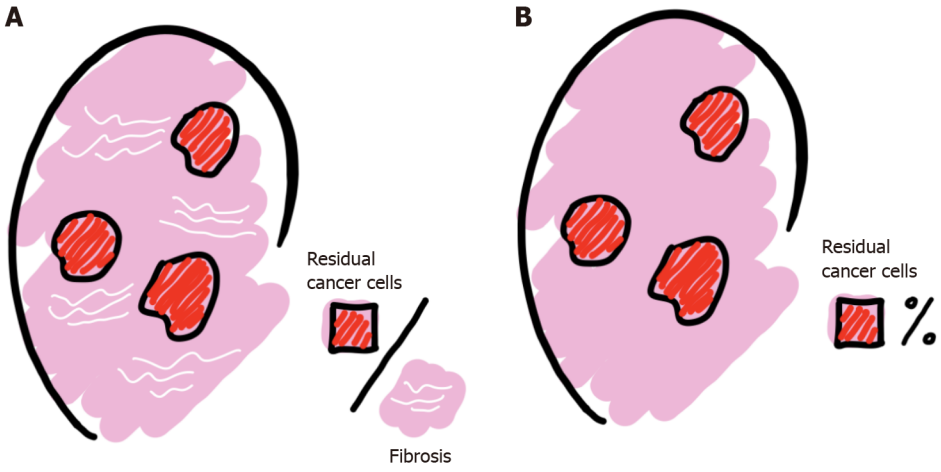


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**Figure 3 Principles of lymph node regression grade assessment.** A: Ratio of residual cancer cells to fibrosis; B: Percentage of residual cancer cells in the lymph nodes.

### *Beppu et al[32]*

In 2015, Beppu *et al*[32] retrospectively analyzed clinical data from 178 patients suffering from LARC who were treated with nCRT preoperatively, investigated the requirement of chemoradiotherapy for positive LNs that had completely regressed, and proposed the following LRG score set: LRG 1 for minor regression, fibrosis outgrown by cancer or no fibrosis with extensive residual cancer; LRG 2 for good regression, residual cancer outgrown by fibrosis; and LRG 3 for total regression, no cancer cells, single cells or small groups of cancer cells. The results showed that the primary tumor response to chemoradiotherapy was related to a positive nodal response. In contrast, for patients with a TRG of 3, the LRG score was associated with positive node size. The conclusion was also drawn that for the complete regression of positive nodes, the requirements were: (1) Degeneration of the primary tumor, with a TRG of 3; and (2) a diameter of < 6 mm for positive nodes.

The following year, Beppu *et al*'s group performed subgroup analyses with 229 patients receiving preoperative nCRT in T3 rectal cancer and showed that total positive node regression following preoperative chemoradiotherapy is the only factor independently associated with favorable overall survival[33]. Therefore, it was concluded that positive nodes showing complete regression after preoperative chemoradiotherapy could improve the prognosis of rectal cancer patients with positive LNs before treatment.

### *Lee et al[34]*

In 2019, Lee *et al*[34] evaluated postoperative LNs in 389 patients with rectal cancer treated with nCRT and then received radical resection. Lee defined the degree of regression of metastatic LNs after nCRT according to tumor cell percentage and degree of fibrosis and proposed a system for grading pathological LRG (pLRG) as follows: pLRG0 is a LN with normal nodal architecture, and without evidence of cancer cells or fibrosis, pLRG1 is a LN with 100% fibrosis, pLRG2 is a LN with < 25% cancer cells, pLRG3 has scattered glandular elements with fibrosis, pLRG4 is a LN with > 50% cancer cells, and pLRG5 is a complete replacement with cancer cells. The results showed that: (1) The LRG-sum distribution correlated significantly with the TRG in primary tumors; and (2) In the multivariate analysis, LRG-sum was the factor most related to RFS among the LN-related variables, in addition to ypT staging. According to the findings from this study, LRG was an influential factor for tumor prognosis in patients with rectal cancer following nCRT and surgical resection. It was shown that LRG was associated with a completely regressed primary tumor; accordingly, predicting LN regression based upon completely regressed primary tumors was beneficial, especially in patients considering a nonsurgical approach after nCRT.

### *Sun et al[35]*

In 2020, Sun *et al*[35] retrospectively analyzed the clinical data of 257 LARC patients receiving nCRT and proposed the following LRG scoring system: LRG 0, normal LN architecture without evidence of regression or cancer cells; LRG 1, 100% fibrosis; LRG 2, < 25% remaining cancer cells; LRG 3, 25–50% scattered glandular elements with fibrosis; LRG 4, > 50% viable cancer cells; and LRG 5, complete replacement with cancer cells. Sun *et al*[35] suggested that, to some extent, LRG was associated with the primary tumor response. In addition, it may help predict clinical complete remission (the cCR) and determine LN regression in patients based on preservation strategies (*e.g.*, local excision or an approach of "watch and wait"[36,37]. Furthermore, higher LRG scores were correlated with higher TRG, later ypN

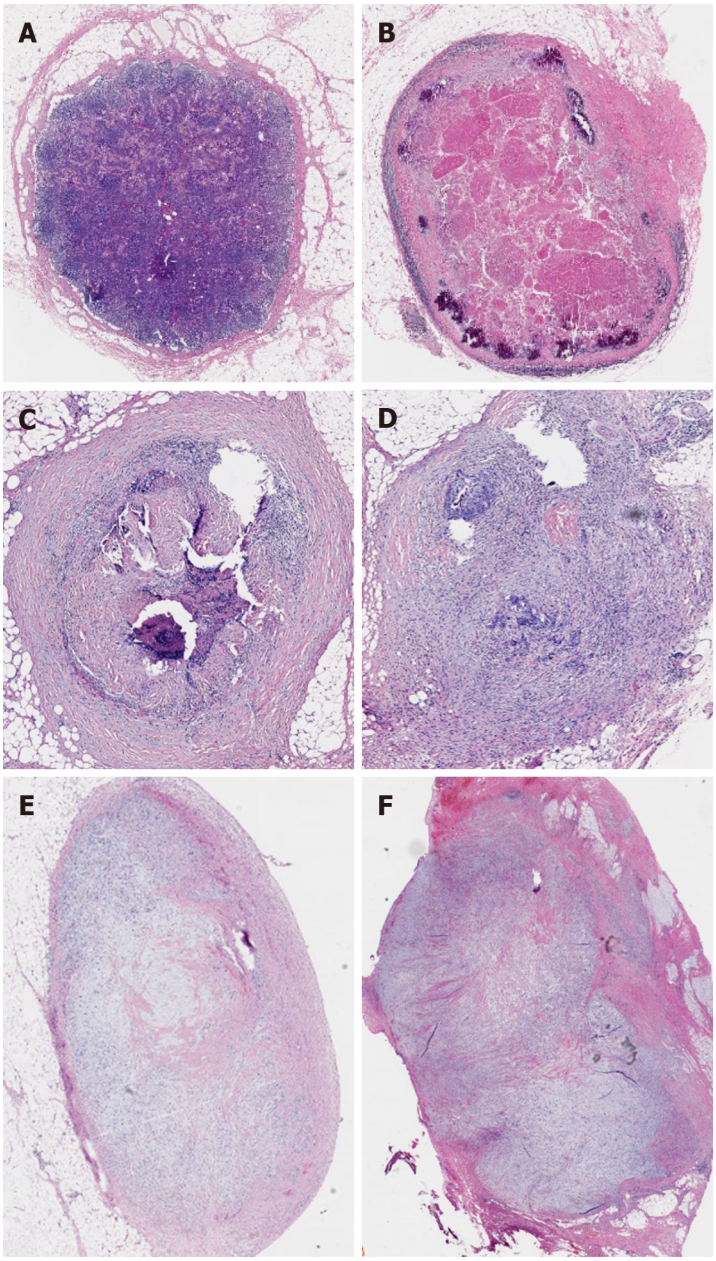


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**Figure 4 Examples of lymph node regression grades according to Mirbagheri *et al*[28] (10 ×).** A: Lymph node regression grade (LRG) 0: Normal lymph node; B: LRG1: 100% fibrosis, no residual cancer; C: LRG2: 75%-100% fibrosis, 0-25% cancer; D: LRG3: 50%-75% fibrosis, 25%-50% cancer; E: LRG4: 25%-50% fibrosis, 50%-75% cancer; F: LRG5: 0-25% fibrosis, 75%-100% cancer.

and ypT staging, and poorer DFS and OS.

### *Cui et al[38]*

In 2020, Cui *et al*[38] retrospectively analyzed the clinical data of 358 patients with LARC who received nCRT and proposed the following set of LRG scores: LRG0, negative LN; LRG1, complete regression with no residual tumor cells; LRG2, rare residual tumor cells; LRG3, fibrosis outgrown by residual tumor cells; LRG4, residual tumor cell outgrown by fibrosis; and LRG5, absence of regression with no fibrosis. The results showed that in the univariate analysis, the factors that correlated with DFS were ypN, ypT, the number of negative LNs (NLN), LN ratio (LNR), TRG, m-TTRG (modifying ypT stage by combining ypT and TRG), LRG-sum, LRG-max, M-NLRG (modifying ypN stage by combining LNR and LRG-max) and the LRG ratio (average of LRG-sum). M-NLRG and M-TTRG were significantly related to DFS in the multivariate Cox regression analysis. It was concluded that LRG significantly contributes to the prognosis in rectal cancer patients receiving nCRT and can improve the ypTNM staging system. A modified ypTNM staging system combining TRG, LRG-max and LNR could enhance DFS prediction for various subgroups of patients.

## CORRELATION BETWEEN LRG AND TRG

The relationship between primary tumors and LRG is still controversial among studies[39,40]. Most of these differences could be accounted for by different treatment plans, varied diagnostic standards for LRG, small sample sizes and patient heterogeneity.

Several studies[18,21,34] have reported that LRG was significantly correlated with TRG in primary tumors. Lee *et al*[34] evaluated postoperative LNs in 389 patients with rectal cancer treated with nCRT and concluded that LRG-sum distribution correlated significantly with the TRG in primary tumors ($P < 0.001$). LRG was associated with a completely regressed primary tumor. Accordingly, predicting LN regression based upon completely regressed primary tumors is beneficial, especially for patients considering a nonsurgical approach after nCRT. There are also studies[28,35] that suggest that higher TRG scores are correlated with higher LRG scores. Sun *et al*[35] retrospectively analyzed the clinical data of 257 LARC patients who were receiving nCRT and found that in the TRG 1, 2 and 3 groups, LRG scores were significantly increased. Higher scores of LRG were also found to be associated with more advanced stages of ypT and ypN. Considering these results, Sun *et al*[35] suggested that, to some extent, LRG may help predict the clinical complete response (the cCR) and determine LN regression in patients based on preservation strategies (*e.g.*, local excision or an approach of "watch and wait"). Additional studies have suggested that LRG is associated with TRG only under specific conditions, and the study by Beppu *et al*[32] concluded that: (1) Primary tumor radiosensitivity was associated with positive LNs; and that (2) LRG scores were associated with positive LN size only if the primary tumor had TRG 3 response.

Others[31] have argued that primary tumor TRG does not predict the LN presence of residual lesions. In 2006, Hughes *et al*[31] examined a total of 211 clinical-stage T3-T4 patients receiving preoperative CRT treatment outcomes and treatment details and concluded that primary tumor pathologic complete response failed to predict the circumrectal LN response, and the extent of the primary tumor response was a predictor of LN response.

Nevertheless, it is significant to note that different diagnostic standards for LRG were used in these previous studies, including the subgrouping of patients, which introduces some heterogeneity. Therefore, no conclusions concerning the association between TRG and LRG can be drawn at this time, and future large-scale research is needed with more homogeneous population groups to clarify this relationship.

## PROGNOSTIC SIGNIFICANCE OF LRG

Most studies[33,34,41] have suggested that LRG is a factor in the prognosis of rectal cancer patients receiving radical resection after nCRT. The study by Beppu *et al*[32] concluded that patients with completely regressed LNs typically had the best outcome. Beppu *et al*'s, Lee *et al*'s, Cui *et al*'s subgroup review of 229 patients receiving preoperative nCRT in T3 rectal cancer showed that total positive node regression following preoperative chemoradiotherapy is the only factor independently related to favorable overall survival[32,34,38]. While complete LN regression has been consistently correlated with improved DFS and OS as well as reduced local and distal recurrence risk, the impact of partial and subtotal LN regression [which is expected to be the main advantage of LRG *vs* TNM and American Joint Committee on Cancer (AJCC) grade] remains poorly understood. Studies from Mirbagheri *et al*[28] and Sun *et al*[35] concluded that a higher LRG was correlated with poorer DFS and OS. Mirbagheri *et al*[28] used multivariate Cox proportional hazard regression analysis and did not find that the LRG score was a factor for mortality, but it was an important predictor of relapse. However, the assumption that patients who had LN complete regression (LRG1) might fare better than LRG0 patients was not adequately tested, considering the small sample size of LRG1 patients. Tominaga *et al*[41] retrospectively analyzed 421 rectal cancer patients receiving preoperative nCRT, and the results indicated that LRG1 is a significant and independent factor for predicting recurrence-free survival. However, their results indicated that patients with grade 1 LN regression had similar local recurrence rates (LR) and 5-year recurrence-free survival rates as patients with LRG 0. However, in 120 patients with grade 2-5 LN regression, the 5-year recurrence-free survival rate and the LR resembled those of patients with LRG0, and the LR and the 5-year recurrence-free survival rate were poor irrespective of LRG (LR of 8.4%-14.0% and recurrence-free survival rate of 38.1%-61.1%). In addition, a large number of studies[28,34,35] have concluded that LRG-max and/or LRG-sum are significantly associated with prognosis. Lee *et al*[34] evaluated postoperative LNs in 389 patients with rectal cancer treated with nCRT and then received radical resection. In the multivariate analysis, LRG-sum was the most related contributor to RFS in LN-related variables alongside ypT staging. In 2020, Cui *et al*[38] suggested that in the univariate analysis, the contributors correlated with DFS were LRG-sum, LRG-max, M-NLRG and the LRG ratio.

However, in 2016, Fernández-Aceñero *et al*[21] retrospectively analyzed 106 rectal cancer patients receiving treatment at a single institution and concluded that there was no remarkable correlation between any factors or DSS and the LN tumor regression model in terms of prognosis.

In summary, we consider LRG to be an independent predictor of DFS for patients with LARC receiving nCRT and radical surgery. Since LN regression is highly correlated with other significant variables (*e.g.*, LVI and TRG), this characteristic might lose its statistical significance in some computational models, explaining the failure of certain studies to show that LRG has independent prognostic value relative to these other parameters[28].

## CRITICAL ISSUES OF LRG

### *Necessity of LRGs*

With the increasing development of comprehensive therapy for rectal cancer, the National Comprehensive Cancer Network has suggested that the therapy criteria for LARC are nCRT and TME[42-46], whose application has brought tremendous prognostic improvement for LARC patients with lower LR [47-50] as well as better anal preservation for patients with low rectal cancer[51,52]. A subset of LARC patients treated with nCRT can achieve complete tumor regression and are thus candidates for nonsurgical treatment[53]. NCRT leads to different degrees of tumor regression, with some patients achieving pCR for the primary tumor[27,54-56]. The LR was low in this patient group, and the tumor-free survival and overall rates were high[27,57,58]. Furthermore, numerous studies have demonstrated that TRG is significantly correlated with patient outcomes[13,24] and is an important prognostic factor for patients with LARC. LRG, like TRG, reflects the response of locally metastatic LNs to nCRT treatment based on postoperative patient histopathology[9,28]. In relevant studies, it is fully documented that residual tumor cells may still be present in local LNs despite the complete regression of primary tumors[30]. Currently, no single histopathological feature of colorectal cancer can reliably predict LN metastasis[59]. Some studies have demonstrated that different responses may exist between primary tumors and mesenteric LNs of the rectum[60]. Despite complete tumor regression, LN involvement may still occur. This was found in up to 17% of cases in some studies[31], especially when a watch-and-wait strategy was chosen after nCRT, likely leading to recurrence and treatment failure. Therefore, the pathologic evaluation of LNs in patients treated with surgery after nCRT could help to accurately determine the clinical staging of tumors and the response of metastatic LNs to nCRT.

The status of TDLN was the most significant factor in the prognosis of patients who have rectal cancer[61-63]. The number of metastatic LNs is currently the only basis for ypN staging, and several studies have demonstrated that nCRT leads to a decrease in the total number of LNs detected and the number of positive LNs[64,65]. Thus, the accuracy of staging ypN can be affected[13,14].

Several studies[66] have shown that current AJCC staging systems cannot accurately evaluate patient prognosis following nCRT because nCRT decreases the tumor stage and leads to varying degrees of treatment response. However, others argue that good prediction and assessment of regression during nCRT treatment and multidisciplinary consultation can allow for more individualized clinical decision making and treatment. The vast majority of studies on tumor response to therapy have focused on the primary tumor, while the effect of LRG on tumor treatment response and prognosis has not yet been fully appreciated.

**TRG:** The assessment of nCRT treatment regression in clinical practice relies mainly on postsurgical pathological examination results. Tumors were also graded by TRG according to the relative proportions of resident tumor cells in pathological specimens and the degree of fibrosis after treatment. Mandard *et al*[24] proposed the following: TRG1 for the absence of residual cancer and fibrosis - complete regression; TRG2 for the presence of rare residual cancer; TRG3 for an increase in the number of residual cancer cells but predominantly fibrosis; TRG4 for residual cancer outgrowing fibrosis; and TRG5 for the absence of regressive changes. Dworak *et al*[25] proposed a TRG staging system in 1997, which classified regression into stages 0 to 4 based on better to worse tumor regression. The seventh edition of the 2010 AJCC Cancer Stage Manual, put forward by the American Joint Committee on Cancer, reads as follows[29,67]: TRG0 for no viable cells present – complete; TRG1 for small groups of cancer cells/moderate-single cells – minimal; TRG2 for residual cancer outgrown by fibrosis; and TRG3 for no tumor-killing or poor/minimal killing, extensive residual cancer (Figure 5). Siddiqui *et al*[68] showed a strong association between patient prognosis and postoperative TRG grade, and they defined Dworak grades 3 and 4 and Mandard grades 1 and 2 as a better prognosis and Dworak grades 0 to 2 and Mandard grades 3 to 5 as a worse prognosis.

### *Limitations of ypN staging*

Currently, the AJCC 8$^{th}$ edition staging system, based solely upon the number of positive LNs for ypN staging, still follows the same ypN staging criteria for patients receiving nCRT and those undergoing surgery alone. Of the currently available TNM staging systems, ypN staging is classified according to the absolute number of positive LNs (PLNs). The guideline is based on little evidence and is largely derived from the historic view that evaluating a smaller number of nodes results in understaging[69, 70]. In addition, although it has been determined that increases in nodal harvest are related to improved survival, generally accepted staging theories explaining this relationship are unsupported by the

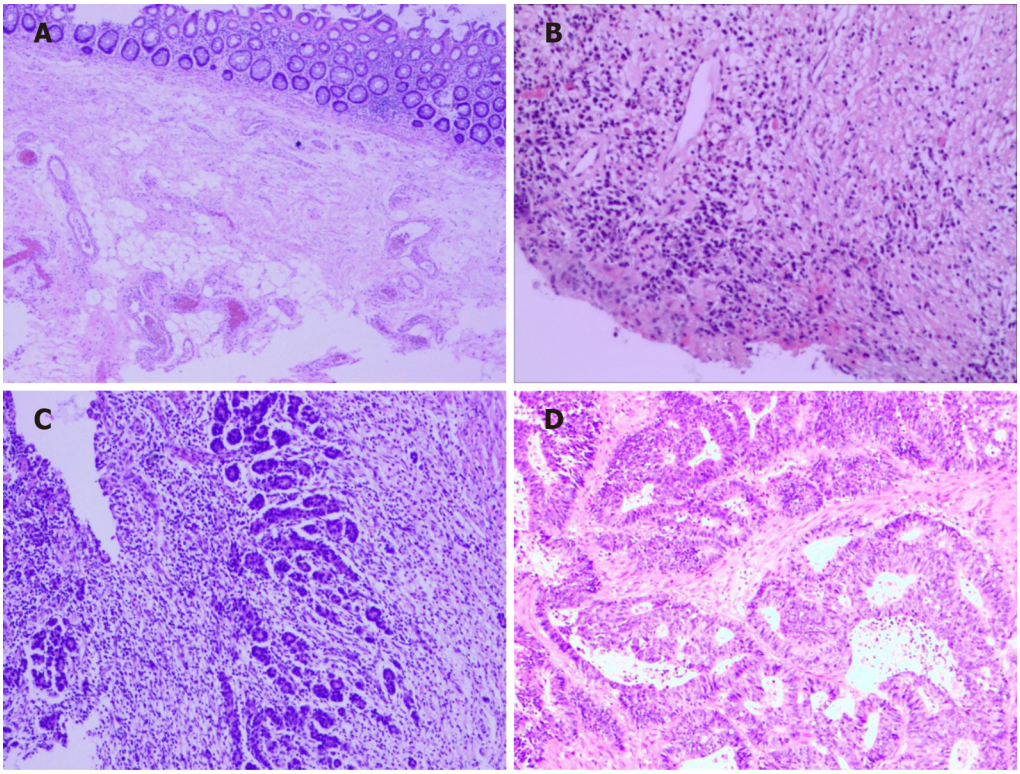


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**Figure 5 Examples of tumor regression grades according to American Joint Committee on Cancer.** A: Tumor regression grade (TRG) 0: complete-no viable cells present; B: TRG1: moderate-single cells/small groups of cancer cells; C: TRG2: minimal-residual cancer outgrown by fibrosis; D: TRG3: poor-minimal or no tumor cell death, extensive residual cancer.

evidence, and several authors have suggested that the higher number of LNs may indicate immune competence in individual patients instead of an improved means of detecting metastatic nodes[71,72]. A large population study in the United States showed that less than 50% of patients achieved the recommended number of LNs[73,74]. Thus, there are two main reasons why the AJCC guidelines have been questioned. First, recommendations for staging guidelines and treatment of rectal cancer depend heavily on data collected from colon cancer patients who are thought to be appropriate for rectal cancer [75,76]. Moreover, LNs found in rectal specimens were smaller in number and size than those found in colonic specimens[70,77]. Second, LNs detected after nCRT was significantly decreased[78,79]. Due to the increasing use of preoperative treatment of rectal cancer, pathology reports demonstrating low counts of LNs are increasingly being received by colorectal surgeons.

This ypN staging system only focuses on the numbers of metastatic LNs regardless of the tumor load in LNs following nCRT. The relevant literature suggests that LN regression should also be considered when assessing LN status. The main reasons for this may be twofold. First, the current ypN staging ignores the influence of LN treatment response on prognosis. A similar number of LN-positive patients might have a different number of LN metastases and a different metastatic load before treatment. The degrees of LN metastatic tumor regression following nCRT may reflect the different biological behaviors of tumors in different individuals, leading to different prognoses. Second, a decrease in the detection of positive LNs and the total number of positive LNs following nCRT can result in a bias in ypN staging based on using the number of positive LNs as grouping criteria[80,81].

One meta-analysis[82] demonstrated that patients receiving nCRT had a mean decrease of 3.9 total LNs detected and 0.7 PLN. Patients treated with neoadjuvant radiotherapy had 2.1 fewer total LNs detected. Ceelen *et al*[83] retrospectively analyzed 4037 patients who have rectal cancer registered in the Belgian Rectal Cancer Registry (Project for Rectal Cancer, PROCARE) between 2006 and 2012 who received nCRT and demonstrated a 12.3% reduction in the total number of detected LNs after short-range radiotherapy and a 31.3% reduction after long-range radiotherapy or long-range simultaneous radiotherapy. For each 1 Gy increase in the radiation dose, the number of detected LNs decreased by 0.21%[84]. Each additional LN detected was related to a 2.7% reduction in the risk of death in patients undergoing surgery alone, a 1.5% reduction in the risk of death in patients with short-range preoperative radiotherapy, and no reduced risk of death in patients with long-range simultaneous preoperative radiotherapy. Data from the publicly available SEER database[85,86] also revealed no significant difference between the two groups in terms of tumor-specific survival rates when the TLN cutoff number was 12, so the criterion of at least 12 LNs may not apply to patients receiving nCRT.

In summary, nCRT can reduce LN retrieval, decrease the N stage, and encourage downstaging of the primary tumor[87] and pN stage migration, leading to staging bias. This bias could affect the ypN staging system and decrease the accuracy in assessing patient prognosis after nCRT for rectal cancer[88, 89]. Therefore, the current ypN staging grouping in TNM staging is probably not applicable to patients receiving nCRT.

## DISCUSSION AND OUTLOOK FOR THE FUTURE

The evaluation and grading of LN regression are feasible for rectal cancer patients following nCRT by the histopathological examination of specimens excised after treatment. Thus, the implementation of LRG in histopathology reports for rectal cancer patients undergoing neoadjuvant radiotherapy is strongly recommended. LRG may even have more prognostic value than currently used staging systems (*e.g.*, TNM stage), primarily derived from untreated or unspecified tumor data. Suppose an apparently regressing LN also shows evidence of residual tumor. In that case, that LN is designated as a positive LN (ypN+), despite the good prognostic value for LN regression.

Lee *et al*[34] evaluated postoperative LNs in 389 patients with rectal cancer treated with nCRT followed by radical resection. In the multivariate analysis, LRG-sum was the most related contributor to RFS in LN-related variables alongside ypT staging. In 2020, Cui *et al*[38] In the univariate analysis, the factors that correlated with DFS were LRG-sum, LRG-max, M-NLRG and the LRG ratio.

However, considering a large number of LRG systems, the main focus of international and interdisciplinary committees should be to determine a consensus that can be applied to LRG reports. Critical concerns such as interobserver variability can also be resolved by individual and institutional training. Efforts should be made by both pathologists and clinicians alike to standardize specimen handling and LRG reporting. Although LRG can be used as a morphologic "biomarker," evidence for clinical trials could not be produced from studies with larger cohorts. The primary purpose of clinical trials should never be to compare different LRG systems but rather to scrutinize the histology and identify a standardized reporting method for LRG, which may further enhance the evidence of the value of LRG for the management of nCRT-treated LARC patients.

Recommendations for the standardized macroscopic and histopathological examination of LNs from rectal cancer excision specimens following nCRT are as follows: We prefer a 5-tier grading system and use the Mirbagheri system[28] in our daily work, which is very similar to the 4-tier modified Dworak TRG system[90]. A reproducible and easy-to-apply grading system for predicting clinical outcomes at a systematic level (comparing adequacy of various therapies) and for the individual patient (assessing their response to treatment, guiding further management, insight into prognosis) are useful. We consider this to be a good option. Based on this concept, additional data from evidence-based studies on the prognostic impact of LRG have confirmed that it is a strong prognostic morphological "biomarker" for guiding clinical decisions, modifying postoperative adjuvant therapy, improving operative strategies and monitoring intensities, and providing potential endpoints and alternative markers of prognosis for research programs and patients within clinical trials, which have yet to be presented.

Moreover, in addition to traditional radiotherapy, chemotherapy and surgery, some new oncological treatment methods have emerged recently, such as *Her-2, MSI,* and *BRAF* targeting for rectal cancer or the recently introduced immune checkpoint inhibitors[91]. Although immunotherapy has made considerable advances for a range of cancers, including non-small-cell lung cancer[92], the advances have not yet been extended to most rectal cancer patients[93]. The majority of rectal cancers are microsatellite stable, where immunotherapies targeting cytotoxic T lymphocyte-associated protein 4, programmed death-1 and programmed death-ligand 1 are currently recommended only for patients with high *MSI-H* [55,94]. Despite this, evidence suggests that it is important for the immune system to combat rectal cancer, as several studies have demonstrated that pretreatment densities of tumor-infiltrating lymphocytes predict better oncologic outcomes[95-97]. Furthermore, increasing numbers of preclinical models demonstrate that current chemotherapy and radiotherapy protocols can activate and synergize the immune system using immunotherapy[98-100]. Nevertheless, there is poor knowledge of the tissue alterations resulting from such emerging therapeutic strategies. Careful histopathological examination of posttreatment tissues and LNs could offer significant insight into the impact of these new agents and resistance mechanisms. Such research is expected to clarify the value of both TRG and LRG and additional detailed histological discoveries equivalent to those reported in the research originally used to introduce TRG into pathology.

## CONCLUSION

In summary, LRG should be recognized as an indicator of the response to nCRT and considered a determinant of prognosis for rectal cancer patients and should be included in pathology reports. With further and more extensive evidence-based validation, LRG may become a strong prognostic morphological "biomarker" that can be used to guide clinical decisions, modify postoperative adjuvant therapy,

and improve operative strategies and monitoring radiation intensities, as well as provide potential endpoints and alternative markers of prognosis for research programs and patients in clinical trials.

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## FOOTNOTES

**Author contributions:** He L performed the literature review and wrote the paper; He L, Xiao J, Zhong L, and Zheng P collected the pathology data; all authors have read and approved the final manuscript.

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*MINIREVIEWS*

# Immunotherapy in biliary tract cancers: Current evidence and future perspectives

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## Abstract

Bile duct tumors are comprised of tumors that originate from both intrahepatic and extrahepatic bile ducts and gallbladder tumors. These are aggressive tumors and chemotherapy is still the main treatment for advanced-stage disease and most of these cases have a poor overall survival. Strategies are aimed at treatments with better outcomes and less toxicity which makes immunotherapy an area of significant importance. Recent Food and Drug Administration approvals of immune checkpoint inhibitors (ICI) for agnostic tumors based on biomarkers such as microsatellite instability-high and tumor mutation burden-high are important steps in the treatment of patients with advanced bile duct tumors. Despite limited responses with isolated checkpoint inhibitors in later lines of systemic treatment in advanced disease, drug combination strategies have been demonstrating encouraging results to enhance ICI efficacy.

**Key Words:** Biliary tract cancer; Cholangiocarcinoma; Anti-programmed cell death protein-1; Anti-programmed death ligand-1; Microsatellite instability high; Tumor mutational burden high

**Core Tip:** Chemotherapy remains the main treatment for advanced bile duct tumors regardless of tumor aggressiveness and poor overall survival rates. The Food and Drug Administration has approved immune checkpoint inhibitors for agnostic tumors based on biomarkers such as microsatellite instability-high and tumor mutation burden-high. They are important steps in combined treatment with systemic chemotherapy for patients with advanced disease and show encouraging results.

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## INTRODUCTION

More than 905677 new cases of hepatobiliary cancer were estimated worldwide including hepatocellular carcinoma (HCC) in 2020. In addition, more than 115949 new cases of gallbladder cancer (GC) were reported in the same period[1]. In the United States, the incidence of patients diagnosed with liver and intra-hepatic tumors was estimated to be 42810 cases in 2020. Additionally, around 11980 patients were diagnosed with gallbladder and other biliary cancers[2,3]. The overall survival remains dismal with less than 10% of patients diagnosed with cholangiocarcinoma surviving 5 years after diagnosis[4,5]. Biliary tract cancers (BTC) comprise of a set of malignant tumors that can arise from any part of the bile ducts. BTC can be divided in intra-hepatic cholangiocarcinoma (ICC), peri-hilar cholangiocarcinoma, extra-hepatic cholangiocarcinoma (EHC) and GC.

In the last few years, several advances have been made in the management of BTC particularly in relation to ICC. Furthermore, the personalized medicine aligned with new molecules have brought about new hope for patients with advanced disease. For patients with tumors harboring isocitrate dehydrogenase 1 (IDH-1) mutations, reported in about 25% of ICC, the IDH-1 mutant inhibitor ivosidenib was evaluated in a randomized phase 3 trial including patients with disease in progress after at least one previous treatment. Ivosidenib delayed progression of the disease compared to placebo[6,7]. For tumors harboring fibroblast growth factor receptor 2 (FGFR2) fusions, United States Food and Drug Administration (FDA) has just approved the inhibitor pemigatinib that showed an objective response rate of 36% in a single arm cohort of 107 patients whom disease had progressed from previous chemotherapy treatment and of these patients, 3 patients had a complete response[8]. Multiple other FGFR inhibitors are under development and new agents will be incorporated into the landscape in the next few years[9,10].

Biliary cancers are desmoplastic tumors with an immunoresistant tumor microenvironment[4,11]. The liver has a great capacity of immunotolerance related to a continuous exposure to antigens derived from intestinal flora and a large population of macrophages. This microenvironment actively contributes to the limited effect of checkpoint inhibitors in these tumors[4,12]. Hepatobiliary cancers have unique characteristics and components related to immune evasion. Tumor-associated macrophages were associated with higher tumor recurrence in a retrospective analysis in a small group of surgically resected hilar cholangiocarcinoma specimens which also showed a worse overall survival[13]. However, more precise and comprehensive evaluation of cellular components, including myeloid-derived suppressor cells and natural-killer cells, are necessary to drive conclusions of the impact of targeting to these cells in biliary cancers[4]. Immune checkpoint inhibitors (ICI), particularly anti-programmed cell death protein-1/programmed death ligand-1 (PD1/PD-L1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) antibodies have shown excellent results in HCC[14,15]. For this reason, most of the recent clinical trials in biliary cancers have been conducted with single ICI or combinations[16,17]. More recently, durvalumab also was evaluated along with chemotherapy and exhibited exciting results in a randomized phase 3 trial in advanced biliary cancers[18,19]. The aim of this review is to cover the main studies of immunotherapy in biliary cancers, biomarkers of efficacy, future combinations and strategies in advanced-stage disease.

## TUMOR-AGNOSTIC BIOMARKERS

### *Microsatellite instability high*

Microsatellite-instability is a phenotype presented in cells with defective mismatch repair genes that result in a hypermutation state and the prevalence of microsatellite instability is high (MSI-H) in biliary tract cancers (BTC) ranging between 5%-10%[20]. The MSI-H phenotype can be a germline (Lynch syndrome) or somatic[21,22]. Multiples studies have evaluated the effect of ICI in patients harboring

MSI-H tumors. Recently, a randomized phase III trial demonstrated better outcomes in MSI-H metastatic colorectal cancer treated with upfront front-line therapy with pembrolizumab compared with the standard chemotherapy regimen[23]. Pembrolizumab is an anti PD-1 antibody. PD-1 is a cell surface protein presented in most activated T cells. When PD-L1 bound to the PD-1, this binding facilitates apoptosis and dysfunction of activated T cells and mediates an immune suppressive microenvironment [24,25]. Therefore, binding PD-1 with an antibody may promote functional enhancement of activated T cells repairing anti-tumor immunity[24].

In BTC, the efficacy of pembrolizumab in MSI-H was evaluated in different cohorts. In one study including 11 patients with advanced BTC MSI-H, the response rate (RR) was 27% (3/11) with response duration ranging between 11.6 to 19.6 mo[26]. Data from microsatellite instability in high BTC patients treated with the Keynote-158 with pembrolizumab showed better response rates. In a cohort of 22 patients, the objective response rate (ORR) was 40.9% (20.7-63.6) with a complete response in 2 patients. The median progression-free survival (PFS) was 4.2 mo with a median overall survival (OS) of 24.3 mo; however, the median duration of response was not reached[27]. A report of 4 BTC MSI-H patients was also included in a cohort of solid tumors treated with pembrolizumab. Of these patients, one had a complete response and the remaining three had stable disease[28]. Based on this evidence, immunotherapy should be considered for the treatment of advanced BTC patients with the microsatellite instability high phenotype. All of these studies included patients previously exposed to chemotherapy regimens and evaluation of ICI in previous lines of systemic treatment in MSI-H BTC should be prospectively evaluated. Currently, pembrolizumab is approved by the FDA to treat MSI-H BTC in cases that progressed following prior treatment and those with no satisfactory alternative treatment options.

### *Tumor mutational burden high*

Tumor mutational burden (TMB) is a biomarker that measures and quantifies the total mutation load in tumors. In biliary tract cancers, the TMB was evaluated retrospectively in a group of 156 patients. Of these, 133 tumoral tissues were assessed for the TMB with next-generating sequencing. Forty-eight cases were GC, 58 were ICC and 50 were EHC. The mean TMB value was high among patients with GC, followed by EHC and ICC [7.1 *vs* 5.5 *vs* 3.9 mutations/megabase (muts/Mb), $P < 0.05$]. The proportion of the TMB high (TMB-H), is defined in the study as 9.0 muts/Mb and was higher in GC than in ICC and EHC[29]. In another cohort including 803 BTC patients, 160 patients were evaluated with whole-exome sequencing and 643 patients with hybrid capture–based comprehensive genomic profiling. The mean TMB was 3.0 (IQR: 0.8-6.1) mut/Mb. In this cohort, 4 of 6 patients with MSI-H phenotype had a response to the disease after immunotherapy of the PD1 inhibitor[30].

In Keynote-158, a phase II basket study of pembrolizumab monotherapy for patients with advanced solid tumors, the relationship between activity of pembrolizumab and TMB was an exploratory endpoint. TMB-H was considered ≥ 10 mut/Mb. In the study, 790 patients had evaluable TMB included for efficacy analysis and 102 patients (13%) were TMB-H. The ORR observed was 29% (21-39) for TMB-H and 6% (5-8) for TMB-low. In addition, median duration of response in months was not reached for TMB-H and was 33 mo in TMB-low. The median OS for TMB-H and low was 11.7 mo (9.1-19.1) and 12.8 mo (11.1-14.1), respectively[31]. Based on these results, on June 16$^{th}$, 2020, the FDA approved pembrolizumab for the treatment of adult and pediatric patients with unresectable or metastatic TMB-H (≥ 10 mut/Mb) solid tumors that showed progression following the prior treatment and for those who have no satisfactory alternative treatment options. Despite being an important approval and option in the management of patients with advanced BTC, no BTC patients were evaluated in the TMB-H cohort. With the approval and clinical utilization in practice, future cohorts will bring insights about response rate and efficacy for this group of patients.

## IMMUNE CHECKPOINT INHIBITORS CLINICAL TRIALS

ICI were first evaluated in metastatic BTC patients whose disease had progressed on chemotherapy and the pembrolizumab was evaluated in two cohorts[32,33]. In the Keynote-028 PD-L1–positivity (membranous PD-L1 expression in ≥ 1% of tumor and associated inflammatory cells or positive staining in stroma) was required for eligibility and at the median follow-up time of 6.5 mo, the ORR was 13% in 23 patients. The median OS was 6.2 mo (3.8-10.3) with a 12-mo OS rate of 27.6%. The grade ≥ 3 adverse events (AE) occurred in 16.7% of the patients[33]. In the Keynote-158, a total of 104 patients with advanced BTC whose disease progressed on any lines of systemic treatment were treated with pembrolizumab. In that study, PD-L1 positivity was not mandatory and 61 patients had PD-L1 combined positive scores (CPS) ≥ 1. The overall RR was 5.8% (2.1-12.1) with six partial responses. Among patients with tumors with PD-L1 CPS ≥ 1 ($n$ = 61) the ORR was 6.6% (1.8-15.9) and in patients with tumors with PD-L1 CPS < 1 ($n$ = 34), the ORR was 2.9% (0.1-15.3). The median OS was compared in patients with PD-L1 CPS ≥ 1 *vs* < 1, 7.2 mo (5.3-11.0) *vs* 9.6 mo (5.4-12.8), respectively. Overall, 13% of patients had a Grade ≥ 3 AE[32].

Equivalent results were obtained in another single center cohort including 40 patients. In this prospective cohort pembrolizumab was evaluated in advanced PD-L1 ≥ 1% BTC patients who radiologically progressed after receiving first-line gemcitabine plus cisplatin. In the study, 47.5% of the patients included had ECOG performance status ≥ 2. The ORR by Response Evaluation Criteria in Solid Tumor (RECIST) v1.1 was 10% and by immune-modified RECIST was 12.5%, and the median OS was 4.3 mo (3.5-5.1). OS did not statistically differ between groups in different biomarker analyses, including CPS or tumor proportion score[34]. Anti-PD-1 nivolumab was evaluated in a multi-site phase II study including 54 advanced BTC patients whose disease progressed while undergoing treatment with at least 1 Line but no more than 3 Lines of systemic therapy. Central independent review RR was 11% (5/46), and investigator-assessed (IA) review response rate was 22% (10/46). Among the intention-to-treat population, median PFS was 3.68 mo (2.3-5.69) and median OS was 14.24 mo (5.98 to not reached). In this study, samples with 1% or more tumor cells for PD-L1 and any tumor infiltrating lymphocytes (TILs) for PD-1 exhibiting membranous staining were considered biomarker positive. PD-L1 expression was associated with prolonged PFS ($P < 0.001$). The PD-1 expression on TILs in this study had no correlation with clinical outcomes[35]. Although some responses were seen in these studies, immunotherapy with anti-PD-1 alone in unselected biomarker patients did not show great efficacy.

## IMMUNE CHECKPOINT INHIBITORS COMBINED WITH CHEMOTHERAPY

Combination of chemotherapy with ICI in BTCs are the subject of study. A phase II study conducted in China evaluated the anti-PD-1 camrelizumab with FOLFOX4 (fluorouracil plus oxaliplatin) in 47 chemotherapy naïve advanced BTC patients. In 43 patients available for RR analysis, the confirmed response was achieved in 7%, with a disease control rate of 67.4%. Median PFS and OS were not reached. A Grade ≥ 3 AE occurred in 57.4% of the patients, the most commonly occurring AE was hematological count decreases[36]. Another study conducted in China evaluated the addition of anti-PD-1 antibodies to nab-paclitaxel and S1 in 32 patients with advanced BTC who were treated with anti-PD-1 inhibitors (pembrolizumab, nivolumab, sintilimab or toripalimab) plus nab-paclitaxel and S1, and in 26 patients who were treated with the combination chemotherapy alone. ORR were higher in the PD-1 inhibitors combination group (25%) compared with chemotherapy alone (15.3%). The median PFS was 5.4 mo in the group treated with anti-PD-1 compared to 2.82 mo in the group with chemotherapy alone ( $P = 0.01$). The median OS is not mature[37].

Multiple mechanisms related to the synergism between chemotherapy and immunotherapy have been established. Chemotherapy can induce PD-L1 expression in cancer cells, facilitates infiltration of cytotoxic T cells in the tumor microenvironment and increases neoantigens and antigen-presenting cells. All of these effects can enhance the PD-1 inhibition[38]. Further evaluation of this synergism, preliminary safety and efficacy results of toripalimab and gemcitabine plus S-1 were presented[39]. In this phase II study, 34 patients were treated with the combination. Overall RR was 20.6% with a disease control rate (DCR) of 85.3%. In this study, patients with a mutation on TP53 or ATM had shorter PFS than the wild type[39].

The TOPAZ-1 trial is a randomized phase 3 trial that evaluated the combination of durvalumab, an anti-PD-L1, with chemotherapy against chemotherapy alone in advanced BTC. The study presented at the 2022 ASCO Gastrointestinal Cancers Symposium met its primary endpoint by improving OS in this subgroup of patients. Median OS was 12.8 mo with the combination *vs* 11.6 mo with gemcitabine and cisplatin alone (HR 0.8, $P = 0.021$). Furthermore, the addition of durvalumab to gemcitabine and cisplatin also improved PFS, 7.2 mo *vs* 5.7 mo (HR 0.75, $P = 0.001$) and tumor responses, 26.7% *vs* 18.7% [19].

## IMMUNE CHECKPOINT INHIBITORS COMBINATIONS

Another strategy to enhance anti-PD1/PD-L1 efficacy is combination with CTLA-4 inhibitors. This strategy has proposed that these combinations can enhance the activity and infiltration of cytotoxic T cells in tumor microenvironment in BTC[4]. Nivolumab plus ipilimumab was evaluated in a group of 39 metastatic BTC patients included in the CA 209-538 clinical trial for rare cancers. The primary endpoint was clinical benefit rate, exploratory endpoints included correlation of efficacy with biomarkers including PD-L1 expression and TMB. A total of thirty-three patients (85%) received at least one prior line of systemic treatment (0-2 Lines). The ORR was 24% and the clinical benefit rate was 45%. Responses were observed in all subgroups of BTC. The median OS and PFS were 6.1 and 3.1 mo, respectively. The other twenty-two (56%) patients experienced an immune–related adverse event of grade ≥ 3 AE were observed in 8 (20%) patients[40].

The combination was further evaluated in a multi-institutional phase 2 trial with patients who had advanced BTC but without previous systemic therapy. Patients were distributed into two groups of treatment, the arm A was treated with gemcitabine, cisplatin and nivolumab and patients received this combination every 3 wk for 6 mo, followed by nivolumab monotherapy that patients received every 2

wk for a total duration of 2 years. The arm B consisted of nivolumab given for patients every 2 wk and ipilimumab every 6 wk for 2 years in case of no disease progression. The primary endpoint was a PFS rate at 6 mo, and of all the patients, 35 were treated in arm A and 36 patients treated in arm B. Progression-free survival rate at 6 mo was 70% in arm A and 18.6% in arm B. In addition, the observed PFS rates at 6 mo were insufficient to reject the null hypothesis in both arms[41].

In another phase II study, combination of durvalumab, an anti-PD-L1 antibody, with tremelimumab, an anti-CTLA-4 antibody, with chemotherapy were evaluated in 121 chemotherapy naïve metastatic BTC patients[42]. In this study, patients were allocated in three groups of treatment. The first group, biomarker cohort (BMC) in which thirty patients were treated with cisplatin and gemcitabine for one cycle, following the next cycles with gemcitabine plus cisplatin, and durvalumab plus tremelimumab every 3 wk until the disease progression. The second group, three combo cohort (3C), a total of 45 patients were treated with gemcitabine, cisplatin and durvalumab. The latter group, in the four-combo cohort (4C) including 46 patients, were treated with all the four drugs, chemotherapy with gemcitabine and cisplatin plus durvalumab and tremelimumab until disease progression or unacceptable toxicity. Overall, the addition of immunotherapy associated with chemotherapy on the first-line therapy was well tolerated and demonstrated to be a promising activity. The ORR was 50% (32.1-67.9) in the BMC group, 73.4% (60.5-86.3) in the 3C group and 73.3% (60.4-86.2) in the 4C group. In addition, the DCR ranged between 96.7%-100%. The median PFS was 13 mo in the BMC group, 11 mo in the 3C group and 11.9 mo in the 4C group. Median OS in the 4C group was 20.7 mo, 18.1 mo in the 3C group, and 15 mo in the BMC group. PD-L1 analysis before treatment did not show any association with PFS or OS. Interestingly, in the BMC group, a trend in higher PFS was observed after gemcitabine and cisplatin first cycle, in patients whose tumor had high expression of PD-L1 compared with patients with lower PD-L1. The main studies described are included on Table 1.

## IMMUNE CHECKPOINT INHIBITORS COMBINED WITH ANTIANGIOGENICS

Combination with antiangiogenics can enhance the immunotherapy effect, contributing to cytotoxic effect of lymphocytes on the tumor microenvironment. After promising results in HCC, studies combining these agents are another option for refractory patients. In a non-randomized phase II study, 31 patients with chemotherapy refractory BTC were treated with Lenvatinib, a tyrosine kinase inhibitor (TKI) with antiangiogenics properties, associated with pembrolizumab[43]. The combination showed an ORR of 10% (2-26). These patients had a median PFS of 6.1 mo and a median OS of 8.6 mo. The most frequent treatment-related AEs, as expected, included hypertension and immune mediated adverse events[43]. Similar results were observed with the combination of regorafenib, another TKI, with anti-PD-L1 avelumab[44]. In this phase II study, 34 heavily pretreated patients with advanced BTC were treated. Four (13.8%) achieved partial response. Hypertension and fatigue were the most common grade 3/4 AE.

## FUTURE DIRECTIONS

Most of the recruiting studies in advanced BTC tend to combine ICI with chemotherapy in first-line systemic treatment or later lines. This combination has additional effects in enhancing ICI efficacy, as previously discussed[40,19]. Local ablative therapies and liver directed therapies are also strategies focused in enhancing neoantigens presentation and/or developing abscopal effect[45,46]. The main ongoing recruiting studies with different strategies and combinations with ICI are summarized in Table 2. In October 2021, interim analysis of the randomized phase III study TOPAZ-1, evaluated cisplatin, gemcitabine and durvalumab, against standard-of-care chemotherapy, and demonstrated a significant OS benefit as a 1st-line treatment for patients with advanced biliary tract cancer[47]. Recently, a final report confirmed that the addition of durvalumab to cisplatin and gemcitabine improved progression-free survival and overall survival compared to chemotherapy alone with no safety concerns.

Based on the results of the TOPAZ-1 trial, upfront combination of chemotherapy with durvalumab could be considered a standard of care however some concerns need to be further evaluated. First, it is clear that based on the curves of overall survival they began to separate around 6 mo. We need to consider that after 6 mo, the control arm is placebo not chemotherapy, the HR was 0.91 for up to 6 mo and 0.74 thereafter. We don't know if continuing chemotherapy with the same regimen or a maintenance strategy would affect the results of the study. Second, more than half of patients included in the study had intrahepatic cholangiocarcinoma and more than half had a PD-L1 score TAP (tumor are positivity) above 1. It is unclear if the study would have different results with more patients with PD-L1 score 0 or other underrepresented sites including extrahepatic cholangiocarcinoma or gallbladder. Third, in the subgroup analysis it seems that Asian patients derived more benefit from the combination than non-Asian patients. Asian patients comprised half of the patients included in the study. Lastly, no molecular analysis was presented, nether evaluation of underlying liver diseases including viral

**Table 1 Immunotherapy studies including biliary cancer patients, biomarkers, response rate and Food and Drug Administration approvals**

| Ref. | Patients | *n* | Biomarker | RR | Drug | FDA approval |
|---|---|---|---|---|---|---|
| Lemery *et al*[26], 2017 | Previously treated | 11 | MSI-H | 27% | Pembrolizumab | 5/23/2017 |
| Marabelle *et al*[27], 2020 (KN158) | Previously treated | 22 | MSI-H | 40.9% | Pembrolizumab | 5/23/2017 |
| Marabelle *et al*[31], 2020 (KN158) | No TMB-H in cholangiocarcinoma cohort | 0 | TMB-H (≥ 10 mut/Mb) | ? | Pembrolizumab | 6/16/2020 |
| Ueno *et al*[32], 2018 (KN158) | Previously treated | 104 | PD-L1 (CPS ≥ 1) | CPS ≥ 1: 6.6%CPS < 1: 2.9% | Pembrolizumab | - |
| Bang *et al*[33], 2019 (KN028) | Previously treated | 24 | PD-L1 ≥ 1 | 13% | Pembrolizumab | - |
| Kim *et al*[35], 2020 | Previously treated | 54 | - | IA: 22%CIR: 11% | Nivolumab | - |
| Klein *et al*[40], 2020 (CA 209-538) | Previously treated | 39 | - | 24% | Nivolumab + Ipilimumab | - |
| Oh *et al*[42], 2020 | Chemo-naïve | 121 | - | 50%-73% | Gem + Cis + Durvalumab ± Tremelimumab | - |
| Oh *et al*[19], 2022 (TOPAZ-1) | Chemo-naive | 344 | PD-L1 (TAP) | 18.7% | Gem + Cis | - |
| | | 341 | | 26.7% | Gem + Cis + Durvalumab | |

KN: Keynote; RR: Response rate; MSI-H: Microsatellite instability-high; TMB-H: Tumor mutational burden-high; mut/Mb: Mutations/megabase; PD-L1: Programmed death-ligand 1; CPS: Combined positive score; IA: Investigator-assessed; CIR: Central independent review; Gem: Gemcitabine; Cis: Cisplatin; TAP: Tumor area positivity.

**Table 2 Recruiting trials with checkpoint inhibitors in biliary tract cancers**

| ClinicalTrials.gov identifier | Study | Intervention | Patients included | State |
|---|---|---|---|---|
| Immune checkpoint inhibitors combined with chemotherapy | | | | |
| NCT03796429 | Phase II | Gemcitabine + S1 + Toripalimab | Chemo naïve | Recruiting |
| NCT04172402 | Phase II | Gemcitabine + TS-1 + Nivolumab | Chemo naïve | Recruiting |
| NCT04027764 | Phase II | Nab-paclitaxel + S1 + Toripalimab | Chemo naïve | Recruiting |
| NCT04300959 | Phase II | Gemcitabine + Cisplatin + Anlotinib + Sintilimab | Chemo naïve | Recruiting |
| NCT03785873 | Phase Ib/II | Nanoliposomal-irinotecan + 5-Fluorouracil + Nivolumab | Advanced disease | Recruiting |
| NCT04066491 | Phase II/III | Gemcitabine + Cisplatin + Bintrafusp alfa | Chemo naïve | Recruiting |
| NCT04004234 | Phase I/II | Gemcitabine + Nab-paclitaxel + Manganese primed anti-PD-1 antibody | Advanced disease | Recruiting |
| NCT04308174 | Phase II | Gemcitabine + Cisplatin + Durvalumab | Resectable disease | Recruiting |
| NCT03875235 | Phase III | Gemcitabine + Cisplatin + Durvalumab | Chemo naïve | Recruiting |
| NCT03046862 | Phase II | Gemcitabine + Cisplatin + Durvalumab + Tremelimumab | Chemo naïve | Recruiting |
| NCT03478488 | Phase III | Gemcitabine + Cisplatin + KN035 | Chemo naïve | Recruiting |
| NCT04191343 | Phase II | Gemcitabine + Oxaliplatin + Toripalimab | Chemo naïve | Recruiting |
| NCT03111732 | Phase II | Capecitabine + Oxaliplatin + Pembrolizumab | Advanced disease | Recruiting |
| NCT03704480 | Phase II | Durvalumab + Tremelimumab + Paclitaxel | Second-line systemic treatment | Recruiting |
| NCT03260712 | Phase II | Gemcitabine + Cisplatin + Pembrolizumab | Chemo naïve | Recruiting |
| Immune checkpoint inhibitors combined with targeted therapy | | | | |

| | | | | |
|---|---|---|---|---|
| NCT03639935 | Phase II | Rucaparib + Nivolumab | Advanced disease | Recruiting |
| NCT04211168 | Phase II | Toripalimab + Lenvatinib | Second-line systemic treatment | Not yet recruiting |
| NCT04057365 | Phase II | Nivolumab + DKN-01 | Advanced disease | Recruiting |
| NCT04298008 | Phase II | Durvalumab + AZD6738 | Advanced disease | Recruiting |
| NCT04298021 | Phase II | Durvalumab + AZD6738 + Olaparib | Second-line systemic treatment | Recruiting |
| NCT03475953 | Phase I/II | Regorafenib + Avelumab | Advanced disease | Recruiting |
| NCT04234113 | Phase I | SO-C101 + Pembrolizumab | Advanced disease | Recruiting |
| NCT04010071 | Phase II | Toripalimab + Axitinib | Advanced disease | Not yet recruiting |
| NCT03829436 | Phase I | TPST-1120 + Nivolumab | Advanced disease | Recruiting |
| NCT03095781 | Phase I | Pembrolizumab + XL888 | Advanced disease | Recruiting |
| NCT03250273 | Phase II | Entinostat + Pembrolizumab | Advanced disease | Recruiting |
| NCT03895970 | Phase II | Lenvatinib + Pembrolizumab | Advanced disease | Recruiting |
| NCT03825705 | Phase Ib/II | Anlotinib + TQB2450 | Advanced disease | Recruiting |
| Immune checkpoint inhibitors combined with local therapy | | | | |
| NCT03482102 | Phase II | Durvalumab + Tremelimumab + Radiotherapy | Advanced disease | Recruiting |
| NCT02866383 | Phase II | Nivolumab + Ipilimumab + Radiotherapy | Second-line systemic treatment | Recruiting |
| NCT04238637 | Phase II | Durvalumab + Tremelimumab + Y-90 SIRT | Intrahepatic biliary cancer | Recruiting |
| NCT02821754 | Phase II | Durvalumab + Tremelimumab + Ablative therapies | Advanced disease | Recruiting |
| NCT03898895 | Phase II | Camrelizumab + Radiotherapy | Unresectable disease | Recruiting |
| Immune checkpoint inhibitors combined with cell therapy | | | | |
| NCT03937895 | Phase I/IIa | Allogeneic NK cell (SMT-NK) + Pembrolizumab | Advanced disease | Recruiting |

Ongoing clinical trials identified in ClinicalTrials.gov using the term "Biliar".

hepatitis or NASH[19]. This question opens up future strategies of research to improve the care and understanding of immunotherapy in advanced biliary cancer patients.

## CONCLUSION

Immune checkpoint agnostic approvals for advanced-stage cancer with biomarkers such as MSI-H and TMB-H are important treatment options for patients with advanced BTC who currently have limited options for treatment of refractory disease. Drug combinations such as anti-PD-1/PD-L1 with anti-CTLA-4 and/or chemotherapy have the potential to establish the standard of care for these patients and benefit a larger proportion of individuals if adopted in earlier lines of systemic treatment, however, more studies are necessary to better identify subgroups of patients that will most benefit from these strategies and treatments.

## FOOTNOTES

**Author contributions:** Araujo RLC and Uson Junior PLS performed the literature review, collected all the data and wrote the manuscript; All authors have read and approved the final manuscript.

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*MINIREVIEWS*

# Crosstalk between gut microbiota and COVID-19 impacts pancreatic cancer progression

Chun-Ye Zhang, Shuai Liu, Ming Yang

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## Abstract

Pancreatic cancer (PC) is one of the most common causes of cancer-associated death worldwide, with a low rate of 5-year survival. Currently, the pathogenesis of PC is complicated, with no efficient therapy. Coronavirus disease 2019 (COVID-19) disease caused by severe acute respiratory syndrome coronavirus 2 further exacerbates the challenge of patients with PC. The alteration of gut microbiota caused by COVID-19 infection may impact PC progression in patients *via* immune regulation. The expression of inflammatory immune mediators such as interleukin (IL)-6, IL-8, and IL-10 has been found to increase in both PC and COVID-19 patients, which is associated with the disease severity and prognostic outcome. Gut microbiome serves as a critical connector between viral infection and PC. It can regulate host systemic immune response and impact the efficacy of immunotherapy. Here, we first demonstrated the features of inflammatory cytokines in both diseases and their impact on disease outcomes. Then, we demonstrated the importance of immunotherapeutic strategies. This includes the immune modulation that targets a single or dual receptors using a single agent or their combinations for the treatment of PC in patients who get infected with COVID-19. Additionally, we explored the possibility of managing the disease by regulating gut microbiome. Overall, modulation of the lung-gut-pancreases axis can boost anti-cancer immunotherapy and reduce adverse prognostic outcomes.

**Key Words:** COVID-19; SARS-CoV-2; Gut microbiota; Pancreatic cancer; Interleukin-6; Interleukin-8; Interleukin-10; Monoclonal antibodies; Modulatory treatment

**Core Tip:** Pancreatic cancer (PC) is a leading cause of cancer-associated death worldwide. Currently, the pathogenesis of this disease is complicated without efficient therapy. Coronavirus disease 2019 (COVID-19) disease exacerbates the challenge of PC patients. The gut microbiome serves as a critical connector between viral infection and PC through the regulation of host systemic immune response. Therefore, by targeting the lung-gut-pancreases axis, we can modulate both cytokine storm and inflammation in patients with PC and COVID-19 infection.

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## INTRODUCTION

Pancreatic cancer (PC) is the leading cause of cancer-associated death globally, only about 9% of patients can survive more than 5 years according to the American Cancer Society's report (February 2021)[1,2]. The major type of PC is pancreatic ductal adenocarcinoma (PDAC), about 90% of all PC cases[3], which is caused by tumor growth of the cell that lines in the pancreatic ducts[4,5]. The pancreatic ducts play a key role in the transportation of pancreas-produced digestive enzymes to the duodenum (the proximal part of the small intestine). This process is critical for digestion[6,7]. Although the pathogenesis of PC is still under intensive investigation, there is a lot of progress has been made. Several factors such as smoking, diabetes, alcohol abuse, and dietary factors have been identified as contributors. They are closely associated with cancer development. Those are the potential factors that contribute to the higher risk of PC development[8-11].

Coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus infection may worsen the disease progression in patients with PC. Here, we summarize the role of gut microbiota, which functions as an important connector for COVID-19 and PC.

## PC AND ITS CLOSE ASSOCIATION WITH GUT MICROBIOME

Accumulating studies showed that gut microbiome plays a vital role in pancreatic diseases, including gut microbiota and their components such as CpG-rich DNAs. For example, dysbiosis of gut microbiota can accelerate the severity of chronic pancreatitis. Chronic pancreatitis is considered as one of the contributing factors that cause PC[12,13]. In addition, the profiles of gut microbiota have been shown to be altered in PC patients compared to that in the cohort controls. However, several studies showed that the treatment with probiotics[14] or synobiotics[15] did not show a significant effect on patients with acute pancreatitis. Recently, a remarkable finding was made by Riquelme *et al*[16], which showed the diversity and composition of gut microbiome were associated with the survival time of patients with PDAC. PDAC patients who survived more than 5 years showed a higher diversity of gut microbiome. In addition, they found the microbiome contains an intra-tumoral unique microbiome component, including *Pseudoxanthomonas*, *Streptomyces*, *Saccharopolyspora*, and *Bacillus clausii*, compared to the cohorts who survived less than 5 years[16]. This study also shows that the associated immune signatures are different between the two cohorts. There was a significant positive correlation between $CD3^+$, $CD8^+$, and $GzmB^+$ cells tissue densities and the overall survival of PDAC patients. The causation role of gut microbiome in the survival of PC patients was further verified. The corresponding microbiome from the patient was colonized into the tumor-bearing germ-free mice, respectively. The result showed a similar pattern of survival between the colonized mice and the clinical patient. In detail, the tumor-bearing germ-free mice that were colonized with the microbiome originated from the long-term survival patients had long-term survival. The mice received microbiome that from the short-term survival patients displayed a short-term survival[16]. In addition, tumor-bearing mice who received fecal microbial transplantation (FMT) from long survival patients have a higher number of $CD8^+$ T cells $CD8^+$ T cells, specifically activated T cells ($CD8^+/IFN\gamma^+$ T cells) in the tumor environment, whereas mice that received FMT from short term survival patients had increased infiltration of $CD4^+FOXP3^+$ regulatory T cells (Tregs) and myeloid-derived suppressor cells in the tumor. In summary, the abovementioned examples demonstrate that gut microbiome contributes a significant role in the pathogenesis of PC and tumor progression through the mechanism of microbial components modulation and associated change of immune activation. Therefore, alteration of gut microbiome could affect the severity and prognostic outcome of PC.

## COIVD-19 INFECTION ALTERNATED GUT MICROBIOME

In the pandemic era of COVID-19, the situation may be even worse for PC patients who get infected by SARS-CoV-2. Besides the major respiratory syndrome, gastrointestinal symptoms such as diarrhea and abdominal pain were also observed, reported, and identified in patients infected with COVID-19[17,18]. In addition, the isolation and detection of SARS-CoV-2 viruses from the gut enterocytes and fecal samples in COVID-19 patients indicated that infection of viruses influences the intestine system. The viruses in the intestine could impact gut microbiome[19]. Notably, angiotensin-converting enzyme II (ACE2) is the important binding receptor for SARS-CoV-2 viruses binding to the host. The ACE2 is broadly expressed in the epithelial cells in the lung, gastrointestinal, vascular endothelial cells, brain, *etc*. The presence of those ACE2 receptors increases the susceptibility of the abovementioned cells to the virus infection[20]. Meanwhile, the other critical enzyme for viral binding and entrying into the cell is transmembrane protease serine 2 (TMRPSS2), which is also expressed in the small intestinal epithelial cells[18]. Thus, the presence and expression of ACE2 and TMRPSS2 in gastrointestinal epithelial cells provide a physiologic foundation for the interaction between COVID-19 and gut microbiome. What's more, studies have demonstrated that the components of gut microbiota are closely associated with the expression level of ACE2. For example, some *Bacteroides* species, such as *Bacteroides dorei* and *Bacteroides thetaiotaomicron*, have the properties of downregulating the ACE2 expression in the murine model[21]. This indicates that gut microbiome plays an important role in the expression level of ACE2. Because of that, gut microbiome is important to host susceptibility and immunity during the COVID-19 infection.

In addition, serving as the binding receptor of coronavirus, ACE2 also plays an essential role in the expression of neutral amino acid transporters. Those transporters can be found in the intestine and the compositions of the gut[22]. The alteration of gut microbiome in COVID-19 patients has been investigated by several studies[23-25]. The results from those studies showed that there were an increased level of opportunistic pathogens and a decreased level of commensal symbionts in the gut of COVID-19 patients. Those commensal symbionts possess the properties of the immunomodulatory function. Butyrate-producing microbiota such as *Faecalibacterium prausnitzii* (*F. prausnitzii*) (phylum Firmicutes), *Eubacterium rectale* (phylum Firmicutes), and *Bifidobacterium adolescentis* (phylum Actinobacteria) are well-known as immunomodulators. They play important role in maintaining intestinal health with anti-inflammatory function[24]. For example, the *F. prausnitzii* has been demonstrated to display anti-inflammatory function and induce polarization of dendritic cells and the priming of interleukin (IL)-10-producing T cells in the human colon. A study showed a significant association between the decreased level of *F. prausnitzii* and the severity of COVID-19 disease in the patients[23,24].

Taken all together, the presence of ACE2 and TMRPSS2 in intestinal epithelium cells is the physiological foundation. The impact of gut microbiome on the expression level of ACE2 provided evidence of their association. Plus, the alteration of gut microbiome happened during COVID-19 occurrence. Additionally, the severity of the COVID-19 was shown to be associated with the level of a certain microbiome. All those above-mentioned aspects illustrate that the gut microbiome is closely associated with COVID-19. Gut microbiome could be the connection for the pancreatic patients infected with SARS-CoV-2[26], through the gut-pancreas axis.

## PANCREATIC INJURY AND ABNORMALITIES IN COVID-19 PATIENTS

Interestingly, pancreatic injury and abnormalities have been reported in SARS-CoV-2 infected patients. However, the mechanism including the cause-effect needs to be further investigated[27]. The statistical analysis was performed for 1378 SARS-CoV-2 infected patients (including both males and females) ranging from mild to severe infection. The result showed that the increased levels of enzyme amylase in serum were significantly related to the COVID-19 severity and the prognosis of infection[28]. Elevated serum enzyme amylase level is also known as an indicator of pancreatic-associated diseases, such as acute pancreatitis and pancreas inflammation[29]. The serum amylase comes from both salivary amylase and pancreatic amylase. The gut serves as a linkage. Through the gut-blood barrier and peritoneal blood barrier, the salivary amylase and the pancreatic amylase are absorbed into the blood vessel. Therefore, both pancreatic inflammation and leakage or damage of gut epithelium integrity can cause an increase in serum amylase[30,31]. Previous analysis of 351 metastatic PC patients showed that there was a positive association between the increased plasma amylase level and negative prognostic outcomes for PC[32]. Thus, the observation of elevated serum amylase levels from the COVID-19 patients highlights the importance to investigate the crosstalk between the SARS-CoV-2 infection, the pancreatic-associated inflammation, and the gut-associated inflammation.

Another analytical study was conducted by a group using the COVID-19 family database (SARS-CoV, SARS-dORF6, SARS-BatSRBD, and influenza A virus subtype H1N1 included) due to the lack of COVID-19 patient databases. They found an upregulated expression level of several genes, such as *CREB1*, *PTEN*, *SMAD3*, and *CASP3* genes in COVID-19 patients. Meanwhile, those genes were also highly expressed in PC. Scientists proposed that there was a potential risk of development of pancreatic severity followed by the SARS-CoV-2 infection[33]. In addition to the data analysis, oncological

treatment procedures should be optimized to provide better outcomes for pancreatic patients in the COVID-19 pandemic era, minimizing morbidity and mortality[34,35].

## LUNG-GUT-PANCREAS AXIS

Gut microbiota plays an essential role in host health and disease through various mechanisms[36,37]. (1) Gut microbiota serves as an extensive metabolic repertoire to help the absorption of nutrition and to provide an energy source to maintain the host homeostasis and health; (2) Gut microbiota plays a crucial role in drug metabolism under the disease condition to facilitate the drug uptake, distribution, absorption, metabolism, excretion, and toxicity modulation; (3) Gut microbiota plays an important role in fighting against infection from bacteria and viruses; and (4) Gut microbiome contributes to maintaining homeostasis and reducing the dysbiosis caused by variable factors from both the endogenous and exogenous antigens. The gut microbiome plays the aforementioned functions through colonization resistance, immunomodulation, and metabolism.

Gut microbiota serves as a central connection between different organs to maintain the balance of the host system[38-40]. The gut-lung axis and gut-pancreas axis are related to each other *via* lymphatics, circulation system, immunomodulatory, *etc.* (Figure 1). Diseases such as SARS-CoV-2 virus infection can cause dysbiosis or gut microbiota alteration through inflammation mediators; meanwhile, pancreas diseases such as PC also can lead to the dysbiosis of gut microbiota. That was mediated by pancreatic hormone (*e.g.*, insulin, glucagon) and digestive enzyme. Similarly, the change or disruption of the stability or equilibrium of gut microbiota also can lead to various severity of the disease. This is contributed by the immunomodulators (*e.g.*, inflammatory cytokines) or bacterial metabolites [*e.g.*, short-chain fatty acids (SCFAs)]. As demonstrated and highlighted by the above-mentioned paragraphs, the alteration of gut microbiota in both COVID-19 and PC patients showed a decreased level of *F. prausnitzii* and *E. rectale*. Both are known as commensal symbionts. They are also known as butyrate-producing microbiota with important immunomodulatory properties in the host[41,42].

In summary, the host acts as a whole system to fight against disease and to maintain homeostasis and health condition. Therefore, it is essential to better understand the disease features such as the underlying mechanism, the immune response, the outcome of prognosis, and their associations with each other. For example, the alteration of some factors in one disease may complicate the another newly occurred disease. The altered microenvironment may cause an adverse influence on the therapeutic efficacy. Especially, caution should be taken, when it is needed to treat both the initial disease and a newly emerging disease in the same patient.

Here, we focus on the case of SARS-CoV-2 infection in PC patients. By investigating the association, correlation, and underlying mechanism, an optimized therapeutic option could be developed to better facilitate the prevention of both diseases. For PC and COVID-19, an immune response is a critical factor that influences the severity of the disease and prognostic outcome. The microenvironment in PC and the change of associated immune profile may positively/negatively influence the severity of COVID-19 in patients, and *vice versa*. Gut microbiome, as a mediator between those two diseases, needs to be further explored. This exploration could be considered from the perspective of improving the host systemic immune response and promoting treatment efficacy for both diseases. In the following discussion, we will focus on the commonality of the immune mediator in both disease and immunotherapy treatment strategies. That includes the single target and dual targets of immune mediators using a single agent or combination therapy.

## COVID-19 INFECTION INFLUENCES THE SEVERITY OF PC *VIA* IMMUNE MODULATION

The change of immune profile due to the SARS-CoV-2 infection could impact the severity of PC patients. A study has demonstrated that the increased levels of inflammatory cytokines are detected in the serum of COVID-19 patients compared with that in normal controls, such as increased levels of ILs (IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17), tumor necrosis factor-α (TNF-α), transforming growth factor-β, interferon-gamma (IFN-γ)[43-46]. In PC, higher serum levels of IL-6, IL-8, and IL-10 are strongly associated with the progression of cancer. They are the prediction of poor prognostic outcomes of PC[47]. Thus, the increased levels of IL-6, IL-8, and IL-10 derived from SARS-CoV-2 infection may further complicate the tumor microenvironment of PC patients.

IL-6 was found as an essential factor that promotes the progression of PC. One study illustrated that the depletion of IL-6 abrogated PC progression regardless of the existence of oncogenic Kras (Kirsten rat sarcoma 2 viral oncogene homolog). The study showed that IL-6 is necessary for activation of the reactive oxygen species detoxification program during PC progression[48]. In addition, IL-6 regulates inflammatory response and results in carcinogenesis. Thus, the increasing level of IL-6 in COVID-19 patients has a negative influence on the disease severity of PC patients. Notably, Il-6 serves as a biomarker for predicting the overall severity of the COVID-19 disease[49]. Taken together, the coronavirus infection may cause an even worse situation or poor prognostic outcome for PC patients

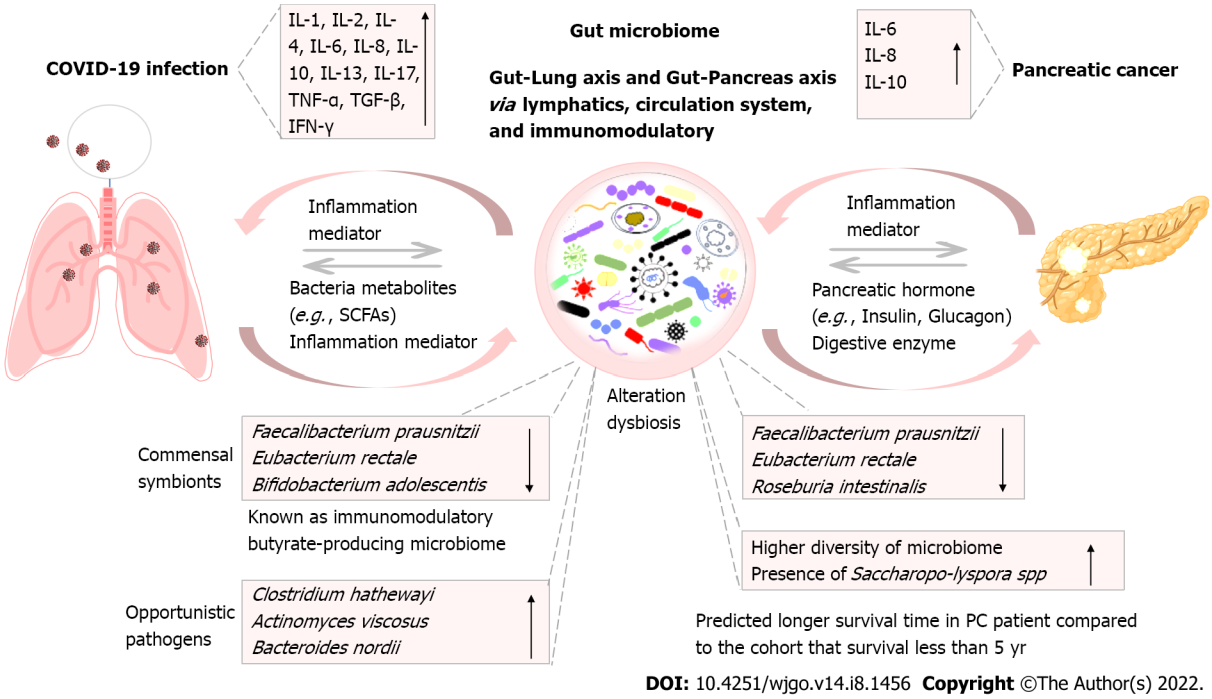


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**Figure 1 The gut-lung axis and gut-pancreas axis connect the interaction of lung infection with pancreatic cancer, *via* altering gut microbiota, systemic inflammation, and immune responses.** COVID-19: Coronavirus disease 2019; IL: Interleukin; TGF-β: Transforming growth factor-β; IFN: Interferon; TNF: Tumor necrosis factor; SCFA: Short-chain fatty acid; PC: Pancreatic cancer.

due to the increased level of IL-6.

IL-8, a neutrophil chemoattractant cytokine with pro-inflammatory function, is broadly produced by monocytes/macrophages[50], smooth muscle cells[51], epithelial cell[52], endothelial cells[53], and other cell types[54]. A study showed that a high serum level of IL-8 was detected in PC patients. That was strongly associated with a higher level of IL-6. Additionally, a higher level of IL-8 showed a significant correlation with a shorter survival time of PC patients ($P < 0.001$, correlation coefficient value -0.414) [47], which indicated that IL-8 could be one of the important biomarkers for the prediction of prognostic outcome in patients with PC. In vivo study showed that nude mice implanted with tumor tissues from the PC patients with higher serum levels of IL-8 grow tumors faster than the mice implanted with the tumor tissues from the patients with a lower level of serum IL-8[55]. Thus, a higher level of IL-8 serves as a predictor of the worse prognostic outcome of PC. Notably, during the SARS-CoV-2 infection, a remarkably higher level of serum IL-8 was also confirmed by several studies from COVID-9 patients[44,56]. In a study that includes 40 COVID-19 patients, the result showed there was a significantly higher level of IL-8 in non-survival patients compared with that in survival patients. This result suggested an association between IL-8 Levels and the fatal outcome of COVID-19 disease[57]. Another study showed that the IL-8 displayed a better correlation with the clinical score of COVID-19 progression compared to IL-6. The study compared the IL-8 and the IL-6 at different time points. This indicated a possibility of using IL-8 as a biomarker to define disease status[56]. Therefore, IL-8 plays a pivotal role in both PC and COVID-19, especially for PC patients infected with SARS-CoV-2.

IL-10, a controversial immunoregulatory cytokine. Up to date, studies have reported that IL-10 displays both tumor-promoting and anti-tumor functions in cancer. Meanwhile, IL-10 also plays a complicated role in viral infection[58-60]. The elevated IL-10 in the serum of COVID-19 patients has been identified and it showed a close association with the severity of the COVID-19 disease[43,61,62].

Overall, the increased levels of inflammatory cytokines IL-6, IL-8, and IL-10 that resulted from the COIVD-19 infection can facilitate the progression of acute pancreatitis. It further promotes PC progression in the patients[43,63]. Thus, it is important to consider immunotherapy as one of the treatment strategies for PC patients who encounter viral infections. Due to the complicated immune response and the commonality of the elevated levels of IL-6, IL-8, and IL-10 in both patients with COVID-19 or PC, or both, the immune mediators for a single target or dual targets could be used as a therapeutic treatment. The treatment agents could also include a single agent and the combination treatments to better improve therapeutic outcomes.

## CLINICAL TREATMENT FOR COVID-19 BY BLOCKADE OF IL-6 AND/OR IL-8 SIGNALING

An anti-inflammatory therapeutic strategy plays an important role in combating viral infections including SARS-CoV-2 infection. Targeting pro-inflammatory cytokines or non-cytokines can be chosen based on their highly elevated levels that are associated with the severity of the disease in patients, as well as the association with prognostic results[64,65]. For instance, targeting IL-6 is an attractive therapeutic option due to its critical role in COVID-19. That has been investigated by multiple studies and was mentioned above[66,67]. Treatment options for blocking IL-6/IL-6 receptors in COVID-19 include monoclonal antibodies and small molecules. Based on the mechanism, they can be divided into three categories: (1) Anti-IL-6 receptor monoclonal antibodies such as Tocilizumab and Sarilumab; (2) Anti-IL-6 monoclonal antibodies such as Siltuximab; and (3) Small molecules such as Furosemide[68-70].

### *Anti-IL-6 monoclonal antibodies*

Multiple clinical trials have been conducted to date with the status of either completed or in progress at different phases. Here, we selected some examples and summarized them in detail in a table (Table 1).

### *Small molecules are targeted to inhibit IL-6 and TNF-α*

Compared to the IL-6 monoclonal antibody that specifically targeted the inflammatory cytokine IL-6, a small molecule has the potential advantage of expanding the targeting range. The treatment targets of the small molecule can be expanded to a broad range for therapeutic efficacy. A preclinical study that aimed to explore the treatment of using small molecules for SARS-CoV-2 infection, was conducted using *in silico* screening method and molecular simulation. As a result, a potential small molecule, Furosemide, was found to have the function of inhibiting both IL-6 and TNF-α. In addition, this inhibiting function was verified by *in vitro* experiment assay. Encouragingly, more investigation and evaluation are needed to screen the small molecules with the properties of dual targets such as Furosemide for COVID-19 treatment[71,72].

### *IL-8 neutralization*

The clinical trial of investigation on the effect of using BMS-986253 (neutralization of inflammatory cytokine IL-8) to treat the COVID-19 patients has been approved for recruiting. The investigation is currently ongoing (Phase 2, NCT04347226).

### *Treatment for PC by blockage of IL-6 and/or IL-8 signaling*

For PC, anti-inflammatory therapy has also been investigated in many studies, including both monotherapy and combinational treatments to improve the efficacy. For instance, an *in vitro* study showed that combinational treatment by blocking both IL-6 (Bazedoxifene) and IL-8 (SCH527123) signaling pathways displayed an enhanced effect on the reduction of cell viability and migration of PC cells[73]. Clinical trials are ongoing to evaluate the treatment efficacy of siltuximab and spartalizumab, such as trials NCT04191421 and NCT04812808 (https://clinicaltrials.gov, accessed on 03/10/2022). The combinational treatment with anti-IL-6R and anti-programmed death 1 (PD-L1)-blocking antibodies showed significant antitumor activity at *in vitro* cell culture. *In vivo* study, this combinational treatment improved therapeutic results and extended the survival time of mice with PC compared to controls[74].

It is worthy to point out that there are some treatments in pre-clinical and clinical studies, such as Tocilizumab[75-79], Sarilumab[80-82], Siltuximab[83,84], and others (Table 1). These above-mentioned treatments are either specifically for PC patients or specifically for COVID-19 patients. Less data is available related to the investigation of the treatment efficacy in SARS-CoC-2-infected PC patients. This shed light on the importance of investigating or documenting the clinical data in the field related to COVID-19 treatment options or strategies in PC patients.

## FURTHER EXPLORATION OF THE IMPACT OF GUT MICROBIOME ON CYTOKINE SECRETION TO ENHANCE THE TREATMENT EFFICACY

Cytokine storm in COVID-19 and inflammatory cytokines in the pancreatic tumor microenvironment are important factors that exacerbate the disease severity. In addition to directly targeting viruses and tumor cells, the exploration of clinical treatments to reduce the inflammation by targeting interleukins such as IL-6 and IL-8 is also required.

Meanwhile, as illustrated early in this paper, gut microbiome reciprocally impacts the severity of PC and SARS-CoV-2 infection. On one hand, the alteration of gut microbiota in COVID-19 may increase the severity of PC. On the other hand, the alteration of gut microbiota resulting from the PC disease could exacerbate the COVID-19 symptoms, increase the susceptibility to the infection, and influence the recovery process due to the weakened immune response. The reciprocal influence of COVID-19 and PC *via* the lung-gut-pancreas axis might be mediated by metabolites and immune modulators. Therefore, the modulation of the gut microbiome could provide a better microenvironment. The enhanced

**Table 1 Clinical and pre-clinical studies in coronavirus disease 2019 and pancreatic cancer**

| Disease | Antibody/drug | Target | Title | ClinicalTrials.gov identifier | Ref. |
|---|---|---|---|---|---|
| COVID-19 | Tocilizumab | IL-6 receptor | Efficacy of Tocilizumab on Patients With COVID-19 | NCT04356937 | [75] |
| COVID-19 | Tocilizumab | IL-6 receptor | A Study to Investigate Intravenous Tocilizumab in Participants with Moderate to Severe COVID-19 Pneumonia | NCT04363736 | [76, 77] |
| COVID-19 | Tocilizumab | IL-6 receptor | RECOVERY Trial: Open-Label RCT of Tocilizumab and Usual Care in Hospitalized Patients With COVID-19 | NCT04381936 | [78, 79] |
| COVID-19 | Sarilumab | IL-6 receptor | Evaluation of the Efficacy and Safety of Sarilumab in Hospitalized Patients With COVID-19 | NCT04315298 | [80, 81] |
| COVID-19 | Sarilumab | IL-6 receptor | Sarilumab COVID-19 | NCT04327388 | [82] |
| COVID-19 | Siltuximab | IL-6 | An Observational Study of the Use of Siltuximab (SYLVANT) in Patients Diagnosed With COVID-19 Infection Who Have Developed Serious Respiratory Complications (SISCO) | NCT04322188 | [83] |
| COVID-19 | Siltuximab | IL-6 | Treatment of COVID-19 Patients with Anti-interleukin Drugs (COV-AID) | NCT04330638 | [84] |
| COVID-19 | Clazakizumab | IL-6 | Study for the Use of the IL-6 Inhibitor Clazakizumab in Patients with Life-threatening COVID-19 Infection | NCT04381052 | None |
| COVID-19 | Clazakizumab | IL-6 | Clazakizumab (Anti-IL-6 Monoclonal) Compared to Placebo for COVID-19 | NCT04348500 | None |
| COVID-19 | Clazakizumab | IL-6 | A Randomized Placebo-controlled Safety and Dose-finding Study for the Use of the IL-6 Inhibitor Clazakizumab in Patients with Life-threatening COVID-19 Infection | NCT04343989 | None |
| COVID-19 | Furosemide | IL-6 and TNF-α | Furosemide as Supportive Therapy for COVID-19 Respiratory Failure | NCT04588792 | [72] |
| COVID-19 | BMS-986253 | IL-8 | Anti-IL-8 for Patients With COVID-19 | NCT04347226 | None |
| Pancreatic cancer | Siltuximab | IL-6 | Siltuximab and Spartalizumab in Patients with Metastatic Pancreatic Cancer | NCT04191421 | None |
| Pancreatic cancer | Bazedoxifene | IL-6 | Bazedoxifene as a Concomitant Treatment of Patients with Metastatic Pancreatic Adenocarcinoma (BAZE) | NCT04812808 | None |
| Pancreatic cancer | Bazedoxifene and Navarixin (SCH527123) | IL-6 and IL-8 | Blocking IL-6 and IL-8 Signaling Inhibits Cell Viability, Colony-forming Activity, and Cell Migration in Human Triple-negative Breast Cancer and Pancreatic Cancer Cells | Pre-clinical research | None |
| Pancreatic cancer | Antibody | IL-6 and PD-L1 | IL-6 and PD-L1 antibody blockade combination therapy reduces tumor progression in murine models of pancreatic cancer | Pre-clinical research | None |
| Pancreatic cancer | Oncolytic vaccinia virus armed with IL-10 | IL-10 | A new role of IL-10 in enhancing the antitumor efficacy of oncolytic vaccinia virus for the treatment of pancreatic cancer | Pre-clinical research | None |

COVID-19: Coronavirus disease 2019; IL: Interleukin; TNF: Tumor necrosis factor; PD-L1: Programmed death 1.

microenvironment is beneficial to promote the treatment efficacy through the modulation of microbiota-associated immunity. There are several strategies to modulate the microbiome. For instance, (1) Supplementing with beneficial microbiota such as butyrate-producing bacteria *F. prausnitzii* with anti-inflammatory and immunoregulatory functions. The decreased abundance of *F. prausnitzii* was found to be associated with a negative prognosis in both COVID-19 and PC patients; and (2) Modulating the gut microbiota to improve the colonization resistance *via* immune modulator or metabolism. This could assist to boost the systemic immune resilience and reduce microbial dysbiosis-induced inflammation. For example, commensal bacteria *Bifidobacterium longum* displayed protective properties against the influenza viruses in a mouse model[85]. Using the fecal microbiota transfer method, scientists transferred the antigen-experienced microbiota from wild mice into germ-free mice. The result showed the enhanced resistance to lethal influenza A virus infection and increased survival in a mouse model [86]. Those studies demonstrated the important roles of gut microbiota conferred against viral infection. Therefore, more investigation is needed to explore and improve host resistance to viruses. In particular, it is necessary to explore the strategy from the perspective of creating a favorited gut microbial environment that is beneficial to the host immune response during viral clarence, disease progression, and treatment efficacy.

From the clinical perspective, accumulating studies and clinical outcomes demonstrated that the gut microbiome influences the response of immune therapy in cancer patients[87-89]. A previous study found that the microbiome, such as *Faecalibacterium* and *Ruminococcaceae*, positively correlated with the better outcome of the anti-PD-1 treatment for melanoma cancer[90]. The gut microbiome also influences the efficacy of PD-1 blockade immunotherapy in epithelial tumors. The low level of commensal bacteria *Akkermansia muciniphila* (*A. muciniphila*) was identified in the non-response patient. Supplemented with *A. muciniphila* could alter the nonresponse response to PD-1 blockade treatment. The underlying mechanism is through the modulation of IL-12[91]. The enriched commensal bacteria *F. prausnitzii* showed close association with a better response to immune therapy. The underlying mechanism is related to the metabolite, SCFA butyrate. *F. prausnitzii* could produce butyrate through metabolism. The concentration of butyrate (high or low) could modulate the production of IFN-γ and IL-10[92,93], respectively. Most recently, a report showed that gut microbiome *Bacteroides*, *Ruminococcus*, and *Faecalibacterium* were associated with the clinical outcome of anti-CD19 CAR T cell treatment[94]. The above-mentioned examples better illustrated that the microbiome has an impact on the clinical treatment efficacy in cancer patients.

It is worth noticing that the clinical data on PC treatment and the influence of the gut microbiome is limited. However, regardless of what kind of cancer, there are commonalities in immunotherapy between cancers. Plus, there are some shared similarities in the underlying mechanism between cancers. Thereby, the clinical investigation of microbiome influence on the response of PC is urgently needed.

Currently, there is limited clinical data on the relationship between treatment efficacy of COVID-19 and gut microbiome. One reason is that only the infected patients who have critical emergency conditions can be hospitalized due to the pandemic. At this critical stage, life-saving medical care is needed. Another reason is that the medicine for COVID-19 is under development. For clinical trials, most efforts were focused on the evaluation of the effectiveness on a large scale. The effort is limited, especially, for further examining the influence of the associated factors on treatment efficacy.

However, there is accumulating data on the association between the COVID-19 vaccination and gut microbiome. Several clinical trials are ongoing. For example, some clinical trials (NCT04884776 and NCT04798677; Clinicaltrials.gov) are focusing on the investigation of gut microbiome influence on COVID-19 vaccination efficacy[95]. In addition, most recently, a report better demonstrated the association between the gut microbiome and clinical vaccination efficacy. This investigation was performed using shotgun metagenomic sequencing in the vaccinated population. They discovered that a gut microbiome community that facilitates the carbohydrate metabolism is beneficial to the efficacy of COVID-19 vaccination. In people with a higher richness of *Bifidobacterium adolescentis*, a higher level of neutralizing antibodies was produced when vaccinated with CoronaVac. People with enriched microbiome such as *Roseburia faecis* showed close association with the BNT162b2 vaccination efficacy [96]. Therefore, the commensal microbiome was correlated with the vaccine-induced neutralization effect. Collectively, gut microbiome plays an important role in host response to the virus (vaccination or treatment). More clinical studies are desired.

The cancer treatment normally causes a weakened immune system in patients. This increases the patient risk and susceptibility to virus infection. Upon the infection, the disease severity could dramatically increase. The management of the clinical care and treatment strategy is a big challenge[97]. Moreover, the application of the COVID-19 vaccine to a cancer patient is another big challenge. The efficacy and safety need to be well-evaluated. Recently, the first safety-related clinical case was reported. The case showed that a cancer patient got the Vaccine-induced thrombotic thrombocytopenia after mRNA-1273 vaccination[98]. In summary, strategies need to be explored to enhance the clinical treatment efficacy for cancer. Meanwhile, exploration should be made to improve the vaccination and treatment efficacy for virus infection. Gut microbiome, serve as an important component in both cancer treatment outcome and vaccination response. Modulation of the gut microbiome could be a potential option to be investigated. The change of microbial environment in the initial disease should be taken into consideration. That consideration helps develop the best options for health care and treatment.

## CONCLUSION

Collectively, the reciprocal influence between COVID-19 and PC disease through the cross-link of gut microbiota may pave the way for the exploration of therapeutic options. For instance, the options include the modulation of gut microbiota *via* dietary intervention, the supplementation of beneficial bacteria, or intake of favored metabolites. Those options can be used to enhance the systemic immune response to battle against both viruses and tumors. The connection of diseases such as COVID-19 and PC through gut microbiota should be investigated to better prepare for a newly emerged disease in the future. Additionally, the efficacy of using synergistic treatment also needs to be explored and evaluated. For instance, it is important to explore the treatment efficacy of using dual agents compared to a single agent. The treatment strategy that aims to target multiple factors in the disease is also favored. For example, in addition to directly controlling the pathogen (*e.g.* virus), it is also critical to control the inflammation-caused damage (*e.g.* Cytokine storm). Therefore, the exploitation of diverse treatment

strategies is urgently needed, especially, for patients with complex disease situations.

## FOOTNOTES

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*MINIREVIEWS*

# Angiogenesis in gastrointestinal stromal tumors: From bench to bedside

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## Abstract

Gastrointestinal stromal tumors (GISTs) are rare neoplasms with an estimated incidence from 0.78 to 1-1.5 patients *per* 100000. They most commonly occur in the elderly during the eighth decade of life affecting predominantly the stomach, but also the small intestine, the omentum, mesentery and rectosigmoid. The available treatments for GIST are associated with a significant rate of recurrent disease and adverse events. Thorough understanding of GIST's pathophysiology and translation of this knowledge into novel regimens or drug repurposing is essential to counter this challenge. The present review summarizes the existing evidence about the role of angiogenesis in GIST's development and progression and discusses its clinical underpinnings.

**Key Words:** Gastrointestinal stromal tumor; Cancer; Oncology; Angiogenesis; Gastrointestinal oncology; Stromal tumors

**Core Tip:** Thorough understanding of gastrointestinal stromal tumors (GISTs)'s pathophysiology and translation of this knowledge into novel regimens or drug repurposing is essential to counter this challenge. The present review summarizes the existing evidence about the role of angiogenesis in GIST's development and progression and discusses its clinical underpinnings.

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## INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare neoplasms with an estimated incidence from 0.78 patients to 2 patients *per* 100000[1,2]. Their highest prevalence is noted during the eight decade of age, when they affect up to 3.06 individuals *per* 100000[3]. GIST typically present as subepithelial masses mainly in stomach (60%) and small intestine (20%-30%) with omentum, mesentery and rectosigmoid areas being less-frequently involved areas[4]. According to their primary location, GISTs could clinically present as gastrointestinal hemorrhage, anemia, dyspepsia or vomiting when the upper gastrointestinal tract is involved and as bowel obstruction, frequent urination or diarrhea in implication of the lower gastrointestinal tract[5]. The metastatic disease principally concerns the liver, omentum, and peritoneum presenting as abdominal pain or constipation while extra-intestinal metastases to lymph nodes (LN) and lungs are infrequent[4]. The pathological diagnosis relies on the tissue's morphological and molecular characteristics. Based on their morphology, GISTs are classified into three groups according to the predominant cell type: Spindle cell type (70%), epithelioid cell type (20%) and a mixed type (10%). CD117 comprises a transmembrane protein which is the end-product of the *c-kit* expression[6]. The KIT (CD117) positivity in immunohistochemistry (IHC) in tissues which are morphologically consistent with GIST establishes the diagnosis in the 95% of the cases. In KIT negative cases, the discovered on GIST 1 (DOG1) and CD34, which is an antigen of the myeloid progenitor cells, staining or the documentation of KIT or *platelet-derived growth factor receptor* (*PDGFRA*) gene mutations are sufficient to institute a diagnosis. Seldom in pediatric and young populations, GIST formation arises in the context of succinate dehydrogenase-deficiency in conjunction with paragangliomas and pulmonary chondromas[7,8].

The pharmacologic targeting of angiogenesis in cancer therapeutics was introduced as a groundbreaking approach. Nevertheless, the anti-vascular endothelial growth factor (VEGF) targeting alone or in conjunction with chemotherapy displayed only modest benefit in overall survival in solid tumors indicating the complexity of the mechanisms that regulate tumor angiogenesis[9]. Thus, the necessity arose to develop a broad spectrum of anti-angiogenic treatments such as: Direct VEGFR2 antagonists (ramucirumab), VEGF-Traps (aflibercept), several receptor tyrosine kinases inhibitors targeting the PDGF-R, CD117 (c-KIT), fibroblast growth factor receptors (FGFR), epidermal growth factor receptor, RET, RAF kinases and the repurposing of drugs like the mammalian target of rapamycin inhibitors and lenalidomide[9,10]. In fact, anti-angiogenetic therapy has gained ground in the management of advanced, unresectable disease. Imatinib, an abl, c-KIT and PDGF-R tyrosine kinase inhibitor (TKI), constitutes the empiric treatment when the mutational status of the disease remains unknown and the first line of treatment in KIT and PDGFRA positive metastatic, inoperable GISTs. The D842V mutation in *PDGFRA* comprises a therapeutic exception and is being treated with avapritinib while *KIT* and *PDGFRA* wild type tumors are treated with sunitinib or regorafinib[11].

All the above mentioned drugs achieve, at least partially, their cytotoxicity disrupting signaling pathways which are implicated in angiogenesis, as it would be further analyzed below. This suggests that angiogenesis might be of paramount importance for the carcinogenesis process in GISTs and an attempt to summarize all the pre-clinical and clinical data would be of great value.

## THE ROLE OF ANGIOGENESIS IN GIST'S DEVELOPMENT AND PROGRESSION

### *The molecular mechanisms of angiogenesis in GISTs–preclinical data*

The regulation of angiogenesis is necessary for cancer cells initially to cope with their increased metabolic needs and in the process to promote their metastatic potential. Its significance was firstly recognized by Folkman[12], which stated that the magnified rate of neovascularization compared with wound healing and inflammation as a result of an interplay between tumor cells and endothelial cells was a prerequisite in order to achieve tumor growth[12]. Presently, it is widely known that the

angiogenic process is being coordinated by the balance of several angiogenesis inducers and inhibitors in tumor's microenvironment. The dominance of the pro-angiogenetic factors, a phenomenon called "angiogenic switch"[13], triggers the angiogenesis and could result either as result of the consequent hypoxia from the increased tumor proliferation or by the immune cell infiltration[14]. The primary induction phase with the undeveloped vessels paves the way for the remodeling phase when the blood vessel generation is sustained[15]. Several models of angiogenesis have been described explaining partially the poor outcomes of the selective angiogenic blockage as certain tumors can utilize alternative modes of angiogenesis[14]. Their analytical presentation has been done elsewhere[14,16,17] and goes beyond the scope of this review but a brief presentation in Table 1 would be helpful.

Xenograft studies in mice constitute an invaluable source of evidence about the angiogenetic mechanisms in GISTs. Our fundamental conceptualization about the orchestration of the angiogenetic process descended from Giner *et al*[18]. They utilized an intensely CD117, DOG1 and CD34-positive GIST with continual Ki-67 expression in about 15% of the tumor's mass. The neovascularization experiments demonstrated the propagation of the induction phase during the first 96 h after implantation which proceeded by the remodeling phase. The induction phase was guided by the *VEGF*, *VEGFC*, *PDGFA*, *PDGFB* gene expression in conformity with their receptors. In more detail, the IHC data indicate that the VEGF ligand and the VEGFR2, VEGFR3 were positive at day 4 after the xenografting. As regards the chemokine expression, CXCL9, CXCL10, GRO and their receptors CXCR3, CXCR2 were stained in tumor cells and stroma soon after the implantation with a slight staining predominance of the chemokine receptors. These effects are possibly orchestrated by hypoxia-inducible factor (HIF)1α and the CXCL12/CXCR4 axis, which are constantly expressed[18].

The angiogenetic process in GIST has been further delineated and several regulatory molecules have been identified. CCL2 represents a chemokine expressed by the tumor cells to attract CCR2-expressing endothelial progenitor cells from the circulation as documented in HER-2/neu-driven breast cancer[19]. On the other hand, the VEGF-induced nuclear factor kappa B (NF-kB) upregulation is frequently utilized to attract inflammatory cell into tumor to stimulate the angiogenesis[20]. The bromodomain and extraterminal domain family mediates immunity regulating several signaling pathways[21]. In GISTs, the BRD4 upregulation enhanced the migratory and invasion processes regulating angiogenesis through the NF-kB/CCL2 signaling pathway. The BRD4-expressing cells attract tumor-associated macrophages *via* the expression of CCL2 potentiating the tumor's microvessel density and secrete various pro-angiogenic molecules such as VEGFA, LOX and MMP9[22,23]. Towards the same direction, mutations of the protein phosphatase 2, regulatory subunit A, alpha (PPP2R1A) affect the carcinogenesis process [24,25]. In GISTs, mutations in *PPP2R1A* gene are found in nearly 20% of the cases and correlate with a more aggressive tumor phenotype. They result in increased growth rate *via* enhancing phosphorylation of c-kit, Akt1/2, ERK1/2 and WNK1. The latter seems to mediate the regulation of the angiogenetic process[26,27]. A further analysis of the specific mechanisms would be of great value and it should be applied.

Furthermore, while the contribution of epigenetic mechanisms in the GIST progression is well established, its impact in the angiogenetic mechanisms could be further delineated. Several gaps in our understanding that remain unaddressed by the subdivisions according to the driver gene mutation status could be further elucidated by the tumor's epigenetic landscape. The alterations in the tumor's methylation profile are associated with a more aggressive phenotype[28] and the methylation status of the CD133 could reshape the management of the disease and it would be presented below in more depth[29]. The KDM4 family members (KDM4A-D) reshaping the structure of chromatin are implicated in the pathogenesis of a wide variety of cancers[30]. In GIST, the upregulation of KDM4D potentiates the angiogenesis in vivo, as indicated by the overexpression of CD31 in IHC. These effects are mediated by the HIF1β/VEGFA pathway in the presence of demethylation in the promoters of the *H3K9me3* and *H3K36me3* genes[31].

Finally, it is worth mentioning that several multi-TKIs exert their anti-tumor efficacy at least partially by the inhibition of angiogenesis. Cabozantinib exerts it's activity inhibiting the receptor tyrosine kinases MET, VEGFR2, Flt-3, c-Kit and RET[32,33] while sorafenib inhibits the signaling of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, Flt-3, c-Kit and the Raf serine/threonine kinases[34]. Both diminish the tumor's microvascular density as evidenced by CD31 IHC[35,36].

### *The association between imaging data and angiogenesis in GISTs*

There have been several classification systems to stratify the malignant potential of GISTs such as: The National Institutes of Healthv consensus criteria (Fletcher's criteria), the Armed Forces Institute of Pathology criteria (Miettinen's criteria) or the International Union against Cancer TNM classification. Their main drawback constitute the inability to validate the tumor's aggressiveness without surgical resection and detailed pathologic examination of the entire tumor to estimate the mitotic count[37,38] . Although taking into consideration the current therapeutic trends, the management of the advanced, unresectable disease is unequivocal, there are margins for improvement in the management of primary localized disease, especially in small-sized tumors. It could not be emphasized enough that even small GISTs could develop malignant behavior. Thereat, it could provide us a wealth of valuable predictive and prognostic information an attempt to incorporate imaging data about the vascularization of the tumor such as the vessels' irregularity or the blood perfusion[39].

**Table 1 The basic mechanisms of angiogenesis**

| Angiogenetic mechanism | Function | Implicated signaling/ pathways |
|---|---|---|
| Sprouting angiogenesis | Vessel formation from a parental one as a sprout outgrowth | VEGF, Dll4/notch pathways and neuropilins |
| Intussusceptive Angiogenesis | Splitting of a parental vessel into two newly formed | VEGF, PDGF pathways and erythropoietin |
| Vasculogenesis/Endothelial progenitor cells | Vessel formation from endothelial progenitor cells differentiating into mature endothelial cells | VEGF pathway, chemokines |
| Vasculogenic mimicry | Vessel-like formations without endothelial cells | HGFR |
| Trans-differentiation of CSCs | CSC give rise to endothelial cells | Tie-2, TGF-, CXCL12/CXCR4 |

PDGF-R: Platelet-derived growth factor receptor; VEGF: Vascular endothelial growth factor; HGFR: Hepatocyte growth factor receptor; TGF-: Transforming growth factor-; CSCs: Cancer stem cells, CXCL12: C-x-c motif chemokine ligand 12; CXCR4: C-x-c motif chemokine receptor 4.

The above mentioned gap was attempted to be filled by a landmark study by Iannicelli *et al*[40], the computed tomography (CT) constitutes the fundamental imaging modality in patients presenting with the clinical manifestations of GIST. Reviewing past literature, several studies have documented that aim to associate certain imaging features with pathologic parameters[41,42]. Iannicelli *et al*[40] presented that GISTs with irregular margins tended to have superior mitotic rate than tumor with regular margins. Furthermore, a heterogonous pattern of contrast enhancement (CE), the angiogenesis and necrosis correlated with an increased tumor size and a more aggressive clinical behavior. It worth mentioning that the intensity of CE although it represents a novel mark of biologic activity, was not correlated with neither the number of mitoses nor the tumor's risk stratification[40]. The above comprise an indirect link between tumor's margins and mitotic rate, which is essential in order to stratify before surgery the clinical behavior of the tumor and highlight the importance of angiogenesis in disease progression. The latter could also be deduced by dynamic positron emission tomography analysis. Strauss *et al*[43] reported an association between the rate in which the F-18-fluorodeoxyglucose diffused into the tumor with the expression of VEGF-A[43]. The main limitation of CT comprises it's low sensitivity as regards the imaging of vascularity in small sized tumors[39]. This divergence could be addressed by the endoscopic ultrasound (EUS) technology.

The utilization of EUS has emerged during the last decades. Its ability to evade the intervention of the abdominal fat and gastrointestinal gas in conjunction with the capability of FNA biopsy render it a useful tool towards a more personalized approach in the management of GIST. In EUS the GISTs are visualized as hypoechoic masses arising from the muscularis propria or the muscularis mucosae. The presence of irregular margins, cystic areas or malignant LN herald bad prognosis[44]. The usage of contrast media enhances further the diagnostic capacity of the EUS and promotes the tumor's vascularity as a valuable prognostic biomarker. The role of CE-EUS in the management has been extensively reviewed elsewhere[45] and we intend to delineate the fundamentals. Sakamoto *et al*[39] classified the tumor's vascularity into two subgroups according to the pattern of perfusion (homogenous or heterogeneous) and vessel appearance (regular or irregular). The homogenous perfusion with regular vessels were considered as signs of mild clinical behavior. Furthermore, they compared the diagnostic sensitivity of contrast-enhanced harmonic US, Power-Doppler EUS and CE-multidetector CT to visualize tumor vessels. In GISTs larger than 3 cm their sensitivities were 100%, 75% and 42% respectively. The differences became more emphatic in tumors less than 3 cm: 100%, 25% and 0%, respectively. It was noteworthy that every malignant lesion less than 3 cm in the cohort had been detected by the CEH-EUS before surgery[39]. The above indicate that CE-US comprises a powerful tool to visualize vascularity. Taking a step further, Yamashita *et al*[46] demonstrated an association between the imaging findings on CE-US and the pathologic risk stratification. In more depth, the large vessels lacked elastic tissue, indicating that neovascularization constitutes the underlying pathogenetic mechanism, and expressed VEGF[46].

It becomes evident that the imaging findings of vascularity might be sensational and practice changing in a subset of patients with small sized tumors (< 3 cm) and aggressive phenotype. A more substantial body of evidence should be collected in order to address properly those dilemmas.

### *Angiogenesis mediators as biomarkers in GIST–clinical data*

The development of biomarkers comprises an essential step towards the individualization of medical practice. Liquid biopsy provides a cutting-edge, non-invasive technology to access predictive information to guide the therapeutic management in a wide variety of diseases[47-51]. It's application in GIST treatment has been started to emerge[52,53]. Reviewing subsequent and more recent literature, an extensive number of studies has been found associating molecules implicated in angiogenesis with pathologic features. Although there are several limitations in the above mentioned research, the

**Table 2 A brief presentation of several angiogenetic molecules in disease progression**

| Ref. | Sample size | Molecule/methods | Outcomes |
|---|---|---|---|
| Zhao *et al*[59] | 124 patients-62, 50% in stomach, 22.6% in small intestine | HIF-1α/IHC | Association with disease-free survival ($P$ = 0.03) |
| | | VEGF/IHC | Association with disease-free survival ($P$ = 0.002) |
| | | MVD/IHC | Association with disease-free survival ($P$ < 0.001) |
| Kang *et al*[60] | 213 patients-63% in stomach, 25.3% in small intestine | 634G/C | Superior OS than 634 G/G ($P$ = 0.054) |
| | | | Superior RFS than 634 G/G ($P$ = 0.082) |
| Mu *et al*[22] | 20 patients | BRD4/mRNA, IHC | Increased BRD4 expression compared with normal tissue |
| | | BRD4/IHC | Associated with poor OS ($P$ < 0.01) |
| | | | Associated with poor DFS ($P$ < 0.01) |
| Toda-Ishii *et al*[61] | 94 patients–mean follow-up period 65 mo | PPP2R1A mutations/PCR | Lower OS ($P$ < 0.05) |
| | | | Lower DFS ($P$ < 0.05) |
| Liu *et al*[62] | 52 patients–27 malignant cases–11 borderline–14 benign | MMP-9, COX-2, VEGF/IHC | Enhance metastasis ($P$ = 0.014, $P$ = 0.010, $P$ = 0.032 respectively) |
| | | | Higher mitotic count ($P$ = 0.021, $P$ = 0.027, $P$ = 0.009 respectively) |
| | | | Higher incidence of central necrosis ($P$ < 0.01) |
| Takahashi *et al*[63] | 53 patients: 21 cases < 30 mm-9 cases with liver metastasis | VEGF/IHC | Association with liver metastasis ($P$ < 0.01) |
| | | VEGF/IHC | Poor 10-yr OS ($P$ < 0.05) |
| | | MVD/IHC | Association with liver metastasis ($P$ < 0.05) |
| Verboom *et al*[64] | 227 patients-36 *SNPs-18* genes, median PFS 39 mo–median OS 86.5 mo | rs1570360 polymorphism in *VEGFA* gene | Association with poorer PFS ($P$ = 0.015) |
| | | rs1870377 polymorphism in *VEGFR2* gene | Association with lower PFS ($P$ = 0.037) |
| Chen *et al*[65] | 62 patients: 31 high risk–31 low risk | HIF-1α/IHC | Association with high risk disease ($P$ < 0.0001) |
| | | | Association with GIST recurrence or metastasis ($P$ = 0.009) |
| Basilio-de-Oliveira and Pannain[66] | 54 patients | VEGF/IHC | Association with survival ($P$ < 0.001) |
| | | CD105/IHC | Association with prognosis ($P$ < 0.001) |
| Imamura *et al*[67] | 95 patients: 64 cases in stomach–31 in small intestine | MVD/IHC | Association with tumor grade ($P$ = 0.036) |
| | | | Association with VEGF expression ($P$ < 0.0001) |
| | | | Association with DFS after surgery ($P$ = 0.0028) |
| Wang *et al*[68] | 68 patients: 20 low risk cases–48 high risk cases | Soluble VEGF | Association with lower DSS ($P$ < 0.05) |
| | | VEGF/IHC | |
| | | MVD/IHC | |

OS: Overall survival; DSS: Disease-specific survival; DFS: Disease-free survival; PFS: Progression-free survival; VEGF: Vascular endothelial growth factor; IHC: Immunohistochemistry; MVD: Microvascular density; HIF: Hypoxia-inducible factor.

importance of angiogenesis in GIST's malignant progression is delineated. In Table 2 are summarized the most significant data.

## CONCLUSION

As highlighted above, angiogenesis mediates an extensive proportion of GIST's malignant dynamics. Several signaling pathways are implicated in the regulation of angiogenesis such as: The VEGF, the fibroblast growth factor-2 (FGF2), the PDGF, the angiopoietins, the Eph/ephrin signaling, the Apelin/APLNR pathway, the HIFs and several chemokines[14]. The VEGF signaling comprises the most well-studied pathway in GIST angiogenesis.

The FGF2/R2 signaling has been extensively studied in GIST as a drug resistance mechanism. Sergei *et al*[54] and Boichuk *et al*[55] demonstrated that the blockage of FGFR2 signaling could enhance the responsiveness to DNA-Topoisomerase II inhibitors[54] while the downregulation of FGF2 signaling might stimulate the response to imatinib[55]. It's contribution in GIST progression has been reviewed [56] but data about potential effects in GIST vascularization process are missing. Towards the same direction, the Eph/ephrin system has been investigated in carcinogenesis[57,58]. It would be of paramount importance an attempt to outline its contribution in GIST angiogenesis.

## FOOTNOTES

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*MINIREVIEWS*

# Stereotactic radiotherapy for intrahepatic cholangiocarcinoma

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## Abstract

Intrahepatic cholangiocarcinoma (iCCA) is an aggressive malignancy with an increasing incidence worldwide and poor prognosis, despite several advances and continuous efforts to develop effective treatments. Complete surgical resection is the mainstay of treatment and offers a potentially curative option, but is only possible in less than a third of patients, owing to advanced disease. Chemotherapy is a well-established treatment in the adjuvant and palliative setting, however, confers limited benefit. Conventional radiotherapy is challenging due to local toxicity. With recent advances in stereotactic ablative radiotherapy (SABR), it is now possible to focus ablative beams of radiotherapy precisely aimed at tumours to minimise damage to surrounding viscera. This review details the history, technical background and application of SABR to iCCA, with directions for future research suggested.

**Key Words:** Cholangiocarcinoma; Intrahepatic; Stereotactic ablative radiotherapy; Stereotactic body radiotherapy; Radiotherapy; Liver cancer; Hepatectomy

**Core Tip:** Intrahepatic cholangiocarcinoma (iCCA) maintains a dismal prognosis despite best available therapy. Complete surgical resection offers a potentially curative option but is feasible in a limited number of cases. This review explores the evolving role of stereotactic ablative radiotherapy (SABR) in the management of iCCA either as an adjuvant to surgical resection, or in cases or recurrent or unresectable disease. Data on the use of SABR as a neoadjuvant/downstaging modality are scarce. Notably, published studies are limited to predominantly retrospective case series. High quality prospective trials evaluating SABR are urgently needed.

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## INTRODUCTION

Cholangiocarcinoma (CCA) is a rare, aggressive malignancy arising from the biliary epithelium. The overall incidence worldwide is less than 6 cases *per* 100000, however, this varies significantly from country to country and is significantly more common in East Asia[1,2], with incidences of up to 90 *per* 100000 reported in Thailand[3].

Prognosis in CCA is dismal with fewer than 10% surviving 5 years after diagnosis. Overall survival (OS) is significantly higher with extrahepatic *vs* intrahepatic tumours (15% *vs* < 5%, respectively)[4]. The reasons for the poor survival are predominantly related to the insidious growth of the tumours, with limited clinical symptoms until the disease is disseminated, by which point surgical resection which is the sole curative option is precluded.

## CLASSIFICATION OF CCA

CCA can be further subdivided by the site of origin in the biliary tract (Figure 1): Intrahepatic CCAs (iCCA) arise from sites proximal to the second order branches of the right or left hepatic duct up to the canals of Hering, while perihilar CCAs (phCCA), also known as Klatskin tumours, arise between the second order branches of the right and/or left hepatic duct and the cystic duct confluence. Distal CCAs (dCCA) arise between the cystic duct confluence and the ampulla of Vater[5-7]. phCCA and dCCAs are collectively termed extrahepatic CCAs (eCCAs) and account for approximately 80% of all diagnoses of CCAs overall, while the remainder are intrahepatic[6,8]. Morphologically, depending on their pattern of growth and appearance, they are categorised in three different types. The mass-forming type, which is the most frequent, accounts for presentation with a mass, the periductal-infiltrating type is characterised by growth along the wall of the bile duct, and the intraductal-growing type by intraluminal growth[7].

Histologically, CCAs can be broadly subdivided into papillary and mucinous carcinomas[9]. iCCAs show greater variability with further subdivision into small and large bile duct cancer. Small bile ducts are lined by cuboidal epithelium and hepatic stem cells, which may be associated with more aggressive tumours and rarely, mixed hepatocellular CCAs. Large bile duct iCCAs are broadly similar to phCCA and dCCA[10].

## PRESENTATION

CCAs are typically asymptomatic in their early stages and manifest clinically only at an advanced stage. Non-specific symptoms such as abdominal pain, night sweats and weight loss may be present in the early stage[11].

Jaundice is a hallmark feature of eCCA as obstruction of large distal bile ducts is needed to obstruct the biliary outflow significantly. Given that iCCAs affect the smaller proximal bile ducts, jaundice is much less frequent, and presentation is more likely to be incidental finding on imaging or after work-up for deranged liver function tests[12].

iCCAs further differ clinically from extrahepatic tumours in that they are more likely to arise on a background of diseased liver parenchyma, much like hepatocellular carcinoma. eCCAs, in contrast, are associated with chronic bile duct inflammation, such as with primary sclerosing cholangitis, choledocholithiasis or, in endemic regions, liver fluke infection[13].

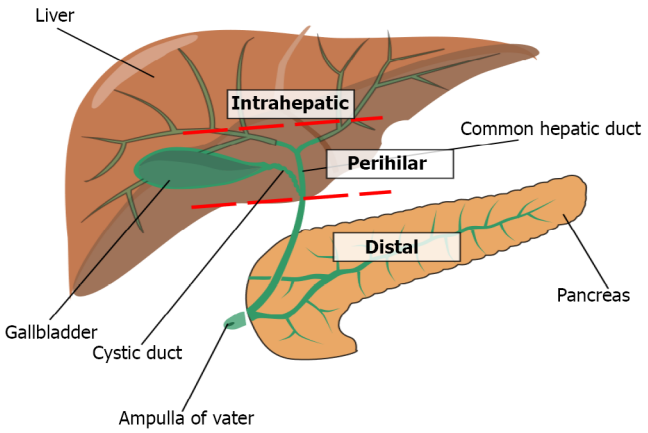


**Figure 1 Anatomical classification of cholangiocarcinoma.** Intrahepatic cholangiocarcinoma-proximal to second order bile ducts; perihilar cholangiocarcinomas-between second order branches of right and/or left hepatic ducts and cystic duct confluence; distal cholangiocarcinoma-between cystic duct confluence and Ampulla of Vater. Citation: Wikimedia Foundation-Licensed under the Creative Commons Attribution-Share Alike 1.0 Generic License. [cited 10 March 2022]. Available from: https://commons.wikimedia.org/wiki/File:Biliary_system_multilingual.svg.

## MANAGEMENT OF CCA

Complete surgical resection is the only prospect for cure in CCA, but this is only possible in < 30% of patients due to advanced disease at presentation[14,15]. Surgery ranges from hepatectomy in iCCA, hepatectomy and/or hilar resection in phCCA, or pancreatoduodenectomy in extrahepatic tumours, to liver transplantation in selected cases of CCA[7,16].

Adjuvant gemcitabine-based chemotherapy is now recommended in most international guidelines [17-20], with evidence of increased disease-free survival (DFS)[21]; overall 5-year survival can reach from 44% in dCCA to 20%-40% in phCCA and iCCA[8,16].

In the palliative setting, data is more robust in supporting chemotherapy with several randomised studies confirming the survival benefit of gemcitabine and platinum-based therapies, with a median progression free survival (PFS) of 8.0 mo[22,23]. Second line chemotherapy with FOLFOX regimens has also been shown to be of limited benefit, with an improvement in OS by 1 mo, although PFS was poor at 8.6% at 1 year[24].

### *Locoregional therapy*

Despite institution of surgery or chemotherapy where appropriate, recurrence rates remain high and, consequently, patient survival is still poor in CCA. Locally advanced disease, oligometastases and medical comorbidities may also preclude surgical intervention. Locoregional therapies such as radiofrequency ablation (RFA)[25] and trans-arterial chemo- or radio-embolization (TACE or TARE, the latter also known as selective internal radiotherapy)[26] have been developed for locally advanced and oligometastatic disease. These therapies have also reduced cancer recurrence as adjuvant therapies along with surgery[27].

Radiotherapy is another alternative treatment modality encompassing standard external beam, brachytherapy and stereotactic forms studied. This has several advantages to RFA and TACE/TARE, in particular being non-invasive and, not requiring the target to be near blood vessels as in TARE/TACE.

Although radiotherapy is not included in guidelines for the treatment of CCA, it has been shown to improve survival *vs* chemotherapy alone for unresectable iCCA in large propensity matched population studies, with reduced hazards of mortality [hazard ratio (HR): 0.80 (95%CI: 0.71-0.91, $P$ = 0.001)][28,29].

Targeted radiotherapy is challenging due to the radiosensitivity of the liver parenchyma and surrounding gastrointestinal tract, which may result in radiation hepatitis, vomiting, diarrhoea and bowel obstruction resulting from stricturing[30,31]. Stereotactic ablative radiotherapy (SABR) allows for high energy beams of radiation focused on target sites avoiding damage to surrounding tissues.

This review gives an overview of the technology of SABR and its application to intrahepatic CCA, which possesses unique characteristics in comparison to other sites.

## SABR

SABR uses multiple beams of radiation focused to a single point in three-dimensional space using a collimation system, as opposed to a single unfocused beam used in conventional radiotherapy. This allows a much larger dose of radiation in a single fraction, whilst avoiding exposure to surrounding tissues[32]. In some cases, the course may be completed in a single fraction. This concept was developed

initially by Phillips *et al*[33] at the Karolinska Institute in Sweden in the 1960s to treat intracranial lesions. Their technology would eventually become known as the Gamma Knife (Elekta Instruments Inc., Tucker GA, United States)[33]. It was not until the early 1990s until similar technology was applied outside the brain. Immobilisation of the patient or tracking of viscera is necessary when targeting the thorax and abdomen to avoid off-target viscera and mitigate against motion such as during respiration [34].

Uematsu *et al*[35] were one of the first to realise the clinical benefits of SABR, in 1998, in patients with locally advanced non-small cell lung cancer who were technically operable but unfit for surgery[35]. Successive studies demonstrated that SABR allowed progression-free survival in 80%-90% of these patients, nearly double that of conventional radiotherapy, with significantly lower toxicity[36].

### SABR in the liver

Following the above reports Herfarth *et al*[37] applied this technology to the liver for unresectable, predominantly metastatic tumours of varying origin. They again showed impressive local control (LC) rates of 81% at 18 mo[37]. Larger, contemporary series of SABR mirror Herfarth's early results in both hepatocellular carcinoma[38] and oligometastatic disease in the liver[39,40]. These series are predominantly observational, and no large-scale interventional trial has been published in this population.

Modern approaches to applying SABR in the liver involve immobilising the abdomen using body moulds or vacuum cushions. Movement from respiration is controlled by using controlled breath holding techniques or respiratory gating or tumour tracking with image guidance. Stereotactic frames and/or implanted fiducial markers may be used to provide a reference for anatomical delineation. The above methods are combined with 4D computed tomography scanning to apply SABR, and accuracy to between 2 and 3 mm is achievable[41,42].

Patients suitable for SABR to the liver, typically have fewer than 3 tumours at no larger than 6cm each, situated greater than 5 mm from adjacent viscera so that ablative doses may be more easily achieved, although these criteria will vary depending on institutional experience[41,42].

The side effect profile of SABR in relation to the liver most commonly consists of nausea and fever, which can be seen within a few hours of treatment. These may be prevented with prophylactic antiemetics[43].

Late side effects include radiation induced liver disease (RILD), which may occur between 2 wk and 8 mo after completion of treatment. This includes clinical symptoms of fatigue, tender anicteric hepatomegaly and ascites. Biochemically, there is elevated alkaline phosphatase, whilst transaminases and bilirubin remain normal[44].

Non-classical RILD (typically in patients with underlying liver disease) occurs within 3 mo of radiotherapy and consists of liver enzymes more than five times the upper limit of normal or a decline in liver function as measured by a worsening Child-Pugh score of 2 or more in the absence of classical RILD.

These occur in less than 5% of patients and are associated with cumulative doses (in conventionally fractionated radiotherapy) higher than 30-32 Gy and 28 Gy in patients with underlying liver disease.

Other specific toxicities are related to off-target effects on the gastrointestinal tract, with nausea, vomiting and diarrhoea being common. Other effects are common to all radiation therapies, and these include skin necrosis (much less common in the era of volumetric modulated arc therapy) and systemic effects such as fatigue and fever. It should be reiterated that these side effects, when they do occur, are typically milder and less frequent than with equivalent conventional radiotherapy[45].

## APPLICATION OF SABR IN ICCA

As mentioned above, the standard of care for curative treatment of iCCA is surgical resection followed by adjuvant chemotherapy. For palliative treatment, chemotherapy with gemcitabine and platinum regimes are recommended[17,18]. We therefore focus on five scenarios where SABR may be useful in the treatment algorithm: (1) Primary therapy in patients with technically resectable disease but precluded from resection due to medical comorbidities; (2) Primary therapy in technically unresectable disease; this may be due to diffuse or metastatic disease; (3) Recurrent disease after surgical resection; (4) Following surgical resection to prevent local recurrence (adjuvant therapy); and (5) As a downstaging modality before surgery (neoadjuvant). Relevant studies are summarised in Table 1.

### SABR as primary therapy in medically unresectable iCCA

Shen *et al*[46] reported data on SABR in inoperable iCCA. In this series 12/28 (42.8%) were inoperable due to medical co-morbidities or advanced age whilst the remainder were technically inoperable. Data was not stratified by the reason for inoperability, although on multivariable analysis, there was no difference in response based on this. The overall disease control rate with SABR was 89.3%, of which 42.9% had stable disease, 35.7% a partial response and 10.7% a complete response at first follow-up (median 16 mo). Predictors of successful response were median biologically effective doses (BED) of > 100 Gy and having solitary lesions. Median OS was 15.0 mo and median PFS was 11.0 mo. OS and PFS

**Table 1 Summary of published studies of stereotactic body radiotherapy in intrahepatic cholangiocarcinoma**

| Ref. | Country | Design | Patient characteristics (reason for inoperability) | Total patients | No. iCCA (%) | Median follow-up/months (range) | Outcomes (1 yr)[1] | | | Major side effects (CTC > 3) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Local control (%) | Progression free survival (%) | Overall survival (%) | |
| Shen *et al* [46], 2017 | China | Retrospective | Unresectable: (1) 7/28 Medical; (2) 16/28 Technical; and (3) 5/28 Advanced age | 28 | 28 (100) | 16 (3-42) | 89.3 | 50.0 | 57.1 | 0 |
| Liu *et al*[47], 2017 | Taiwan | Retrospective | Unresectable: (1) Medical 3/15; and (2) Surgical 12/15 | 15 | 12 (80) | 29.0 | 48.5 | - | 50.3 | 0 |
| Thuehøj *et al* [48], 2022 | Denmark | Retrospective | Unresectable, locally advanced | 41 | 15 (37) | 9.5 (0-66.5) | 85.4 | 31.7 | 48.8 | - |
| Tao *et al*[49], 2016 | United States | Retrospective | Unresectable, locally advanced | 79 | 79 (100) | 24 (4-33) | 81.0 | 91.0 | 87.0 | 0 |
| Tse *et al*[51], 2008 | Canada | Prospective, phase I | Unresectable, locally advanced (includes HCC) | 41 | 10 (24) | 17.6 (range 10.8-39.2) | 65.0 (all patients) | - | 58.0 | 0 |
| Mahadevan *et al*[52], 2015 | United States | Retrospective | Unresectable: (1) Medical 3/34; and (2) Surgical 29/34. R1 Resection: 2/34 | 34 | 31 (91) | 38 (8-71) | 88.0 | - | 58.0 | 0 |
| Barney *et al* [53], 2012 | United States | Retrospective | Unresectable: 6/12 lesions. Recurrent: 6/12 lesions | 10 | 6 (60) | 14 (2-26) | 100% | - | KM 73.0% | 0 |
| Brunner *et al* [54], 2019 | Germany and Switzerland | Retrospective, multicentre | Unresectable, unclear reasons | 64 | 41/82 lesions (50%) | 35 (7-91) for survivors | 89 | - | 81 | 0 |
| Weiner *et al* [55], 2016 | United States | Prospective, phase I | Unresectable, locally advanced (includes HCC) | 26 | 14 (54) including 2 biphenotypic ICCA and HCC | 8.8 (0.3-33) | 91 (all patients) | 68 | 51 | Grade IV lymphopenia-1 patient; Grade V hepatic failure-2 patients |
| Kozak *et al* [56], 2020 | United States | Retrospective | Unresectable disease | 40 | 26 (63) | 18 (1-100) | 70 (all patients) | - | 66 (all patients) | 0 |
| Sebastian *et al* [59], 2019 | United States | Retrospective, population database study, comparative study between SABR, TARE and CRT | Unresected, locally advanced disease | 27-SABR; 52-CRT; TARE-60 | 141 (100%) | 17 | - | - | Propensity matched hazard ratio of overall survival for SABR *vs* CRT-0.22; *vs* TARE 0.58 | Not reported |
| Jung *et al*[60], 2014 | South Korea | Retrospective | Unresectable and recurrent disease after | 28-Unresectable; | 33 (57) | 10 (1-97) | Unresectable-76; Recurrent-91 | Overall-26 | Unresectable-29; Recurrent-53 | 2-Cholangitis; 1-Gastric perforation |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | surgery | 30-Recurrent | | | | | | |
| Franzese *et al* [61], 2020 | Italy | Retrospective | 49/51 (96%) Recurrent metastatic disease after surgical resection | 51 (includes GB adenoCa) | 34 (66)-iCCA and eCCA grouped together | 14 (3-95) | 74.7 | 32.8 | 63.2 | 0 |
| Ibarra *et al* [62], 2012 | United States | Retrospective | Unresectable disease | 21-HCC; 11-iCCA | 11 (34) | 7.8 (1.4-17.9) | 55.5 | - | 45 | 0 |

[1]Survival and control figures are for intrahepatic cholangiocarcinoma subgroup unless otherwise specified.

iCCA: Intrahepatic cholangiocarcinoma; CTC: Common toxicity criteria; HCC: Hepatocellular carcinoma; SBRT: Stereotactic body radiotherapy; TARE: Trans-arterial radio-embolization; GB adenoCa: Gallbladder adenocarcinoma; eCCA: Extrahepatic cholangiocarcinoma.

were 32.1% and 21.4% at 2 years, respectively[46].

A Taiwanese study included patients with solely medically inoperable tumours (14/15 iCCA). 1- and 2-year OS were 50.3 and 14.4%, while LC was achieved in only 48.5% at 1 year. The reason is likely the lower BED used at 45 Gy and the authors reported significantly higher survival with doses at > 75 Gy, with 1-year OS at 58.3%[47]. A Danish study with predominantly patients with eCCA but who were also medically inoperable showed similar OS and LC rates[48].

The largest study of SABR in iCCA (79 patients) showed 1-year OS of 87% and 3-year OS of 44%. LC rates were 81% and 31%, respectively, for the same time period with a PFS of 88% and 39%. Patients in this study were excluded if treatment was directed with palliative intent, which may explain the higher survival rates, although the authors' definition of this is unclear. All patients had favourable performance status: 94% scored at 0 or 1, 6% scored 2 and no patients had performance status > 2. 20% of patients had extrahepatic metastatic disease and 58% had nodal disease, implying a poor prognosis pre-treatment[49]. Nevertheless, the survival figures in this study are similar to curative resection, which according to a recent review confers an overall 3-year survival ranging from 32% to 47% and a similar 3-year recurrence free survival which is between 6 to 47%[50]. Survival also correlated with the radiation dose, with a BED greater than 80.5 Gy associated with 3-year OS of 73% *vs* 38% for patients receiving lower doses.

These results may suggest that SABR could be a suitable alternative to surgical resection in patients unfit for surgery, however comparative studies, in particular, randomized trials are needed to confirm this.

### *SABR as primary therapy in technically unresectable iCCA*

Tse *et al*[51] provided one of the first reports of SABR in iCCA. Their phase I study included 10 patients with iCCA who were unresectable due to metastatic disease, pre-dominantly confined to the liver or with locoregional lymphadenopathy. The median OS was 15.0 mo with 58% 1-year OS[51].

In Mahadevan *et al*'s retrospective study of locally advanced 31 iCCAs (11 further phCCAs or dCCAs), 1-year OS was 58% and 4-year OS was 19%. LC was achieved in 88% at 1 year and 79% at 4 years for the overall cohort. Median PFS was 11 mo after SABR[52].

Barney *et al*[53] performed a retrospective study consisting predominantly of patients with either primary or recurrent oligometastatic disease. OS was 73% at 1 year and LC was achieved in 100% of

patients (of whom 25% had a complete response and 42% a partial response). 40% of patients had PFS [53].

A large multicenter German and Swiss study with 64 patients (41 iCCA) showed 1-year OS of 63% and LC at 89%. After multivariable analysis, as above, improved survival and LC were achieved with higher radiation doses, without a significant increase in toxicity[54].

Weiner *et al*[55] performed a phase II study of SABR in unresectable primary liver lesions of which 14/26 (54%) were iCCA or biphenotypic with HCC. 1-year OS was 51% and PFS was 68% with only 2 of 26 (4%) patients in the study having local progression at the SABR site[55].

Kozak *et al*[56] performed a retrospective study of SABR in 40 patients with unresectable CCA (23 patients iCCA and the remainder phCCA) assessing the location of failure with respect to the radiation field. Median OS for patients with iCCA was 10 mo, 1-year OS for the entire cohort was 66%, and median follow-up was 18 mo. 12 patients (30%) had in-field local failure, whilst seventeen (42.5%) had out of field hepatic failure. Seven patients (17%) experienced regional failure predominantly in perihilar and para-aortic nodes, whilst 15 patients (37.5%) had distant failure of which the lungs were the most common site of progression (7 patients, 46.7%)[56]. Given the high rates of out of field recurrence, the authors proposed elective nodal irradiation in the perihilar space to prevent regional recurrence, however there are no trials on this.

Bisello *et al*[57] proposed a series of guidelines on clinical target volumes for biliary tract cancers, including iCCA, to incorporate sites of potential regional progression. They proposed a margin of 9.8mm from the primary tumour boundary to incorporate all microscopic spread[57]. This is at the cost of potential for increased toxicity, in particular around the central biliary tree with suggested dosing limited to for example 42 Gy in 15 fractions or 35 Gy in 5 fractions[58].

One study compared SABR to TARE and conventional chemoradiotherapy in unresectable iCCA using the United States National Cancer Database. Median OS was 20 mo with SABR and significantly greater than TARE and chemoradiotherapy after adjusting for confounders with propensity weighting and multivariable regression [HR: 0.44 (95%CI: 0.21-0.91)][59].

Of note, Jackson *et al*[28] performed a propensity matched study of patients with inoperable iCCA identified from the United States National Cancer Database comparing patients who received any form of radiotherapy (not specifically SABR). After propensity score matching, they showed that the addition of radiotherapy to the standard chemotherapy regimen significantly reduced the hazards of death [HR: 0.83 (95%CI: 0.71-0.97, $P$ = 0.018)][28].

### *SABR for recurrent iCCA*

Jung *et al*[60] studied patients with unresectable and recurrent disease, of which 57% were iCCAs. 1- and 2-year OS in the recurrent disease group were 53% and 28%, respectively, LC rates were 91% and 81%, respectively, at the same time periods. Overall PFS for all patients were 26% and 23% at 1 and 2 years. Of note, 2 patients developed transient liver failure following SABR in this study[60].

Franzese *et al*[61] performed a retrospective study of SABR in recurrent biliary tract cancer after surgical resection, of which 18/51 (35%) had iCCA. 1-year OS and PFS were 63.2% and 32.8%, respectively, whilst LC rates were 74.7% at 1 year[61].

Ibarra *et al*[62] performed a small multi-centre study of 11 patients undergoing SABR for iCCA, with 50% reported as undergoing this following surgical resection and recurrence (the remainder were for unresectable disease, of whom 45% had distant disease). 1-year survival was 45% and LC was estimated to be 55.5% in this study[62].

### *SABR as adjuvant treatment for incomplete (R1) resection*

Hammad *et al*[63] performed a study using the United States National Cancer Database of patients with iCCA who underwent surgical resection. Of the 525 out of 2897 patients who underwent postoperative conventional radiotherapy, 230 (43.8%) had positive resection margins, compared to 704 (24.3%) in the non-radiotherapy group. There was no significant OS benefit [0.99 (95%CI: 0.84–1.16) $P$ = 0.931] for patients who underwent radiotherapy, after propensity score matching and multivariable Cox regression. LC and PFS were not reported[63].

Kim *et al*[64] published a small case series of 18 patients with incompletely resected iCCA (R1) of whom 7 underwent adjuvant chemoradiotherapy. They found significant increases in OS, LC and PFS with chemoradiotherapy: (LC: 5.6 mo *vs* not reached, $P$ < 0.001, PFS: 5.6 mo *vs* 8.3 mo, $P$ = 0.047, OS: 15.0 mo *vs* 26.6 mo, $P$ = 0.064)[64].

While there are no large studies of SABR specifically, given its advantages over conventional radiotherapy, the above studies could be regarded as showing some promise in its potential use for incomplete resection.

Studies on SABR as standard adjuvant therapy following resection of iCCA are limited, however there is a limited number of studies evaluating conventional radiotherapy following resection.

Jiang *et al*[65] assessed adjuvant conventional radiotherapy where macroscopic regional lymph nodes were identified following surgical resection on imaging. Out of 100 patients, 24 received radiotherapy, whilst 76 did not, but it was not specified whether the latter patients received any further treatment. Median OS was significantly superior at 68.8% in the radiotherapy group and 12.1% in the non-radiotherapy group ($P$ = 0.01). After multivariable analysis, radiotherapy was independently associated

with survival [HR: 0.482 (95%CI: 0.27-0.86)][65]. A further meta-analysis of studies assessing adjuvant radiotherapy in iCCA did not show a significantly improved patient survival[66].

### SABR and locoregional treatments as a neoadjuvant/downstaging modality

Studies assessing SABR for downstaging of iCCA (neoadjuvant therapy) have mainly focused on doing this to allow liver transplantation. Wong *et al*[67] and Sandler *et al*[68] both reported impressive OS of 80 and 75% at 1 year in the few (4 in each study) patients who underwent liver transplantation following successful SABR. However, 18/22 (82%) in Wong's study and 27/31 (87%) patients in Sandler's failed to proceed to transplant, predominantly due to tumour progression.

Conventional chemoradiotherapy has been attempted with promising results in a small case series. Of 7 patients with locally advanced, unresectable iCCA, five (71.4%) became resectable following chemoradiotherapy and one patient remained disease free after resection at 18 mo. 5-year OS was 23.6% [69].

Rayar *et al*[70] reported their experience of using TARE as a downstaging modality for unresectable iCCA. Of 45 patients who underwent downstaging TARE and chemotherapy, eight (17.7%) ultimately underwent surgical resection with curative intent. With a median follow-up of 15.6 mo, only two patients died perioperatively and only one died from unrelated disease. Of the remainder, two were found to have recurrence at follow-up[70]. Similarly, Edeline and colleagues reported a similar proportion of patients with iCCA downstaged to resectability (9/41, 22%) with TARE, a further two patients remained unresectable, but underwent liver transplantation. For the resected patients, 1-year OS was 88.9% and DFS was 66.8%. For both of the patients undergoing liver transplantation, solitary lung recurrence occurred at 15 and 16 mo and both were alive at 19 and 18 mo of follow-up[71].

### Side effects and quality of life

Side effects were shown to be transient and mild in the majority of patients in these studies of SABR. Those studies which reported liver function tests, showed mildly deranged values of all parameters (alkaline phosphatase, alanine transaminase, aspartate transaminase and bilirubin) in most patients following SABR. Very few studies reported greater than 40% of patients having grade II symptoms. Of these, the majority are gastrointestinal side effects with nausea and diarrhoea being common.

Although bowel obstruction and perforation may be complications of radiotherapy, only one case of gastric perforation requiring surgery was found in the studies included in the review. Radiation hepatitis was rare and liver failure was reported in only 2 patients in all the studies included in this review.

One study evaluated the quality of life in patients undergoing SABR in the liver and showed a reduction in quality of life in terms of appetite and fatigue within 1 mo of treatment but returning to baseline after 3 mo. These features demonstrate overall that SABR is tolerated well, relative to other therapies[72].

## CONCLUSION

### Current and future directions for research

A search of the clinicaltrials.gov registry (search terms "cholangiocarcinoma" and "stereotactic") showed 2 actively recruiting trials evaluating stereotactic radiotherapy. Of these two, the CORRECT trial (NCT03898895) is a multicentre randomized trial evaluating a programmed cell death ligand 1 checkpoint inhibitor (Camrelizumab) with either SABR or conventional radiotherapy *vs* standard gemcitabine chemotherapy in unresectable iCCA[73]. The second is a phase II trial of nivolumab with SABR in unresectable iCCA and dCCA[74].

Of the remainder, 4 studies assess all types of liver tumours, 2 assess phCCA only, and the rest assess a mix of extrahepatic and intrahepatic tumours. These are all phase I and II trials.

In addition, the ABC-07 trial is actively recruiting and is a multicentre randomized controlled trial comparing chemotherapy *vs* chemotherapy and SABR in unresectable CCA (of all types) and gallbladder carcinoma[75].

Furthermore, the ACCTICA-1 trial is primarily assessing the superiority of gemcitabine and cisplatin *vs* capecitabine in patients with resected CCA and gallbladder adenocarcinoma. However, within this trial there is a sub-study evaluating conventional radiotherapy in patients with R1 resections[76,77].

Thus far, there have been no published randomized trials of SABR in any subgroup of iCCA, and the majority are retrospective single institution studies. Few studies have compared SABR to a control group or other locoregional therapies. There is limited literature on SABR as a downstaging modality prior to standard surgical resection of iCCA, despite evidence of excellent LC in patients who are inoperable.

High quality prospective clinical trials of SABR are urgently needed in homogeneous groups of iCCA, to explore its role as an adjuvant and neoadjuvant therapy either prior to resection or liver transplantation, and as a treatment modality in recurrent and unresectable disease.

## FOOTNOTES

**Author contributions:** Mavroeidis VK conceptualised and designed the study; Borakati A did the literature search; Borakati A and Froghi F did the analysis, interpretation of data and drafted the original manuscript; Bhogal RH and Mavroeidis VK supervised and made critical revisions; all authors prepared the final draft and approved the final version.

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*MINIREVIEWS*

# How the COVID-19 pandemic has affected the colorectal cancer screening in Italy: A minireview

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## Abstract

The coronavirus disease 2019 (COVID-19) pandemic has caused detrimental effects on many aspects of healthcare practice. Screening programs for the commonest malignancies, namely colorectal cancer (CRC), breast cancer and cervical cancer have been discontinued or interrupted since the beginning of restriction measures aimed to limit transmission of the new coronavirus infection. Robust evidence exists in favour of the role of screening campaigns in reducing mortality from CRC. In fact, the majority of pre-malignant lesions of the colon and rectum can be diagnosed with colonoscopy and treated by endoscopic or surgical resection. Besides, colonoscopy screening allows the diagnosis of CRCs in their pre-clinical stage. Italy was one of the first European countries where a high level of COVID-19 infections and deaths was observed, and one of the first where lockdowns and strict measures were adopted to reduce the risk of COVID-19 diffusion among the population. A systematic review of the literature was performed, including the PubMed, Scopus, Web of Sciences, and Reference Citation Analysis databases, with the aim of critically evaluating the impact of the COVID-19 pandemic on CRC screening in Italy. We found that reduction of CRC screening activity surpassed 50% in most endoscopic units, with almost 600000 fewer CRC screening exams conducted in the first 5 mo of 2020 *vs* the same period of 2019. While the consequences of the discontinuation of endoscopy screening for the prognosis and mortality of CRC will be evident in the next few years, recent data confirm that CRC is currently treated at a more advanced stage than in the pre-COVID-19 era. Since delays in CRC prevention and early diagnosis may

translate to increased CRC-specific mortality, world healthcare systems should adopt strategies to maintain the regularity of CRC screening during subsequent peaks of the COVID-19 pandemic, or future events that might hamper screening programs.

**Key Words:** COVID-19; Colorectal cancer screening; Italy; Minireview



**Core Tip:** Screening is a key component of colorectal cancer control. As in the rest of the world, the coronavirus disease 2019 (COVID-19) emergency has interrupted the regular delivery of cancer screening services in Italy. As a consequence, significant delays in the diagnosis and treatment of malignant and pre-malignant lesions have occurred, with possible effects on disease prognosis. Screening activity has gradually resumed after the first wave of the pandemic. The healthcare system is called on to be prepared to prevent the potential suspension of new rounds of screening during the COVID-19 pandemic or future extraordinary events that might hamper screening programs.

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## INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males, the second in females, and the second leading cause of cancer death. Although incidence and mortality vary between countries, according to GLOBOCAN estimates, worldwide, the year 2020 saw 1.93 million new CRC cases diagnosed and 0.94 million deaths caused by CRC. The incidence of the disease is increasing in high-income countries, where it has traditionally been higher, as well as in middle- and low-income countries[1,2]. Robust evidence exists about the role of screening programs in reducing mortality from CRC. CRC screening includes a faecal occult blood test (FOBT) to detect blood in stool that may originate from a neoplastic or pre-neoplastic lesion, as well as colonoscopy. The latter allows either biopsy of early CRC or lesion removal at the time of the test.

In the last 2 years, population screening programs for the commonest cancers have been devastated by the spread of the coronavirus disease 2019 (COVID-19) pandemic[3-7]. In fact, screening has been deprioritized as healthcare resources have been reoriented toward treatment and prevention of the new coronavirus infection. Besides, many people have avoided hospitals and screening services for fear of contracting COVID-19.

Italy was one of the first countries in Europe to be affected by COVID-19, and measures taken to contain the spread of COVID-19 infection were more restrictive than those in other countries from the onset of the pandemic.

This review aims to critically evaluate the impact of the COVID-19 outbreak on CRC screening programs in Italy. We also discuss projected effects of delayed CRC diagnosis and treatment due to discontinuation of screening.

## LITERATURE SEARCH AND STUDY SELECTION

The present review focused on the literature covering the topic of CRC screening in Italy during the COVID-19 era. A systematic literature search using the PubMed, Scopus, Web of Science, and Reference Citation Analysis databases was conducted in February 2022. The following keywords were used and combined for the search: 'colorectal', 'colon', 'rectal', 'cancer', 'carcinoma', 'malignancy', 'screening', 'screening program', 'COVID', 'COVID-19', 'SARS-CoV-2', 'coronavirus', 'Italy' and 'Italian'. Articles published in English from January 1, 2020 to January 31, 2022 were retrieved, screened and selected by two independent authors. Relevant data were extracted into a standardized data collection sheet by three authors. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses[8] guidelines were used to create a flowchart, which is shown in Figure 1.

The final inclusion criteria were observational retrospective studies, surveys or national and regional database-based studies that presented numerical analyses and comparisons of CRC screening results

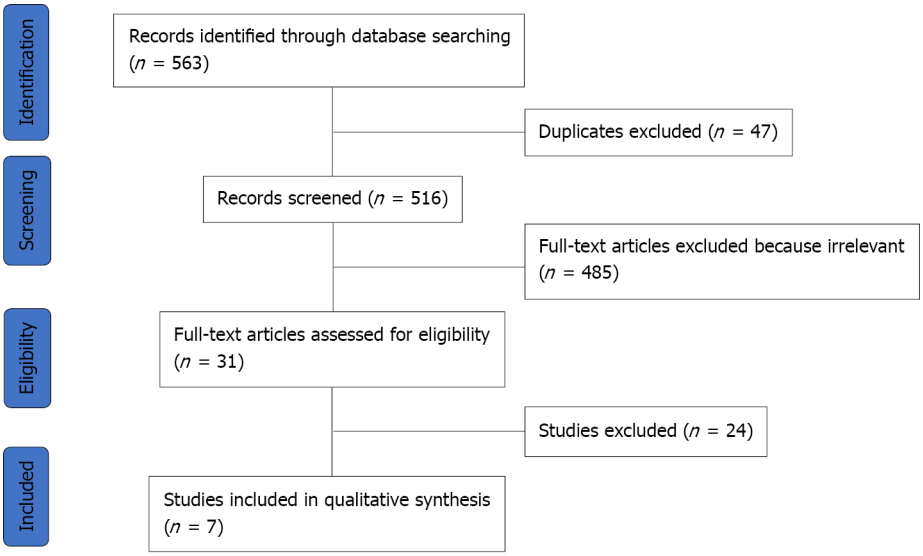


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**Figure 1 PRISMA flow diagram showing selection of studies.**

between the COVID-19 and pre-COVID-19 eras.

At the time of this review, a total of seven articles had been finally selected from a comprehensive number of 563 published studies[6,7,9-13]. The included articles are briefly summarized in Table 1. The outcomes were defined as percentages or overall proportions. Due to the nature of the work (minireview), no formal statistical analyses were conducted. Most of the studies (71%) focused their attention on the first semester of 2020, coinciding with the first COVID-19 burst in Italy[7,9,11-13]. The remaining two articles (29%) analysed a broader time frame of almost the entire year of 2020[6,10]. No articles were found concentrating on the 2021 situation, even though the pandemic was ongoing in its third and fourth waves. The studies ranged from single-unit experiences[7] to nation-wide surveys including all Italian regions[9] or evaluating more than 100 units across the country[12]. All of them focused on the reduction of endoscopic screening exams for CRC and the decrease in CRC new diagnoses in the analysed period with respect to the same temporal window in the previous years, namely 2018 and 2019.

## EPIDEMIOLOGY OF CRC AND SCREENING PROGRAMS IN ITALY

According to the Italian Minister of Health, in the year 2020, about 43700 people were diagnosed with CRC (20282 women and 23420 men), and about 20000 died from the disease[14]. The 5-year survival rate for CRC in Italy is 65.3% in men and 65.3% in women[15]. CRC is one of the most preventable of all cancers, and regular screening is one of the most powerful preventive tools. Screening is the process of looking for cancer or precancerous lesions in people asymptomatic for the disease. The key usefulness of screening is that most CRCs develop following the so called 'adenoma-carcinoma sequence'. Benign adenomatous polyps usually take several years to develop into CRC. With regular screening, most polyps can be detected and safely removed before they turn into forms of invasive carcinoma. Besides, screening can permit the diagnosis and treatment of early forms of CRC, thus increasing the possibility of a cure. The main aim of CRC screening is to decrease mortality from the disease[15-17]. There is evidence that the introduction of CRC screening programs in the early 2000s has substantially reduced mortality rates in European countries[18-21]. In Italy, CRC screening programs organized by the public health system cover the population of the entire country. In most regions, a FOBT by the immuno-chemical technique is offered every 2 years to all men and women aged 50–69 years, who are at the highest risk of developing the disease. In some regions, such as Piedmont, flexible sigmoidoscopy or FOBT are offered once in a time to people aged 58–69 years. The widespread use of FOBT in Italy has led to a progressive reduction in the incidence and mortality from CRC, the latter thanks to detection of CRC in its early stage. Data from 48 cancer registries from 17 Italian regions reported a reduction in the CRC incidence rate from 104.3 and 64.3 *per* 100.000 in the year 2003 to 89.9 and 58.4 *per* 100000 in 2014 in men and women, respectively. Besides, in the same time frame, mortality rates decreased from 41.1 to 39.2 *per* 100000 in men and from 24.6 to 23.1 *per* 100000 in women[15]. Data from randomized studies have demonstrated that both FOBT and flexible sigmoidoscopy have proven efficacy in reducing mortality from CRC by 22% and 28%, respectively[15,22]. In Italy, the most often used approach to CRC screening is to invite the target population by mail to undergo FOBT. Men and women with negative

**Table 1 Studies reporting on the effects of coronavirus disease 2019 pandemic on colorectal screening in Italy**

| Ref. | Centers participating in the study | Time frame | Main conclusions |
|---|---|---|---|
| Armaroli *et al*[9] | 20 out of 21 regions involved | January-May 2020 *vs* January-May 2019 | (1) Cumulative delay of colorectal screening = 585.287 less exams (54.9%); (2) Esteemed delay of diagnosis of 3953 high-risk colonic adenomas and 611 colon cancer cases; and (3) Esteemed delay in diagnosis of 2.7 mo |
| Germana *et al*[10] | Veneto regional screening database | January-November 2020 *vs* same period in 2018-2019 | (1) 453877 people invited to undergo FOBT, within the regional colorectal cancer screening program, 115976 fewer than the previous two years (-20.4%), with an adherence rate that dropped from 65.2% to 54.2%; (2) Colonoscopies fell by 22.2% (67138 in 2020 ss. 86298 for the years 2018-2019); and (3) The reduction was of 13.1% for screening colonoscopies following a positive FOBT, and 24.9% for non-screening colonoscopies |
| Buscarini *et al*[6] | 49 units across Italy: 32 from the North (65.3%), 6 from the Center (12.2%), and 11 from the South (22.4%) | January-October 2020 *vs* same period in 2017, 2018 and 2019 | (1) CRC new diagnoses decreased by 11.9%; and (2) The 2019–2020 comparison showed fewer CRC diagnoses in the North (-13.7%), Center (-16.5%) and South (-4.1%) |
| Ferrara *et al* [11] | 7 Units in Northern-Central Italy | 11$^{th}$-20$^{th}$ week of 2020 *vs* same period in 2018 and 2019 | Decrease of 46.6% of new colorectal cancer diagnosis with screening program (335 in 2018-2019 and only 178 in 2020) |
| De Vincentiis *et al*[7] | Single Unit audit | 11$^{th}$-20$^{th}$ week of 2020 *vs* same period in 2018 and 2019 | CRC new diagnoses fell in 2020 by 62% compared with the average number in 2018 and 2019. CRC was identified as carrying a potentially important diagnostic delay |
| Maida *et al* [12] | 121 Units from 20 Italian regions | Survey between March 30, 2020 and April 7, 2020 | (1) 49 (46.7%) of 105 gastroenterology divisions had suspended their endoscopic screening program for colorectal cancer during the COVID-19 pandemic; (2) Overall, 10.7% Gastroenterology Divisions have been converted to Covid Units; and (3) Endoscopic procedures were limited to urgencies and oncology indications |
| Repici *et al* [13] | 41 EUs across Northern Italy | Survey between March 16, 2020 and March 21, 2020 | (1) 75%–99% reduction in activity in 28% of endoscopic units, a 50%–75% reduction in 9% of units, with only a single unit maintaining its workload unchanged; and (2) Most EUs limited their activity to urgent cases, including patients at high-risk of cancer |

EUs: Endoscopic Units; CRC: Colorectal cancer; COVID-19: Coronavirus disease 2019; FOBT: Faecal occult blood test.

FOBT are recalled to repeat the test 2 years later. Those who do not respond to the first call are contacted by mail a second time within 6 mo. Patients with positive FOBT are contacted by phone to undergo a total or virtual colonoscopy (computed tomography colonography) in the case of incomplete colonoscopy[15]. When colonoscopy or sigmoidoscopy detects neoplasms, patients are directed to surgery or endoscopic surgery and enrolled in a follow-up program. Despite being a less tolerated and operator-dependent examination, colonoscopy leads to a complete exploration of the entire colorectal lumen and is much more sensitive than flexible sigmoidoscopy, based on indirect evidence and observational studies[23].

## EPIDEMIOLOGY OF COVID-19 IN ITALY

Italy was the first European nation to be affected by COVID-19. The first Italian cases of COVID-19 date back to January 30, 2020, when two tourists tested positive by nasopharyngeal COVID-19 Test in Rome. In February 2020, in the city of Codogno, located in the Northern region of Lombardy, a 38-year-old man was hospitalized for respiratory symptoms and tested positive; the day after, 60 cases of COVID in Codogno were diagnosed[24]. During this first COVID-19 wave, the Italian Healthcare Service was near collapse, registering in just 1 mo almost 40000 total cases and 3000 deaths (March 2020)[25]. From then onwards, Italy underwent three further pandemic waves, like most other countries in Europe. In that period, the development of vaccines contributed dramatically to proper management of the pandemic crisis[26]. As of March 14, 2022, 13402905 positive cases were registered in Italy, including 12242669 discharged and healed people, 156997 deaths and 1003239 active cases[27]. Italy ranks 9$^{th}$ in the world and 5$^{th}$ in Europe for the total number of cases, and 8$^{th}$ in the world and 3$^{rd}$ in Europe for the absolute number of deaths. Furthermore, Italy ranks 53$^{rd}$ in the world for total cases *per* capita and 25$^{th}$ for total deaths *per* capita[26].

## EFFECTS OF THE COVID-19 PANDEMIC ON CRC SCREENING IN ITALY

The DECOR-19 DElayed CRC care during the COVID-19 Pandemic was a global perspective from an international survey, where the highest number of respondents (1051) were from Italy. Of note, endoscopic procedures for CRC were the diagnostic techniques most affected by the COVID-19 emergency (73.7% of respondents). CRC surgery was delayed in 58.3% of institutions. For 90% of respondents, the delay was 5–8 wk beyond the normal wait time and for the remaining 10%, more than 8 wk[28].

The Italian National Screening Observatory reported on the accumulated delay experienced by organized screening programs up to May 2020. In the first 5 mo of 2020 *vs* the same period of 2019, 585287 fewer CRC screening exams were conducted, accounting for a 54.9% decrease[9]. Based on these numbers, an estimated 1168 CRCs and 6667 advanced adenomas would have been missed in the period from January 2020 to September 2020[29].

A survey was conducted by the National Centre for Screening Monitoring on cervical, breast and CRC screening activities conducted in 2020. Screening tests for CRC decreased by 45.5% in 2020 compared with 2019, with an estimated 1299 CRC cases going undiagnosed. Interestingly, participation in CRC screening programs decreased by 20%[30].

In a study investigating the Cancer Diagnostic Delay in Northern and Central Italy During the 2020 lockdown, a comparison was made among the number of first pathologic diagnoses of malignancy made from weeks 11 to 20 (April and May) of 2018, 2019 and 2020 at seven pathology units serving secondary care hospitals in Northern-Central Italy. A consistent decrease of 46.6% in new CRC cases diagnosed by screening programs (335 in 2018–2019 and only 178 in 2020) was observed[11].

The number of people who responded to invitations for FOBT screening in the region of Veneto in 2020 was about 16000 less than in the previous 2 years, with an adherence rate that decreased from 65.2% to 54.2%. Colonoscopies fell by 22.2% (67138 in 2020 *vs* 86298 for the period 2018–2019); the rate reached its lowest in April (-70.4%). There was a 13.1% reduction in screening colonoscopies following a positive FOBT and a 24.9% reduction in non-screening colonoscopies ($P < 0.001$)[10].

In a national survey, CRC diagnoses decreased by 11.9% from 2019 to 2020. A comparison between 2019 and 2020 showed fewer CRC diagnoses in the North (-13.7%), Center (-16.5%) and South (-4.1%)[6]. The authors performed an audit to evaluate the impact of COVID-19 pandemic-related delays in the diagnosis of major cancers at a Pathology Unit of a Secondary Care Hospital Network in Italy[7]. Cancer diagnoses fell in 2020 by 39% compared with the average number recorded in 2018 and 2019, and CRC was the tumour type with the greatest decrease.

A multicentric study evaluated the impact of the 2019 outbreak on 41 Italian endoscopic units. In 27 (65.9%) units, endoscopists were relocated to other hospital departments. In 31 (75.6%) units, nurses were relocated to other hospital departments. Most endoscopy units limited their activity to urgent cases, also including patients at high risk of cancer. After the COVID-19 outbreak, 39 endoscopy units (95.1%) continued to perform urgent procedures, 39 (95.1%) continued inpatient procedures and 28 (68.3%) continued screening colonoscopies for CRC. In quantitative terms, this corresponded to a 75%–99% reduction in activity in 28% of endoscopic units and to a 50%–75% reduction in 9% of units, with only a single unit maintaining its workload unchanged. Finally, most EUs limited their activity to urgent cases, including patients at high risk of cancer[13]. Examining Gastroenterology Divisions in Italy, a national survey that analysed data between March and April 2020 underscored that 46.7% of gastroenterology divisions had suspended their endoscopic screening programs for CRC during the COVID-19 pandemic, 10.7% of Gastroenterology Divisions had been converted to COVID units, and endoscopic procedures had been limited to urgencies and oncology cases in 96.2% of units[12].

Similar data were reported from countries outside Italy. In South Australia, the total number of colonoscopies decreased by 51.1% from 2019 to 2020[31]. In the United Kingdom, endoscopic cancer detection was reduced by 58% overall and by 72% for CRC in particular during the period impacted by COVID (March–May 2020)[32]. In France, roughly 250000 fewer colonoscopy preparations were dispensed during the first 6 mo of the COVID-19 pandemic[33]. In Hong Kong of China, the mean number of lower endoscopies performed *per* week decreased by 51.0% after the beginning of the pandemic[34]. The number of obstructive CRCs in Japan has increased during the COVID-19 pandemic, as a possible consequence of CRC screening discontinuation[35].

## CONSEQUENCES OF REDUCED CRC SCREENING ACTIVITY

This review demonstrates the remarkable impact of the pandemic on endoscopic services in Italy. Interruption and discontinuation of CRC screening inevitably translated into a substantial and concerning reduction in CRC detection. It is commonly believed that screening delays beyond 4–6 mo would significantly increase advanced CRC cases and, if lasting beyond 12 mo, mortality as well[15]. In patients with CRC, 3–10-year survival is lower if treatment is started > 90 d from diagnosis, and similar data are reported for other cancers[36]. The ideal timing of resection of colon cancer specifically has been estimated to be between 3 and 6 wk from diagnosis, which is unlikely to be achieved during the

COVID-19 outbreak[37]. In a study where patients who underwent surgery for CRC in the pre-COVID-19 era (October 2019–February 2020) were compared to those who did so after the end of the second wave (January 2021–May 2021), an increase in T4 tumours with higher preoperative levels of CEA and CA 19-9 was observed. These tumours required more extensive lymph node dissection. The authors speculated that this finding could be attributed to the reduced number of colonoscopies performed during the lockdown, as well as to patients' fears of potential infections in the hospital setting[37].

A survey by the Italian Federation of the Digestive Diseases Societies found that in gastroenterology units, 11.9% fewer CRC cases were diagnosed between January 1, 2020 and October 31, 2020, compared with the same period in 2019[6].

Surgical oncology services around the world suffered a remarkable reduction in activity, resulting in a doubling of waiting lists as a result of delays in the screening and diagnosis of CRC due to the restrictions imposed by the pandemic.

A study was designed to evaluate the effects of COVID-19-related delays in CRC screening in 20 hospitals of Northern Italy by comparing 1755 patients who underwent CRC surgery in 2019 *vs* 1481 in 2020. The results showed that CRC s in 2020 (compared to 2019) were more likely to be symptomatic [OR: 1.36 (95%CI: 1.09-1.69)], to be clinical stage T4 [OR: 1.38 (95%CI: 1.03-1.85)] and to have multiple liver metastases [OR: 2.21 (95%CI: 1.24-3.94)], although they were not more likely to be associated with surgical complications [OR: 0.79 (95%CI: 0.68-0.93)][38]. In particular, locally advanced disease, as well as the presence of CRC metastases to the liver, are definite prognostic factors in patients affected by CRC.

Another study evaluated the impact of the COVID-19 emergency on elective oncological surgical activity in 54 surgical units in Italy, including 11 colorectal units. Among the latter, 9 (82%) experienced a reduction of their surgical activity by 60%, with an expected prolongation of 5 wk between multidisciplinary meetings and surgery[39].

In the absence of proper catch-up campaigns aiming to recuperate those who missed their scheduled screening, the prognosis of patients with CRC could worsen. In fact, the long-term effects of the delay in CRC diagnosis due to interruption of screening activity could result in a rise in late-stage CRC cases and eventually in an undesirable loss of life years due to the lack of appropriate treatments for these patients [40].

Based on a procedural model using real-world data, in Italy a significant increase in deaths (12%) can be estimated at 5 years after a delay of longer than 12 mo in access to colonoscopy. In particular, in a study comparing baseline (0–3 mo), moderate (7–12 mo) and long (> 12 mo) delays, a significant increase in advanced CRC (from 26% to 29% and 33%, respectively, was seen. Thus, the authors have estimated a significant increase in the total number of deaths (12.0%) when moving from a 0–3-mo to a > 12-mo delay ($P < 0.005$) and a significant change in the mortality distribution by stage from baseline to > 12 mo ($P < 0.001$)[41,42].

## CONCLUSION

The results of our review confirm that the COVID-19 emergency has caused detrimental effects on CRC screening programs in Italy, similarly to what occurred in other counties on all continents. In most hospitals and territorial healthcare services, a time-limited suspension of CRC screening services was observed. At the time of writing, the situation is different from that observed at the beginning of the COVID-19 crisis. Indeed, advances in the treatment of patients affected by COVID-19, as well as prevention with massive vaccine campaigns, has significantly decreased the growth in the total number cases and rates of hospitalization. As a consequence, screening activity has now resumed in many Italian regions. Nonetheless, sporadic COVID-19 outbreaks due to the diffusion of new variants of the virus continue to modify the activities of healthcare services, and the duration of the effects of the COVID-19 pandemic on social life and healthcare in general is difficult to predict. The delayed diagnoses of CRC cases attributable to screening discontinuation is expected to result in an increase in advanced cancer cases—and possibly deaths—in the coming years. It is of the utmost importance that healthcare services of countries around the world develop reliable policies to maintain standard CRC screening activity in the presence of new pandemic outbreaks or similar extraordinary events.

## FOOTNOTES

**Author contributions:** Fancellu A conceived the paper and drafted the manuscript; Veneroni S made the literature search and contributed to the drafting of the manuscript; Santoru A and Meloni A searched the web sources for relevant issues regarding the topic of the article; Ginesu GC critically resumed the data from the studies; Deiana G contributed to the literature search; Paliogiannis P contributed to critically evaluate the literature data; Ninniri C participated in the analysis and interpretation of the data; Perra T revised the article critically for important intellectual content; Sanna V prepared the figures and tables; Porcu A coordinated and supervised the writing of the paper.

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ORIGINAL ARTICLE

Basic Study

# Safety and feasibility of irreversible electroporation for the pancreatic head in a porcine model

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## Abstract

### *BACKGROUND*

Irreversible electroporation (IRE) is a local non-thermal ablative technique which has been suggested as a potential cancer therapy. However, the specific anatomic characteristics of the pancreatic head make it challenging to perform any local ablation in this region. Therefore, the safety and feasibility of IRE in the pancreatic head region should be further explored.

### *AIM*

To evaluate the safety of IRE in pancreatic head region including its effects on pancreatic ducts, vessels, and adjacent gastrointestinal organs.

### *METHODS*

Eight landrace miniature pigs underwent IRE of pancreatic head tissue successfully, with a total of 16 lesions created. Laboratory testing including white blood cell (WBC) count and serum amylase before IRE with follow-up laboratory analysis and pathological examination at 1, 7, 14, and 28 d postablation were performed.

### *RESULTS*

All pigs tolerated the ablation procedure without serious perioperative complications. Transiently elevated WBC count and amylase were observed at 24 h post-IRE, suggesting an acute pancreatic tissue damage which was confirmed by pathological observations. Vascular endothelial cells and pancreatic duct epithelial cells in ablation zone were also positive in terminal deoxynucleotidyl

transferase dUTP nick end labeling staining. There was extensive duodenum mucosa damage with local hemorrhage 24 h after ablation, while regeneration of new villous structures were observed at 7 and 28 d post-IRE. Masson's trichromatic staining showed that the extracellular matrix was still intact in vessels and pancreatic ducts, and even in the duodenum.

*CONCLUSION*
IRE ablation to the pancreatic head may be safe and feasible without long-term damage to the surrounding vital structures. However, risks of stress injuries in acute phase should be taken into consideration to prevent severe perioperative complications.

**Key Words:** Irreversible electroporation; Pancreatic head; Duodenum; Safety; Feasibility; Stress injury



**Core Tip:** This is a basic experimental research paper on irreversible electroporation (IRE) in the pancreatic head region. To examine the feasibility and safety of this technique in Landrace pigs, we designed a series of research experiments. We found that IRE ablation to the pancreatic head may be safe and feasible without long-term damage to the surrounding vital structures. However, risks of stress injuries in acute phase should be taken into consideration to prevent severe perioperative complications.

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## INTRODUCTION

Irreversible electroporation (IRE) is a novel local ablation technique based on non-thermal damage principle. It mainly causes irreversible perforation of cell membrane through applying instantaneous, high-frequency, and repeated high-voltage pulses to cells, which leads to imbalance of cell homeostasis and induces apoptosis, thus achieving the goal of tumor ablation[1-3]. The risk of thermal damage is significantly reduced due to the non-thermal effect of tumor killing, and there is no heat sink phenomenon that affects the effectiveness of tumor ablation. Therefore, compared with local physical ablations based on the thermal effect such as radiofrequency ablation (RFA), IRE is more suitable for the treatment of locally advanced malignant tumors that cannot be radically resected owing to the invasion of vital vessels and thus has a good application prospect[4,5]. Although the theory of IRE ablation of tumors has been widely accepted, it remains controversial in terms of whether there would be potential damage to tissues and organs adjacent to tumors that are located at special anatomical positions such as pancreatic head cancer[6,7]. Even though the safety of IRE ablation in the pancreas and upper gastrointestinal (GI) tract has been preliminarily validated[8-10], studies on the local and systemic effects of IRE for the pancreatic head of large animals remain limited. Elucidating the short- and long-term effects of IRE on the pancreatic head will be an essential step in demonstrating its safety and feasibility before further implementation in clinical patients. Therefore, our study aimed to investigate the immediate and late complications of IRE on the pancreatic head and evaluate its safety in pancreatic head region including its effects on pancreatic ducts, vessels, and adjacent GI organs.

## MATERIALS AND METHODS

### *Experimental subjects*

Eight Landrace miniature pigs weighing approximately 30 kg were selected with no gender restrictions. The pigs were provided by the Experimental Animal Center of the PLA General Hospital, where they were reared under clean experimental and single-cage standard conditions (22 °C, 12 h/12 h light/dark, 60% humidity, *ad libitum* access to food and water). The experimental procedures were approved by the Institutional Animal Care and Use Committee of PLA General Hospital.

### *Experimental methods*

**Experimental groups:** Eight pigs were randomly divided into four groups (A, B, C, and D), with two pigs *per* group, corresponding to different observation time points (1 h, day 1, day 7, and day 28 after

IRE surgery). The pigs were used to evaluate the effect of IRE (Nanoknife, AngioDynamics, Queensbury, New York, United States) on the pancreatic head and adjacent duodenum to observe the acute and chronic response to IRE ablation of the pancreatic head region. The IRE parameters were set as follows: Fixed pulsed-field intensity of 1500 V/cm, pulse width of 100 μm, frequency of 1 Hz, needle exposure depth of 1 cm, and a preset pulse number of 120. The pancreatic head tissue adjacent to the medial duodenal wall was selected as the target area for ablation.

**IRE ablation of the pancreatic head:** Animals were fasted for 12 h before the operation. Sedazine II (xylazine hydrochloride injection) + midazolam injection (volume ratio: 1:1) at 0.3 mL/kg was used for anesthesia induction by intramuscular injection. After the induction was successful, the animals were intubated with a video laryngoscope, and isoflurane (0.8%) inhalation at a flow rate of 0.7 L/min combined with intravenous injection of 3-5 mg/kg fentanyl citrate through the ear vein was used for anesthesia maintenance. Rocuronium bromide was administered intravenously at a dose of 1-1.5 mg/kg as a muscle relaxant to prevent severe muscle contraction during electrical pulse generation. Vital signs including blood pressure, heart rate, and temperature were monitored during the operation.

Two 19G IRE probes (AngioDynamics) were used to puncture parallelly into the target area with a distance of 1 cm and puncture depth of 1.5 cm. After completing the probe deployment, 20 trial pulses were applied based on the preset parameters, and the remaining 100 pulses were administered after confirming that there was no voltage overload. Then, the pigs' response and changes in pancreatic head tissue and the duodenum in the ablation zone were observed and recorded during IRE ablation. After the ablation was completed, the probes were removed and the abdomen was sutured closed layer by layer after observing no abnormality in the pig's vital signs, and bunarizine hydrochloride injection was used by intramuscular injection (3-5 mg/kg, 1/d) for postoperative analgesia.

### *Postoperative observation indicators*

General condition of the animals was observed and recorded including activity, feeding, bowel movements, and weight changes. The white blood cell (WBC) count and serum amylase level were measured before surgery and 1 h, 1 d, 3 d, 7 d, 14 d, and 28 d after surgery. Tissue specimens were harvested from pigs in the corresponding groups after 1 h and on days 1, 7, and 28 after IRE. The pigs were euthanized *via* intravenous injection of 3% nembutal (100 mg/kg), and pathological examinations were conducted on the ablation and non-ablation zones, including hematoxylin and eosin (HE) staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and Masson trichrome staining and transmission electron microscopy observation.

### *Statistical processing*

SPSS version 22.0 statistical software was used to analyze the experimental results, and measurement data are expressed as the mean ± standard error. The experimental data were subjected to multiple comparisons among groups and the pairwise *t*-test, and the difference was considered statistically significant at $P < 0.05$.

## RESULTS

### *General results*

All animals were subjected to IRE ablation and survived to the respective experimental endpoints. The animals started to be active 6 h after surgery, but their activity was reduced and they did not consume food. Within 24 h after surgery, the animals gradually increased their activity and had a small amount of food and defecation. Then, at 2 d after surgery, the animals' activity, food intake, and defecation essentially returned to normal. No significant change in body weight was observed at the preoperative and postoperative time points in each group.

### *Changes in blood indicators and pathological characteristics of pancreatic head tissue in pigs subjected to IRE ablation*

**Changes in blood indicators:** The results of the laboratory testing showed that the WBC count in the postoperative acute phase of IRE was gradually elevated from the preoperative baseline level (16.2 ± 2.0) × $10^9$/L to the peak (28.2 ± 5.5) × $10^9$/L at 24 h postoperatively and then gradually resolved to normal (Figure 1A). The serum amylase concentration showed a significant increase 1 h after surgery (873.4 ± 118.8 U/L), then reached the highest value at 24 h after surgery (2077.6 ± 637.3 U/L), and essentially returned to normal 3 d after surgery (1383.9 ± 218.8 U/L) (Figure 1B). Statistical comparative analysis showed that the serum amylase concentration at day 1 after surgery were significantly higher ($P < 0.05$) than that at baseline (700.9 ± 88.1 U/L).

**Pathological findings:** The pancreatic tissues after IRE ablation showed different pathological changes over time. At 1 h after surgery, the ablation zone showed distinct acute edema and congestion with clear demarcation from the surrounding area (Figure 2). HE staining showed that some of the pancreatic

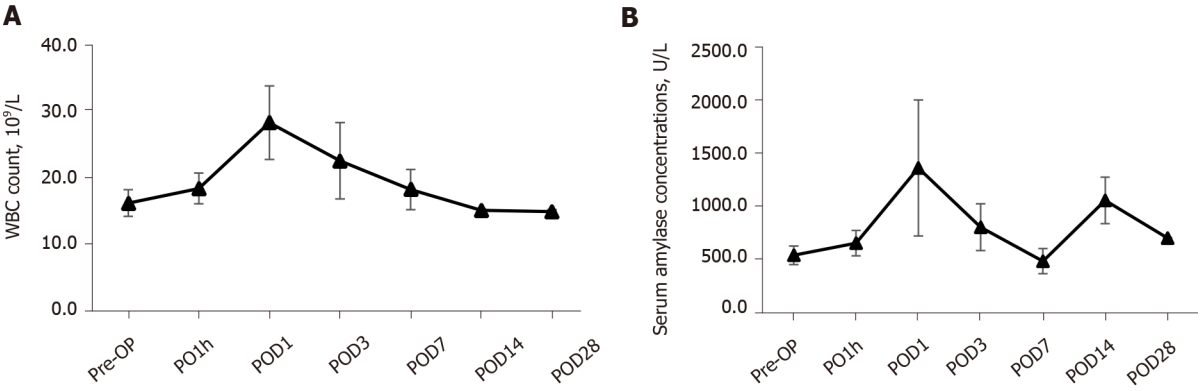


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**Figure 1 Perioperative changes of white blood cell count and serum amylase.** Both of them were elevated immediately postoperatively (1 h) and peaked after 1 d, then gradually resolved by 4 wk post-ablation. A: White blood cells count; B: Serum amylase. WBC: White blood cell; Pre-OP: Pre-operation; PO1h: Post-operative 1 h; POD: Post-operative day.

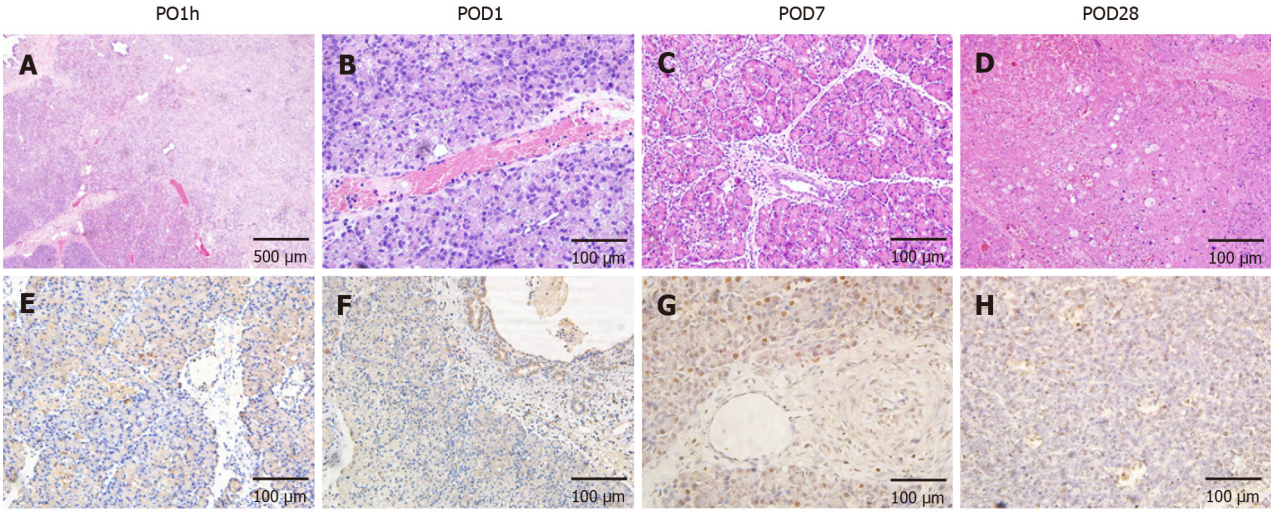


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**Figure 2 Effects of irreversible electroporation on pancreatic head tissue.** A: Hemotoxylin and eosin staining demonstrated extensive tissue damage in the irreversible electroporation (IRE) ablation zones with clear boundaries between ablation area and nonablation area; B-D: Tissue necrosis and immune cell infiltration were noted up to 4 wk post-IRE with gradual resolution and subsequent mild fibrosis; E-H: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining revealed that the area centered on the probes in the ablation zone was strongly positive, and apoptotic expression was also seen in pancreatic ductal cells (F) and vascular endothelial cells (G). Scale bar in A = 500 μm. Scale bar in (B-H) = 100 μm. PO1h: Post-operative 1 h; POD: Post-operative day.

acinar cells were obviously necrotic accompanied by interstitial congestion and edema, and focal hemorrhages were observed locally, but most cells were negative for TUNEL staining (Figure 2A and E). At day 1 after surgery, inflammatory cell infiltration was visible under the microscope, and the pancreatic lobule structure remained intact. A small number of apoptotic cells were seen in TUNEL staining and were mostly concentrated around the probes (Figure 2B and F). At day 7 after surgery, the size of the ablation zone was reduced, and pancreatic tissue edema disappeared. HE staining revealed pancreatic acinar cell atrophy in the ablation zone and increased cell eosinophilia, accompanied by the infiltration of a large number of inflammatory cells and fibrosis (Figure 2C). TUNEL staining revealed that the area centered on the probes in the ablation zone was strongly positive, and apoptotic expression was also seen in pancreatic ductal and vascular endothelial cells (Figure 2G). At day 28 after surgery, severe pancreatic destruction with vacuolation of cells was observed. The positive rate of TUNEL stained cells decreased, while the structure of pancreatic ducts and vessels in the ablation zone was still intact.

Observation by transmission electron microscopy showed that the pancreatic acinar cells in the ablation zone were atrophied, the nucleoli were broken and disappeared, the chromatin of the cells was highly pyknotic and condensed to the edge, and the endoplasmic reticulum appeared vacuolated (Figure 3).

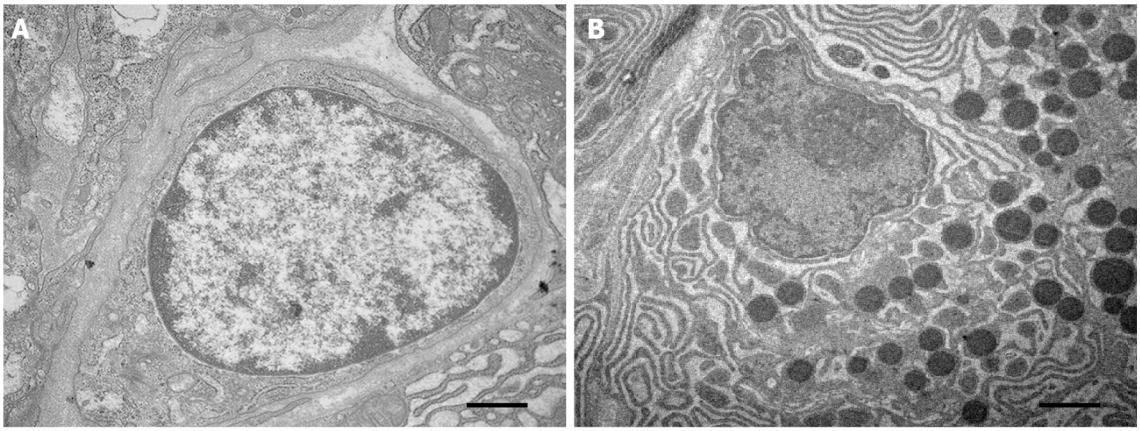


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**Figure 3 Effects of irreversible electroporation on the ultrastructure of pancreatic acinar cells.** A: Pancreatic acinar cells in the ablation zone showed highly agglutinated and marginalized chromatin, fragmented nucleoli, condensed cytoplasm, and disappearance of rough endoplasmic reticulum; B: Normal control. Scale bar = 500 nm.

### *Effect of IRE ablation on the duodenum*

After IRE, the duodenal segments in the ablation zone showed a gradually deepening color with local congestion and edema as the distance from the probes gradually shortened, and the peristalsis of the corresponding segment slowed down. Postoperative observations at different time points showed that there was no perforation or obstruction in the duodenum, and the edema gradually disappeared. The color of the duodenal serosa in the ablation zone was not significantly different from that of the normal segment (Figure 4). Normally rhythmic peristaltic waves were observed.

HE staining (Figure 5) revealed that the mucosal structure of the duodenum in the ablation zone was disorganized at 1 h after surgery, with obvious destruction of the villous structure and congestion of the mucosa with localized focal hemorrhage; no significant changes were observed in the manifestation at day 1 after surgery; at day 7 after surgery, dead mucosal epithelial cells were still visible by microscopy and signs of repair could be seen in all layers of the duodenum; at day 28 after surgery, the duodenal structure did not significantly differ from that before surgery.

Masson trichrome staining showed proliferation of blue-stained fibrous connective tissue in the ablation zone of the pancreas at day 7 after surgery (Figure 6A), and the vascular and pancreatic duct extracellular matrix structures were intact in the ablation area without loss (Figure 6B and C). Continuous blue-stained collagen fibers was seen between the mucosa, submucosa, muscularis, and serosa, and the structure of each tissue was intact and did not differ considerably from that in the non-ablation zone (Figure 6D-F).

## DISCUSSION

Since IRE was first approved for clinical use, its safety and efficacy have been the focus of scholars both at home and abroad. The pancreatic head/neck region has special anatomical and structural characteristics, surrounded by important vascular structures such as the celiac axis, superior mesenteric artery, and portal vein. The invasion of these important vessels is closely related to the unresectability of pancreatic cancer and restricts the application of traditional physical ablation modalities based on thermal effects in the treatment of locally advanced tumors. Previous studies have shown that traditional physical therapies such as RFA can cause severe complications in treating pancreatic cancer, including GI bleeding, pancreatic fistula, biliary fistulas, pancreatitis, and portal vein thrombosis[11,12]. Among these, GI bleeding and secondary infection caused by biliary and pancreatic fistulas are the most common death-related complications. Therefore, apart from the vessels, the preservation of vital surrounding tissue including the bile duct, pancreatic duct, and duodenum should be taken into primary consideration when IRE is performed. This study provides a novel insight into the short- and long-term effects of IRE on pancreatic head region and adjacent structures. We demonstrated that IRE ablation to the pancreatic head may be safe and feasible without long-term damage to the surrounding vital structures but risks of stress injuries in acute phase should be taken into consideration to prevent severe perioperative complications.

### *Selection of experimental animals*

Studies on IRE ablation of hollow organs have been carried out by scholars long before the application of this technique in clinical practice. Phillips *et al*[13] preliminarily validated the safety of IRE ablation of

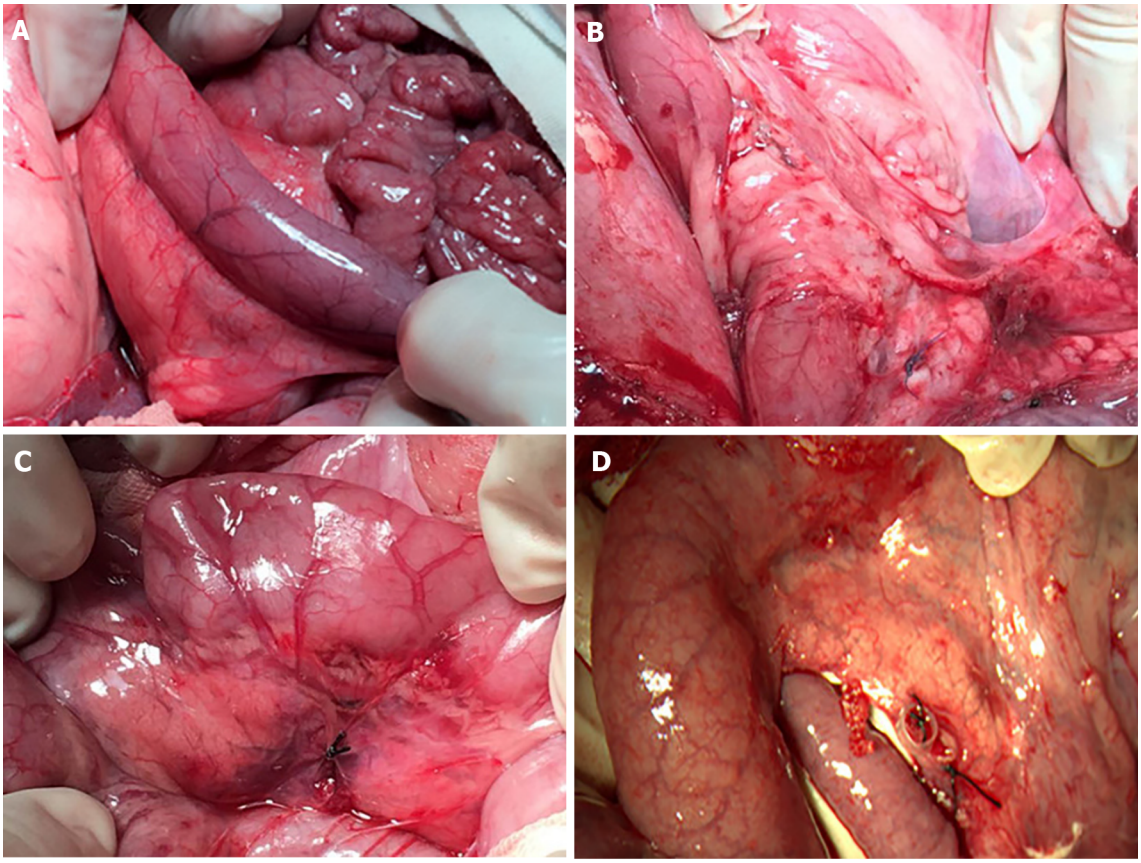


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**Figure 4 Gross pathology of the duodenum wall after irreversible electroporation.** A: The duodenal wall deepened in color with local congestion and edema 1 h after irreversible electroporation (IRE); B: Postoperative adhesion was observed 24 h after IRE without gastroduodenal obstruction; C and D: The duodenum went back to normal at 1 and 4 wk post-IRE.

hollow organs using the small intestine of Sprague Dawley rats as the target organ; however, the differences in anatomical structure and ablation protocols limit the reference significance of this study for the safety assessment of IRE ablation in the pancreatic head. Subsequently, Schoellnast *et al*[14], Srimathveeravalli *et al*[15], and Luo *et al*[16] investigated the feasibility of colorectal IRE ablation using pigs as experimental animals, indicating that it was feasible to use hollow organs of miniature pigs as IRE target organs. This has guiding significance for simulating the application of IRE for tumors from corresponding human organs. However, due to the different target organs and anatomical positions, these studies did not provide meaningful clinical references for assessing the safety of IRE ablation of pancreatic head cancer on adjacent hollow organs. Therefore, the anatomical structure and position as well as the tolerance of the experimental animals to IRE were the main considerations in selecting the experimental subjects. The pancreas of miniature pigs is flat and attached to the inner mesentery of the duodenum in a "herringbone" shape; this anatomical position is similar to that of humans. Therefore, compared with rats, pigs are a relatively more ideal animal model for IRE ablation experiments in the pancreatic head region.

### *Effect of IRE ablation on the duodenum*

In our experiments, the duodenum in the IRE ablation area gradually deepened in color as the distance from the probes gradually shortened, and the peristaltic rhythm slowed down, indicating that although the duodenum was not a direct target organ for IRE ablation, the tissues within a certain range of the IRE probes were affected by the pulsed electric field, which resulted in an acute stress response. The microscopic changes at day 1 after IRE showed that IRE ablation with conventional parameter settings could cause irreversible tissue death in the mucosa, submucosa, and muscularis of the duodenum. Nevertheless, the microscopic changes at day 7 after surgery showed structural repair of new villi in the small intestine, and the duodenal structures in the ablation zone gradually resolved to normal up to day 28 after surgery, suggesting that the effects of IRE ablation of the pancreatic head on the duodenum may be limited to acute stress injury without long-term effects.

Consistent with reports in the literature, IRE ablation of the pancreatic head did not cause severe duodenal-related injury for the following possible reasons: (1) The IRE effect targets the cell membrane and does not affect the extracellular matrix and other skeletal structures; thus, the structural integrity of

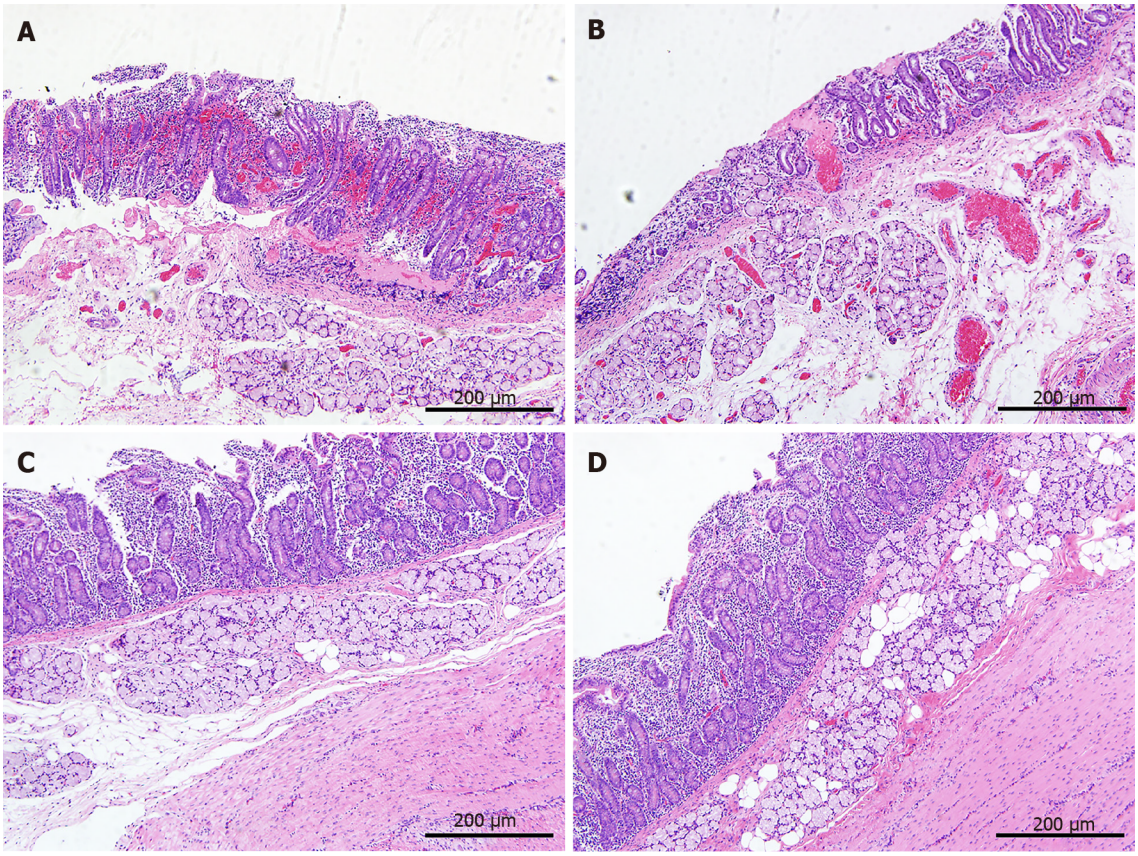


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**Figure 5 Histopathology of the duodenum wall after irreversible electroporation.** A: The duodenum wall showed necrosis and focal hemorrhage in the mucosa, submucosa, and muscle layers 1 h after irreversible electroporation (IRE); B: The mucosal layer was congested with massive infiltration of inflammatory cells 24 h after IRE; C: On 7 d after IRE new villous structures were observed in the mucosal layer, and immature muscle cells were seen in the muscle layer; D: Twenty-eight days after IRE, the structure of the duodenum appeared intact, and the mucosa layer returned to normal thickness. Scale bars in A-D = 200 μm.

the duodenum is preserved, providing the basis for subsequent injury repair[17]; (2) The principle of IRE killing cells is based on inducing apoptosis, thereby causing a mild local inflammatory response, which is conducive to the growth and migration of new cells; (3) The vasoprotective effect of IRE did not significantly affect the blood supply to any layer of the duodenum; (4) The high renewal rate of mucosal epithelial cells in the small intestine allows rapid repair of the damaged duodenum; and (5) A study showed[18] that the pluripotent stem cells of duodenal glands can be induced to differentiate into epithelial cells to form new villous structures in the small intestine, promoting the recovery of duodenal structure and function.

Notably, in the present study, when IRE was used to ablate the head of the pancreas, we found extensive congestive changes in the mucosa and submucosa of the duodenum early after surgery, localized mucosal tissue detachment, and hemorrhagic manifestations; such acute stress changes suggested the risk of stress ulcer bleeding in the GI tract after IRE of tumors in the head of the pancreas. Consistent with the actual clinical situation, it is common for pancreatic head adenocarcinoma to invade the duodenum, and there have been clinical reports on GI bleeding after IRE[5,19]. Therefore, although experimental animal studies have shown that IRE ablation of the pancreatic head does not result in severe long-term complications after ablation, such as duodenal perforation, the reference significance of its acute stress changes for the safety of IRE ablation for pancreatic head cancer in clinical practice still warrants further investigation of the clinical application of this emerging technology in this special region.

### *Effect of IRE on pancreatic tissue and ductal structure in ablation zone of the pancreatic head*

Due to the pancreatic head's special anatomical position and structure, safety has always been the primary consideration in applying physical ablation modalities in this area. Conventional physical ablation modalities kill tumors by causing cell necrosis *via* thermal effects. Although some studies have reported that they can achieve pain relief and improve survival quality, the high incidence of severe complications dramatically limited their application in treating pancreatic cancer[11,12]. Unlike conventional physical ablations, IRE does not rely on the thermal effect to kill tumors, leading to its acceptance as an ideal ablation modality for pancreatic tumors. Nevertheless, the fragility of the pancreas makes it

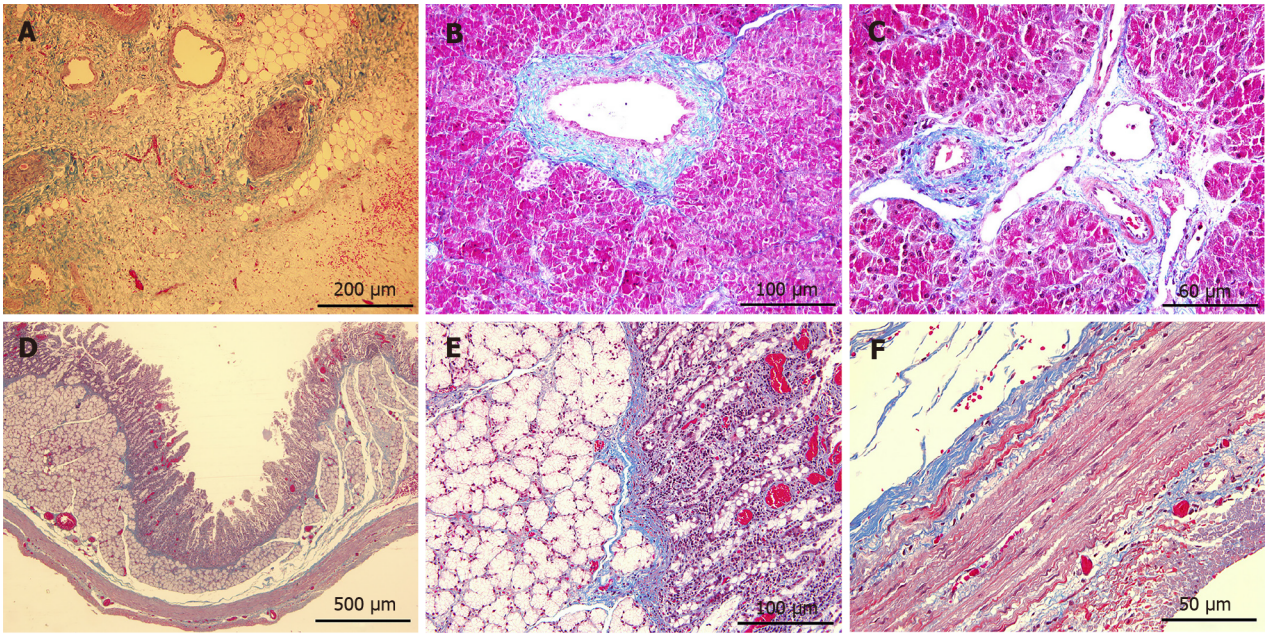


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**Figure 6 Masson trichrome staining of tissues in the ablation zone.** A-C: Mild fibrosis (blue stained) was observed in pancreatic parenchyma on 7 d post-ablation (A), and the structures of pancreatic ducts (B) and vessels (C) remained intact; D-F: Staining of the duodenum wall showed that the structure of all layers was preserved although minimal injury to the mucosa layer was noted. Scale bars in (A) = 200 μm. Scale bars in (D) = 500 μm. Scale bars in (B and E) = 100 μm. Scale bars in (C and F) = 50 μm.

more vulnerable to any surgical manipulation or local physical ablation compared to other solid organs such as the liver or kidney. Therefore, assessment of the local and systemic effects of IRE ablation in the pancreatic head region remains necessary.

In our study, no abdominal necrosis or exudation was observed in the gross specimen at any time point, suggesting that no significant pancreatic fistula occurred after IRE ablation. The blood test results suggested that IRE can cause an inflammatory response in the first 24 h after surgery, and the WBC count would return to normal 3 d after surgery, indicating that this inflammatory response caused by IRE is only a stress response to this procedure during the acute phase of trauma. Additionally, the trend of the WBC count also suggested that IRE does not increase the risk of perioperative abdominal infection, thus validating the safety of IRE ablation in the head of the pancreas from another perspective. Notably, the trend of postoperative serum amylase also only showed a transient increase in the acute phase, while the long-term serum amylase level suggested that there was no evidence showing that IRE ablation of the pancreatic head could induce chronic pancreatitis. Combined with previous reports in the literature[20,21], we analyzed the reasons why IRE ablation of the pancreatic head did not induce severe pancreatitis, which may be as follows: (1) IRE ablation of the pancreas has a precise and limited scope, and its damage to the pancreatic tissue is limited to a localized area; (2) The ablation using a fine needle probe (19G) is less traumatic to the pancreas and can effectively prevent direct damage to the pancreatic duct; and (3) Unlike other thermal ablation methods such as RFA or cryoablation, IRE does not damage the extracellular matrix, effectively protecting the integrity of the pancreatic duct structure in the ablation zone and avoiding pancreatic fistula.

The histopathological findings corroborated these results. The ablation zone of the pancreatic head showed different changes at different time points during 4 wk after IRE ablation, which was consistent with the findings of Lee *et al*[8]. Necrosis of pancreatic acinar cells was found 1 h after ablation, suggesting that IRE ablation can cause morphological changes of cells in the ablation zone at an early stage after the procedure. From day 1 to day 28 after ablation, the ablation zone showed a series of pathological changes from massive accumulation of inflammatory cells to gradual regression and from atrophy and death of pancreatic acinar cells to proliferation of fibrous connective tissue, which confirmed that IRE could produce irreversible damage to pancreatic tissues. However, such damage was not coagulation necrosis but apoptosis. This mechanism of IRE was confirmed by results of TUNEL staining. We found that pancreatic ductal and vascular endothelial cells were also positive for TUNEL staining, suggesting that IRE ablation also induced apoptotic effects on cells of ductal structures, such as vessels and pancreatic ducts. Nevertheless, IRE did not damage their structural integrity and function, demonstrating that important ductal structures in the pancreatic head could be preserved while target cells were destroyed.

Although the effectiveness of IRE ablation of the pancreatic head and the safety of vital ductal structures and adjacent organs were validated in this study, it was limited as the study aimed to

generate an IRE model in normal pancreatic tissue of pigs, which failed to truly simulate the tumor model that invades the peripheral vessels of the pancreatic head and duodenum. Additionally, owing to the difference between the microenvironment of tumor and that of normal tissues, there is still uncertainty on whether the same results would be obtained if tumor cells are present.

## CONCLUSION

In conclusion, IRE ablation to the pancreatic head may be safe and feasible without long-term damage to the surrounding vital structures. However, risks of stress injuries in acute phase should be brought to our attention. In the future, *in vitro* studies of IRE ablation on various human pancreatic cancer cell types should be conducted to optimize parameters and techniques of pancreatic IRE ablation in clinical settings, and further studies are needed to investigate the mechanism of tissue repair and regeneration after IRE.

## ARTICLE HIGHLIGHTS

### *Research background*

Irreversible electroporation (IRE) is a relatively novel local ablation technique based on the delivery of repeated and high-frequency microsecond- to millisecond-long electrical pulses to a target tissue. It is characterized by non-thermal damage and no heat sink effect, thus able to protect vital anatomic structures such as pancreatic ducts and vessels in close proximity within the targeted organs. These qualities make IRE an attractive alternative for treatment of locally advanced pancreatic cancer. Recently, this novel ablation has been tested successfully on normal and malignant lesions in the prostate, liver, lung, and pancreas in animal models, and even in human subjects; however, the safety and feasibility of IRE for lesions in the pancreatic head are still controversial.

### *Research motivation*

Studies on the local and systemic effects of IRE for pancreatic head of large animals remain limited. Elucidating the short- and long-term effects of IRE on the pancreatic head will be an essential step in demonstrating its safety and feasibility before further implementation in clinical patients. We carried out an animal experiment to examine this procedure.

### *Research objectives*

This study aimed to examine the safety and feasibility of IRE for the pancreatic head in a porcine model.

### *Research methods*

In total, eight Landrace pigs were randomly divided into four groups, with two pigs *per* group, corresponding to different observation time points (1 h, day 1, day 7, and day 28 after IRE surgery), and underwent IRE ablation of the pancreatic head successfully. Laboratory testing including white blood cell (WBC) count and serum amylase before IRE with follow-up laboratory analysis and pathological examination at 1, 7, 14, and 28 d postablation were performed.

### *Research results*

The effects of IRE on the pancreatic head were characterized by transiently elevated WBC and amylase, and acute damage to targeted area including pancreatic tissue and the duodenum which was confirmed by pathological observations in the early phase after ablation. Vascular endothelial cells and pancreatic duct epithelial cells in ablation zone were also positive for terminal deoxynucleotidyl transferase dUTP nick end labeling staining while the structure was still intact in long-term observation, indicating that the risk of short-term damage should be paid more attention to prevent severe perioperative complications.

### *Research conclusions*

IRE ablation to the pancreatic head is safe and feasible without long-term damage to the surrounding vital structures while risks of stress injuries in acute phase should be brought to our attention.

### *Research perspectives*

*In vitro* studies of IRE ablation on various human pancreatic cancer cell types should be conducted to optimize parameters and techniques of pancreatic IRE ablation in clinical settings to keep safe and further studies are needed to investigate the mechanism of tissue repair and regeneration after IRE.

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## FOOTNOTES

**Author contributions:** Yan L and Chen YL designed and coordinated the study; Yan L, Liang B, and Feng J performed the experiments, and acquired and analyzed the data; Zhang HY, Chang HS, and Liu B interpreted the data; Yan L and Feng J wrote the manuscript; Liang B and Feng J should be regarded as co-first authors; all authors approved the final version of the article.

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ORIGINAL ARTICLE

Retrospective Cohort Study

# Second-line therapy for advanced hepatocellular carcinoma with regorafenib or cabozantinib: Multicenter French clinical experience in real-life after matching

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## Abstract

### *BACKGROUND*

Starting a second-line systemic treatment for hepatocellular carcinoma (HCC) is a common situation. The only therapeutic options in France are two broad-spectrum tyrosine kinase inhibitors (TKIs), regorafenib (REG) and cabozantinib (CBZ), but no comparative real-life studies are available.

### *AIM*

To evaluate the progression-free survival (PFS) of patients treated with REG or CBZ, we investigated the disease control rate (DCR), overall survival (OS), and safety of both drugs. To identify the variables associated with disease progression over time.

### *METHODS*

A retrospective multicenter study was performed on the clinical data of patients attending one of three referral centers (Avignon, Marseille, and Nice) between January 2017 and March 2021 using propensity score matching. PFS and OS were

assessed using the Kaplan-Meier method. Multivariate analysis (MA) of progression risk factors over time was performed in matched-pair groups.

***RESULTS***

Fifty-eight patients 68 (62-74) years old with HCC, Barcelona clinic liver cancer (BCLC) B/C (86%), Child-Pugh (CP)-A/B (24%) received REG for 3.4 (1.4-10.5) mo as second-line therapy. Twenty-eight patients 68 (60-73) years, BCLC B/C (75%), CP-A/B (25%) received CBZ for 3.7 (1.8-4.9) mo after first-line treatment with sorafenib [3 (2-4) (CBZ) *vs* 4 (2.9-11.8) mo (REG), $P = 0.0226$]. Twenty percent of patients received third-line therapy. After matching, PFS and DCR were not significantly different after a median follow-up of 6.2 (2.7-11.7) mo (REG) *vs* 5.2 (4-7.2) mo (CBZ), $P = 0.6925$. There was no difference in grade 3/4 toxicities, dose reductions, or interruptions. The OS of CP-A patients was 8.3 (5.2-24.8) *vs* 4.9 (1.6-11.7) mo (CP-B), $P = 0.0468$. The MA of risk factors for progression over time identified C-reactive protein (CRP) > 10 mg/L, neutrophil-to-lymphocyte ratio (NLR) > 3, and aspartate aminotransferase (AST) > 45 IU as predictive factors.

***CONCLUSION***

This multicenter indirect comparative study found no significant difference in PFS between REG and CBZ as second-line therapy for advanced HCC. Elevated levels of inflammatory markers (CRP and NLR) and AST were associated with non-control of TKIs over time. A 2-mo online progression risk calculation is proposed.

**Key Words:** Hepatocellular carcinoma; Regorafenib; Cabozantinib; C-reactive protein; Neutrophil-lymphocyte ratio



**Core Tip:** One limited population of advanced hepatocellular carcinoma patients has sustained disease control using tyrosine kinase inhibitors (TKIs) as first-line systemic therapy. Patients with preserved liver function and performance status progress to second-line systemic therapy. Only two broad-spectrum TKIs are approved for this indication in France, and no direct comparative studies are available. Immune checkpoint inhibitors are currently the standard of care as first-line therapy in combination with an anti-angiogenic agent and will most likely change the treatment strategy of second-line therapy. No biomarkers are available to guide treatment, but serum inflammation-related factors may provide additional support.

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## INTRODUCTION

Most patients with advanced hepatocellular carcinoma (HCC) do not have sustained disease control with first-line systemic therapy, particularly after tyrosine kinase inhibitors (TKIs)[1], due to failure, secondary progression and/or intolerance to therapy. Switching to a second line of systemic therapy has become a common situation. During the last decade, several phase II/III trials evaluated different protein kinase inhibitors after sorafenib[2,3], including one targeting an overexpressed oncogene (tivantinib, a MET pathway inhibitor)[4], and all of these trials were negative. However, there have been important therapeutic advances in the treatment of HCC over the past four years with various anti-cancer agents, multi-kinase inhibitors[5,6], monoclonal antibodies targeting vascular endothelial growth factor receptor 2 (VEGF-R2)[7], and antibodies directed against the immune checkpoint molecules human cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed death receptor-1 (PD-1)[8,9] and its ligand PD-L1, after progression ± intolerance to sorafenib, similar to other cancer types. The most significant results were achieved with the combination of a monoclonal antibody against PD-L1 and an anti-angiogenic agent targeting VEGF-A[10]. Angiogenesis contributes to immunosuppression *via* a direct effect of the VEGF-VEGFR interaction or from the tumor microenvironment[11]. Combination therapies have become the standard of care in first-line systemic treatment of HCC. Therefore, the therapeutic landscape in second-line treatment is expected to change in the future. All of these advances

should not omit HCC specificity, which is generally linked to a chronic liver disease with cirrhosis of various etiologies. The strict selection criteria of clinical trials have resulted in a lack of data for a large number of patients in routine practice. Two multi-targeted TKIs regorafenib (REG) and cabozantinib (CBZ) are the only treatment options available in France based on phase III trials after sorafenib. Notably, no controlled trials of second-line treatment were performed after first-line treatment with atezolizumab-bevacizumab (lenvatinib[12] is not approved for this indication in France). There are also no direct comparison studies between the "approved" second-line molecules or any predictive biomarker correlated with treatment activity[13]. Elevated pre-treatment inflammation-related factors, such as C-reactive protein (CRP) and neutrophil-to-lymphocyte ratio (NLR) are clearly associated with poor survival outcomes in various tumor types and across all stages[14,15]. Inflammation supports tumor development and metastasis[16] because inflammatory cells [particularly macrophages, mast cells, neutrophils, myeloid-derived suppressive cells (MDSCs) and selected lymphocytes] release various mediators, such as growth factors, pro-inflammatory cytokines and metalloproteinases, which result in stromal remodeling and tumor growth and spread.

The present study (1) Evaluated the survival of advanced HCC patients treated with second-line systemic therapy in a real-life cohort; (2) Evaluated the progression-free survival (PFS) of patients treated with REG or CBZ, the disease control rate (DCR), overall survival (OS) and the safety of both drugs after matching; and (3) Identified factors associated with disease progression over time, with a focus on inflammatory markers recorded at baseline and longitudinally during treatment.

## MATERIALS AND METHODS

### *Study design-eligibility*

This study was a retrospective multicenter study in three institutions from southern France (Nice, Marseille, and Avignon). All patients with advanced HCC (radiologically proven according to the European Association for the Study of the Liver[17]/Association for the Study of Liver Diseases[18] criteria or with histology) who received a second-line treatment with REG or CBZ from January 2017-March 2021 were included. Eligible patients included patients with prior first-line systemic sorafenib treatment that was discontinued after failure and/or intolerability. Patients who received REG or CBZ in combination with other therapies were excluded. The decision of second-line treatment for HCC in all three centers followed a multidisciplinary team discussion. Selected patients were Barcelona clinic liver cancer (BCLC) HCC stage B or C, without curative options: Evolutive multinodular HCC, refractory transarterial chemoembolization patients, or patients with vascular invasion and/or metastatic disease, Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) 0/1/2, Child–Pugh (CP)-A or B grade. Baseline and follow-up demographic, clinical, and biological characteristics, including full blood count (neutrophils, lymphocytes, hemoglobin and platelets), biochemical blood tests [particularly alpha-fetoprotein (AFP) and CRP levels] and radiological features, were collected prospectively and analyzed retrospectively following a similar process in all three centers. Only patients with full available data were included. The local institutional review board in each center approved the study protocol. Informed consent from patients was waived by the IRBs because of the retrospective nature of this study.

### *REG / CBZ cohorts*

**Procedure and assessments:** Before starting TKI treatment, patients were informed of potential adverse events (AEs) and useful prophylactic measures to prevent or reduce these events[19]. Monitoring included clinical evaluation twice monthly during the first two cycles then at each treatment cycle, focusing on TKI treatment tolerance. Radiological assessment included initial cross-sectional imaging (computed tomography and/or magnetic resonance imaging) 8 to 12 wk after the initiation of therapy then every 2 to 3 mo, using Response Evaluation Criteria in Solid Tumors version 1.1 for the grading of tumor responses. Patients with controlled disease included patients with radiological response and stable disease as the best response. Liver function or AFP serum levels and inflammation-related factors (NLR and CRP) were also assessed at each treatment cycle.

**Treatment schemes:** REG: Patients received 160 mg once daily during the first 3 wk of each 4-wk cycle. REG was continued until progression or intolerable AE occurrence. Interruptions and dose reductions were based on the severity and nature of AEs, which were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) version 4. REG was first started at 80 mg/d in the setting of ECOG PS 2 or CP-B cirrhosis, with subsequent dose escalation in cases of adequate safety.

Cabometyx: Patients received 60 mg once daily continuously until progression or intolerable toxicities. Interruptions and dose reductions were also determined by the severity and nature of AEs graded according to NCI CTCAE version 4. Treatment was started at 40 mg/d in the setting of ECOG PS 2 or CP-B cirrhosis, with subsequent dose escalation when the treatment was well tolerated.

Dose reductions were used for grade 2 toxicity that was not controlled by symptomatic treatment. Treatment interruption was used for any grade ≥ 3 toxicity until recovery to ≤ grade 1 severity.

### Statistics

Quantitative data are reported using medians and interquartile ranges. Qualitative data are reported using frequencies and percentages. Crude comparisons between CBZ and REG were performed using the nonparametric Wilcoxon test for median comparisons and the chi-squared test or Fisher's test for frequency comparisons. The Mantel-Haenszel chi-squared test was performed to compare ordinal scale data.

OS was defined as the time interval between the initiation of CBZ or REG and death or the time of last follow-up for patients who were still alive. PFS was defined as the time interval between the initiation of CBZ or REG and the time until first progression or the time of last follow-up for patients with no progression. Survival between groups was compared using the log-rank test.

Paired analysis between CBZ and REG was performed using propensity score matching (PSM) on BCLC staging, CP grade, vascular invasion, metastasis, and AFP. Risk factors for tumor progression were analyzed using univariate logistic regression analysis followed by multivariate logistic regression analysis. Factors with significant results in univariate analysis were included in multivariate model analysis. All *P* values were considered significant at α-level = 0.05. All calculations were performed using SAS V9.1 statistical software (SAS Institute Inc., Cary, NC).

## RESULTS

### Patient characteristics (entire cohort)

Table 1 shows the patients' characteristics. There were 86 patients with a median age of 68 (60-74) years at the start of treatment, mostly men, with an ECOG PS of 0/1 (78%) or 2 (22%). Patients had cirrhosis due to viral, alcoholic or metabolic etiology in most cases and CP grade A or B liver function. The tumors were classified as stage B or C (83%) according to the BCLC system. Macroscopic vascular invasion was present in 48% of cases, and metastasis was present in 43% of cases. AFP elevation ≥ 400 ng/mL was found in 48% of cases. The largest tumor diameter was 69 (40-100) mm. The baseline CRP serum level was 22 (8-51) mg/L, and 53% of patients had an NLR > 3. The median duration of prior treatment with sorafenib was 3.5 (2.7-9.2) mo Fifty-eight patients received REG as second-line therapy, and 28 patients received CBZ as second-line therapy (Figure 1). The median second-line treatment duration was 3.5 (1.6, 8.3) mo. Twenty percent of patients received third-line therapy. After a median follow-up of 6.9 (4.0-13.7) mo, 79% of patients died, and the median OS was 7.1 (4.2, 17.0) mo (Figure 2A). PFS was 3.6 (1.6, 10.9) mo and the DCR was 37% at the end of follow-up.

### OS according to Child–Pugh grade, presence of macrovascular invasion, and extrahepatic disease

After matching CP grade, BCLC staging, vascular invasion, metastasis and AFP level < ≥ 400 ng/mL, the median OS of HCC patients classified CP-A was 8.3 (5.2-24.8) mo *vs* 4.9 (1.6-11.7) mo for CP-B (*P* = 0.0468). The median OS of HCC patients without vascular invasion was 12.0 (5.2-24.8) mo *vs* 6.3 (3.4-23.0) mo for patients with vascular invasion (*P* = 0.3471). The survival time of patients with and without metastases was 8.3 (4.2-24.8) *vs* 8.2 (4.9-17.0) mo, respectively (*P* = 0.8902).

### REG vs CBZ as second-line therapy: Non-adjusted indirect comparative analysis

Fifty-eight patients, who were 68 (62-74) years old, with BCLC stage B/C (86%) HCC and CP-A/B (24%) received REG for 3.4 (1.4-10.5) mo as second-line therapy (Figure 1). Twenty-eight patients, who were 68 (60-73) years old, with BCLC stage B/C (75%) HCC and CP-A/B (25%) received CBZ for 3.7 (1.8-4.9) mo as second-line therapy. The median time on sorafenib was 3 (2-4) mo in the CBZ group and 4 (2.9-11.8) mo in the REG group (*P* = 0.0226). The median PFS was not significantly different [3.6 (1.4-11.7) mo REG *vs* 4.0 (1.8-10.9) mo CBZ, *P* = 0.7495], and the DCR was not different [24% (REG) *vs* 32% (CBZ), *P* = 0.4466] after a median follow-up period of 7.8 (3.6-14.1) mo (REG) *vs* 5.2 (4.1-9.4) mo (CBZ) (*P* = 0.3049) (Table 2).

### REG vs CBZ as second-line therapy: Matched-pair analysis

Only patients with two lines of systemic TKI therapy were considered (Figure 1). A total of 42 patients received REG as second-line therapy, and 27 patients received CBZ without subsequent treatment (Figure 1). After PSM, there were 25 patients in each group. The main characteristics of these patients are shown in Table 3. There was no significant difference between the two groups in PS, liver function, cirrhosis etiology, tumor burden, CRP level, or the number of patients with AFP ≥ 400 ng or NLR > 3. The median duration of prior sorafenib treatment was 3.2 (2.7-10.9) mo (REG) *vs* 3 (1.7-4.1) mo (CBZ) (*P* = 0.1865). After a median follow-up period of 6.2 (2.7-11.7) mo (REG) *vs* 5.2 (4-7.2) mo (CBZ) (*P* = 0.6925), 92% of patients receiving REG died compared to 64% of patients receiving CBZ (*P* = 0.0374). PFS was not significantly different [2.9 (1.4-10.7) mo (REG) *vs* 3.6 (1.8-5.9) mo (CBZ), *P* = 0.7896] (Figure 2B),

**Table 1 Patient characteristics prior to second-line treatment (entire cohort)**

| Characteristics at baseline | *n* = 86 |
|---|---|
| Age–median (Q1Q3), yr | 68.0 (60-74) |
| **Gender, *n* (%)** | |
| Male | 77 (90) |
| Female | 9 (10) |
| **Etiology of HCC, *n* (%)** | |
| Alcohol use | 30 (35) |
| Virus/Virus + Alcohol | 26 (30)/8 (9) |
| Non-alcoholic steatohepatitis | 13 (15) |
| Other | 9 (10) |
| **ECOG performance status, *n* (%)** | |
| 0 | 33 (38) |
| 1 | 34 (40) |
| 2 | 19 (22) |
| Esophageal varices[1], *n* (%) | 36 (45) |
| Macrovascular invasion, *n* (%) | 41 (48) |
| Extrahepatic disease, *n* (%) | 37 (43) |
| **Child-Pugh class, *n* (%)** | |
| A | 65 (76) |
| B[2] | 21 (24) |
| **BCLC stage, *n* (%)** | |
| B | 15 (17) |
| C | 71 (83) |
| **AFP, ng/mL, *n* (%)** | |
| < 400 | 45 (52) |
| ≥ 400 | 41 (48) |
| **HCC morphology[3], *n* (%)** | |
| Diffuse | 15 (18) |
| Mass forming | 24 (29) |
| Multinodular | 44 (53) |
| Maximal tumor diameter, mm–median (Q1Q3) | 69 (40-100) |
| Hemoglobin, g/dL–median (Q1Q3) | 13 (12-14) |
| Platelet's count (× 100/L)–median (Q1Q3) | 153 (95-213) |
| Neutrophil count/L–median (Q1Q3) | 3675 (2700-4600) |
| Lymphocyte count/L–median (Q1Q3) | 1118 (810-1650) |
| **Neutrophil-to-lymphocyte ratio, *n* (%)** | |
| ≤ 3 | 40 (47) |
| > 3 | 46 (53) |
| CRP, mg/L–median (Q1Q3) | 22 (8-51) |
| AST, IU/L–median (Q1Q3) | 62 (46-117) |
| ALT, IU/L–median (Q1Q3) | 40 (28-64) |
| GGT, IU/L–median (Q1Q3) | 187 (112-360) |

| | |
|---|---|
| ALP, IU/L–median (Q1Q3) | 166 (128-267) |
| Total bilirubin, μmol/L–median (Q1Q3) | 17 (12-27) |
| Albumin, g/L–median (Q1Q3) | 35 (29-39) |
| Creatinine, μmol/L–median (Q1Q3) | 70 (57-85) |
| Prothrombin time, %–median (Q1Q3) | 79 (68-93) |
| Duration of prior Sorafenib treatment, months–median (Q1Q3) | 3.5 (2.7-9.2) |

[1]Esophageal varices, missing data *n* = 6.
[2]Child–Pugh grade B: Child–Pugh (CP)-B7 *n* = 14, CP-B8 *n* = 4, CP-B9 *n* = 3.
[3]Hepatocellular carcinoma morphology, patients with metastatic recurrence and without intrahepatic tumor, *n* = 3.
HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis; PS: Performance status; BCLC: Barcelona clinic liver cancer; AFP: Alfa-fetoprotein; CRP: C-reactive protein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyl transpeptidase; ALP: Alkaline phosphatase.

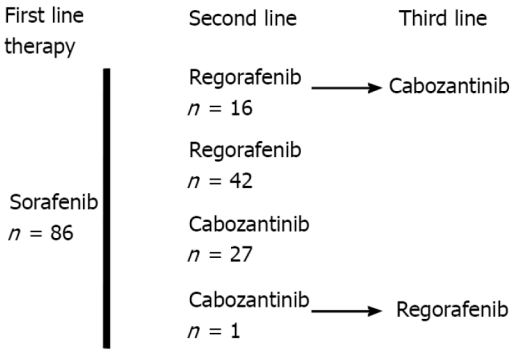


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**Figure 1 Study flowchart.**

and the DCR was not different [28% (REG) *vs* 32% (CBZ), *P* = 1.0000] (Table 3).

### *Characteristics and survival of patients treated with cabometyx as third-line systemic treatment*

Sixteen patients received CBZ as third-line systemic therapy. The median age was 68 (64-75) years at the start of treatment, and HCCs were classified as BCLC stage B/C (81%). Vascular invasion was present in 38% of cases, and metastasis was present in 37% of cases. Fifty percent of the patients had an AFP ≥ 400 ng/mL. After a median follow-up of 5.2 (3.1-16.6) mo, 63% of patients died, and the median OS was 8.1 (3.8-24.3) mo. There was no significant difference in PFS between patients who received CBZ as second-line or third-line therapy (*P* = 0.7044) (Figure 2C) after a comparable follow-up period [5.2 (4.0-8.2) mo *vs* 5.2 (3.1-16.6) mo, respectively, *P* = 0.8907] (Table 4).

### *Adverse events associated with REG and cabometyx as second-line therapy*

Adverse events, such as fatigue, anorexia and weight loss, were observed in both treatment groups with no significant difference between groups, primarily grades 1 and 2 toxicities. There was no significant difference in other common adverse events associated with TKIs, such as diarrhea, hand-foot skin reaction, increased blood bilirubin, increased Aspartate aminotransferase (AST)/Alanine aminotransferase, or hypertension. Drug-related AEs leading to interruptions or dose reduction were reported in greater than 40% of cases without a significant difference between groups (Table 5).

### *Univariate and multivariate analyses of baseline variables and tumor progression over time in matched-pair groups*

Univariate analysis of risk factors for tumor progression over time identified the following baseline variables: Bilirubin > 17 μmol, increased AST > 45 IU, increased CRP > 10 mg/L, and NLR > 3 (Table 6).

Multivariate analysis (MA) of risk factors for progression identified NLR > 3, increased CRP > 10 mg/L, and increased AST > 45 IU as independent variables over time (Table 6).

Based on these results, we defined a progression risk score at two months that was calculated at T0 before REG or CBZ: Score 2 M = - 0.1849 + 0.1943 × (1 if NLR ratio > 3, and 0 if < 3) + 0.3053 × (1 if CRP > 10, and 0 if < 10) + 0.4962 × (1 if AST > 45, and 0 if < 45). Scores approaching 1 indicate a higher the risk of progression (*i.e.*, score > 0.50 indicates increased risk of progression), and scores approaching 0, indicate a low risk of progression (*i.e.*, score < 0.50 indicates low risk).

**Table 2 Patient characteristics prior to second-line treatment with cabometyx or regorafenib (without matching)**

| Characteristics at baseline | Cabozantinib (*n* = 28) | Regorafenib (*n* = 58) | *P* value |
|---|---|---|---|
| Age–median (Q1Q3), yr | 68 (60-73) | 68 (62.74) | 0.6828 |
| **Gender, *n* (%)** | | | 0.4645 |
| Male | 24 (86) | 53 (91) | |
| Female | 4 (14) | 5 (9) | |
| **Etiology of HCC, *n* (%)** | | | 0.4219 |
| Alcohol use | 6 (21) | 24 (41) | |
| Virus/virus + alcohol | 10 (36)/4 (14) | 16 (28)/4 (7) | |
| NASH | 5 (18) | 8 (14) | |
| Other | 3 (11) | 6 (10) | |
| **PS, *n* (%)** | | | 0.6286 |
| 0 | 12 (44) | 21 (38) | |
| 1 | 9 (32) | 25 (43) | |
| 2 | 7 (24) | 12 (20) | |
| Esophageal varices[1], *n* (%) | 12 (44) | 24 (45) | 0.9432 |
| Macrovascular invasion, *n* (%) | 13 (46) | 28 (51) | 0.6995 |
| Extrahepatic disease, *n* (%) | 10 (36) | 27 (50) | 0.2177 |
| **Child-Pugh class, *n* (%)** | | | 1.0000 |
| A | 21 (75) | 44 (76) | |
| B | 7 (25) | 14 (24) | |
| **BCLC, *n* (%)** | | | 0.2375 |
| B | 7 (25) | 8 (14) | |
| C | 21 (75) | 50 (86) | |
| **AFP, ng/mL, *n* (%)** | | | 0.4468 |
| < 400 | 13 (46) | 32 (55) | |
| ≥ 400 | 15 (54) | 26 (45) | |
| **Morphology[2], *n* (%)** | | | 0.0830 |
| Diffuse | 7 (26) | 8 (14) | |
| Mass forming | 4 (15) | 20 (36) | |
| Multinodular | 16 (59) | 28 (50) | |
| Maximal tumor diameter, mm–median (Q1Q3) | 69.5 (37.5-118.5) | 68.5 (40-100) | 0.7495 |
| Hemoglobin, g/dL–median (Q1Q3) | 13.3 (12-14) | 13 (11-14.7) | 0.9730 |
| Platelet's count (× 100/L)–median (Q1Q3) | 136 (94-197) | 173 (97-215) | 0.2582 |
| Neutrophil count/L-median (Q1Q3) | 3118 (2120-3720) | 4081 (3000-5668) | 0.0042 |
| Lymphocyte count/L–median (Q1Q3) | 1130 (820-1675) | 1105 (810-1643) | 0.7723 |
| **Neutrophil-to- lymphocyte ratio, *n* (%)** | | | 0.0217 |
| ≤ 3 | 18 (64) | 22 (38) | |
| > 3 | 10 (36) | 36 (62) | |
| CRP, mg/L–median (Q1Q3) | 14.5 (6.7-36.2) | 29.7 (8.3-58) | 0.1665 |
| AST, IU/L–median (Q1Q3) | 73 (52-132) | 59 (41-95) | 0.0681 |
| ALT, IU/L–median (Q1Q3) | 47 (33-73) | 37 (26-51) | 0.0970 |
| GGT, IU/L–median (Q1Q3) | 150 (100-350) | 194 (117-362) | 0.3538 |

| | | | |
|---|---|---|---|
| ALP, IU/L–median (Q1Q3) | 159 (137-231) | 182 (122-269) | 0.6232 |
| Total bilirubin, µmol/L–median (Q1Q3) | 21 (14-29) | 17 (11-25) | 0.1135 |
| Albumin, g/L–median (Q1Q3) | 36 (31-39) | 34 (29-40) | 0.7476 |
| Creatinine, µmol/L–median (Q1Q3) | 67 (55-87) | 71 (57-84) | 0.6051 |
| Prothrombin time, %–median (Q1Q3) | 81 (68-99) | 78 (68-88) | 0.1878 |
| Duration of prior Sorafenib treatment, months–median (Q1Q3) | 3 (2-4) | 4 (2.9-11.8) | 0.0226 |

[1]Esophageal varices, Cabozantinib (CBZ): Missing data $n = 1$; Regorafenib (REG): Missing data $n = 5$.
[2]Hepatocellular carcinoma morphology, patients with metastatic recurrence and without intrahepatic tumor, CBZ $n = 1$, REG $n = 2$.
HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis; PS: Performance Status; BCLC: Barcelona clinic liver cancer; AFP: Alpha-fetoprotein; CRP: C-reactive protein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyl transpeptidase; ALP: Alkaline phosphatase.

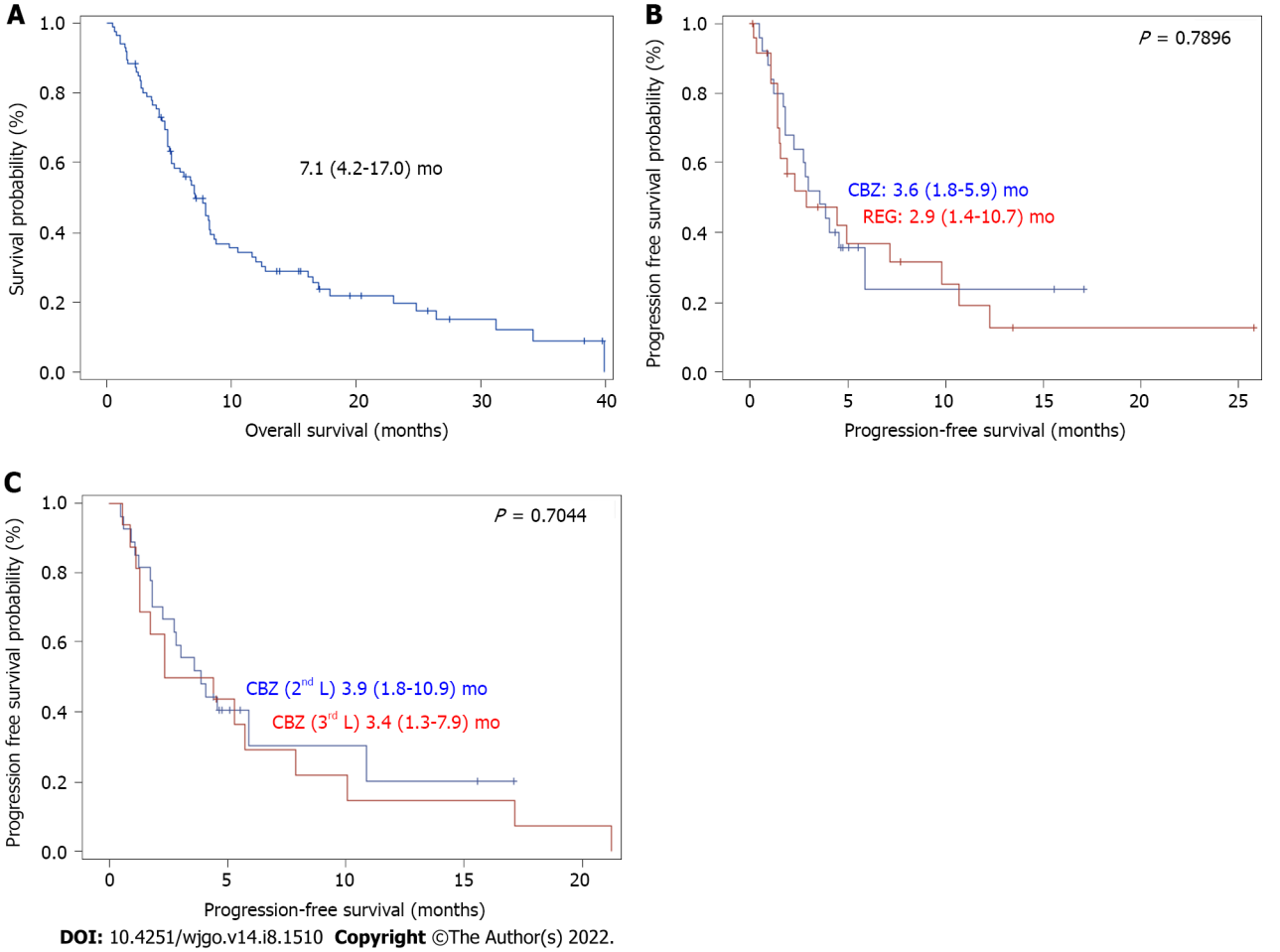


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**Figure 2 Kaplan-Meier curves.** A: Median overall survival in hepatocellular carcinoma (HCC) patients receiving regorafenib (REG) or cabozantinib (CBZ) (entire cohort $n = 86$); B: Median progression-free survival in HCC patients receiving REG *vs* CBZ as second-line therapy: matching-adjusted indirect comparison study; C: Median progression-free survival in HCC patients receiving second- or third-line CBZ. HCC: Hepatocellular carcinoma; REG: Regorafenib; CBZ: Cabozantinib.

To simplify the calculation, we used the following online application: https://jscalc.io/calc/3nzmguiJK5QIn8eQ#%7B%221%22:null,%222%22:null,%223%22:null%7D.

## DISCUSSION

The present real-life multicenter cohort studied the use of second-line therapy with TKIs for advanced HCC and found PFS of 3.6 (1.6-10.9) mo, which was similar to phase III studies with TKI[5] and anti-PD-1 monotherapy[8]. The median OS of 7.1 (4.2, 17.0) mo was naturally lower, despite an equivalent

**Table 3 Patient characteristics prior to second-line treatment with cabometyx or regorafenib: Matching-adjusted comparison study**

| Characteristics at baseline | Cabozantinib (*n* = 25) | Regorafenib (*n* = 25) | *P* value |
|---|---|---|---|
| Age–median (Q1Q3), yr | 69 (60-74) | 68 (58-72) | 0.7870 |
| **Gender, *n* (%)** | | | 1.0000 |
| Male | 23 (92) | 22 (88) | |
| Female | 2 (8) | 3 (12) | |
| **Etiology of HCC, *n* (%)** | | | 0.6370 |
| Alcohol | 6 (24) | 10 (40) | |
| Virus/virus + alcohol | 8 (32)/4 (16) | 7 (28)/2 (8) | |
| NASH | 4 (16) | 2 (8) | |
| Other | 3 (12) | 4 (16) | |
| **PS, *n* (%)** | | | 0.4591 |
| 0 | 9 (36) | 8 (29) | |
| 1 | 9 (36) | 10 (42) | |
| 2 | 7 (28) | 7 (29) | |
| Esophageal varices[1], *n* (%) | 10 (42) | 10 (45) | 0.7957 |
| Macrovascular invasion, *n* (%) | 12 (48) | 14 (58) | 0.4687 |
| Extrahepatic disease, *n* (%) | 10 (40) | 13 (54) | 0.3206 |
| **Child-Pugh class, *n* (%)** | | | 0.5512 |
| A | 18 (72) | 15 (60) | |
| B | 7 (28) | 10 (40) | |
| **BCLC, *n* (%)** | | | 0.7585 |
| B | 5 (20) | 5 (20) | |
| C | 20 (80) | 20 (80) | |
| **AFP, ng/mL, *n* (%)** | | | 0.5713 |
| < 400 | 12 (48) | 14 (56) | |
| ≥ 400 | 13 (52) | 11 (44) | |
| **Morphology[2], *n* (%)** | | | 0.2393 |
| Diffuse | 7 (29) | 4 (14) | |
| Mass | 4 (17) | 9 (36) | |
| Multinodular | 13 (54) | 12 (50) | |
| Maximal tumor diameter, mm–median (Q1Q3) | 74 (38-130) | 70 (40-94) | 0.6067 |
| Hemoglobin g/dL–median (Q1Q3) | 13 (12-13.9) | 12.5 (10-13.7) | 0.2875 |
| Platelet's count (× 100/L)–median (Q1Q3) | 148 (95-193) | 152 (97-206) | 0.6229 |
| Neutrophil count/L–median (Q1Q3) | 3150 (1970-3760) | 4100 (3000-5676) | 0.0276 |
| Lymphocyte count/L–median (Q1Q3) | 1140 (810-1700) | 940 (739-1600) | 0.5828 |
| **Neutrophil-to-lymphocyteratio, *n* (%)** | | | 0.1564 |
| ≤ 3 | 16 (64) | 10 (40) | |
| > 3 | 9 (36) | 15 (60) | |
| CRP, mg/L–median (Q1Q3) | 14.5 (7.2-41.1) | 32 (8-65) | 0.2900 |
| AST, IU/L–median (Q1Q3) | 75 (56-134) | 64 (4 6-79) | 0.0940 |
| ALT, IU/L–median (Q1Q3) | 48 (33-77) | 30 (26-47) | 0.0556 |
| GGT, IU/L–median (Q1Q3) | 179 (99-360) | 187 (112-322) | 0.7925 |

| | | | |
|---|---|---|---|
| ALP, IU/L–median (Q1Q3) | 162 (138-252) | 203 (122-269) | 0.6094 |
| Total bilirubin, µmol/L–median (Q1Q3) | 17.5 (14-29) | 15.6 (12-27) | 0.5123 |
| Albumin, g/L–median (Q1Q3) | 36 (29-39) | 31.6 (28-35) | 0.1772 |
| Creatinine, µmol/L–median (Q1Q3) | 69 (57-89) | 72 (58-91) | 0.4996 |
| Prothrombin time, %–median (Q1Q3) | 80 (68-100) | 71 (61-78) | 0.0792 |
| Duration of prior Sorafenib treatment, months–median (Q1Q3) | 3 (1.7-4.1) | 3.2 (2.7-10.9) | 0.1865 |

[1]Esophageal varices, Cabozantinib (CBZ): Missing data $n = 1$; Regorafenib (REG): Missing data $n = 3$.

[2]Hepatocellular carcinoma morphology, patients with metastatic recurrence and without intrahepatic tumor, CBZ $n = 1$.

HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis; PS: Performance status; BCLC: Barcelona clinic liver cancer; AFP: Alpha-fetoprotein; CRP: C-reactive protein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-Glutamyl transpeptidase; ALP: Alkaline phosphatase.

duration of treatment [3.5 (1.6-8.3) mo] as the phase III studies with TKIs[5,6], which is consistent with our cohort's features, including PS 2 patients or patients classified as CP-B grade, but inconsistent with the RESORCE[5], CELESTIAL[6], REACH-2[7] trials. These randomized controlled studies included CP-A patients, PS 0/1, mostly with viral disease, except for the KEYNOTE-240[8] study. Vascular invasion (recognized as a significant aggressive feature) was found in 13% to 36% of all patients in these studies [5-8], as opposed to one of two patients in our cohort. The median OS of HCC patients without vascular invasion in our cohort at 12 mo was comparable to real-life studies with REG[20] or CBZ[21]. The Korean ($n = 440$) and Italian ($n = 96$) cohorts included CP-A patients, and the Refine[22] study ($n = 498$) included 11% CP-B patients. These three studies reported a high proportion of metastatic patients (> 60%) with lower vascular invasion in 30%-35% of patients. Other evidence of cohort differences is that the prior duration of sorafenib treatment in our study was reduced compared to phase III studies, except for REACH-2[7] (4 mo), which included only patients with AFP levels ≥ 400 ng/mL. The median treatment duration was 5.0 mo in the CELESTIAL[6] study (with 43% of patients receiving more than 6 mo of sorafenib[23]). It was also 5 mo in the KEYNOTE-240[8] study and 7.8 mo in the RESORCE[5] study. Yoo *et al*[20] found that the time to progression on prior sorafenib < median was an independent outcome factor that adversely affected survival. Another indirect comparison study with CBZ and REG in real life that arose from the CELESTIAL study reported that the OS of patients on REG was 6.5 mo (IQR: 4.7-10.9) for a prior duration of sorafenib treatment < 3 mo[24].

Clinicians are dealing with populations that do not fit the phase III trials, and moving phase III trial results to real-life patients in clinical practice is challenging. This difficulty highlights the importance of real-life cohorts. Patients with preserved liver function in our cohort had better OS than CP-B patients. Consistent with other studies of TKIs[25,26], the OS of CP-B patients was low at less than 5 mo. Kim *et al*[26] did not find any difference in PFS or OS between CP-B7 patients and CP-B 8/9 patients. Our study included too few patients to make this distinction. CP-B liver function was an independent prognostic variable in MA that adversely affected PFS and OS in Kim *et al*[26] Therefore, preserved liver function is an essential criterion for first- or second-line systemic TKI therapy eligibility. Real-life studies with immune checkpoint inhibitors also suggest caution[27]. A multicenter retrospective cohort study assessing antibodies targeting the immune checkpoint molecule PD-1 in advanced HCC patients with or without prior systemic therapy found a comparable rate of side effects but a significant difference in survival between patients classified as CP-A and CP-B [16.7 (8.2-25.2) mo *vs* 8.6 (4.8-12.4) mo, respectively, $P = 0.065$].

Switching to a second-line systemic therapy is now a common situation, although it occurs in fewer than half of patients in the TKI era[28,29]. The only therapeutic options in France in this situation are REG or CBZ, but no head-to-head phase III trial is available for reference. We do not have ramucirumab (which is recommended for HCC patients with AFP > 400 ng/mL) or an immune checkpoint inhibitor against PD-1, although pembrolizumab is approved by the Food Drug Administration in this setting. This French multicenter series is one of the first indirect real-life comparison studies between REG and CBZ as second-line systemic treatment for HCC. Despite different mechanisms of action, this study found no difference in efficacy before and after matching, which contrasts the indirect comparison studies from CELESTIAL and RESORCE populations on PFS[30]. REG[31] is a multiple protein kinase inhibitor that targets angiogenesis (the VEGFR 1-3 and the angiopoietin 1 receptor TIE2) more intensively than sorafenib, tumor cells (especially the oncogenic kinases KIT and RET and the intracellular kinases Raf), and fibroblast growth factor receptors in contrast to sorafenib. CBZ[32] also targets key angiogenesis receptors, including VEGFR-2, AXL, and MET, which exhibits expression increased after sorafenib as an escape mechanism driven by hypoxia inducible factor (HIF)-1. The mortality rate was higher in the REG group most likely because the initiation of CBZ treatment was more recent (molecule available in France from July 2019 *vs* November 2017 for REG). Therefore, an OS assessment would be biased, especially because patients on REG as second line received CBZ as 3rd

**Table 4 Patient characteristics prior to third-line treatment with cabometyx**

| Characteristics at baseline | Cabozantinib (*n* = 16) |
|---|---|
| Age–median (Q1Q3), yr | 68 (64-75) |
| **Gender, *n* (%)** | |
| Male | 15 (94) |
| Female | 1 (6) |
| **PS, *n* (%)** | |
| 0 | 8 (50) |
| 1 | 7 (44) |
| 2 | 1 (6) |
| Macrovascular invasion, *n* (%) | 6 (38) |
| Extrahepatic disease, *n* (%) | 10 (62) |
| **Child-Pugh class, *n* (%)** | |
| A | 10 (62) |
| B | 6 (38) |
| **BCLC, *n* (%)** | |
| B | 3 (19) |
| C | 13 (81) |
| **AFP, ng/mL, *n* (%)** | |
| < 400 | 8 (50) |
| ≥ 400 | 8 (50) |
| Maximal tumor diameter, mm–median (Q1Q3) | 60 (32-106) |
| Hemoglobin, g/dL–median (Q1Q3) | 13.7 (11.4-14.7) |
| Platelet's count (× 100/L)–median (Q1Q3) | 159 (107-246) |
| Neutrophil count/L–median (Q1Q3) | 4690 (3128-7463) |
| Lymphocyte count/L–median (Q1Q3) | 1063 (783-1359) |
| **Neutrophil–lymphocyte ratio, *n* (%)** | |
| ≤ 3 | 3 (19) |
| > 3 | 13 (81) |
| CRP, mg/L–median (Q1Q3) | 33 (7-78) |
| AST, IU/L–median (Q1Q3) | 51 (33-76) |
| ALT, IU/L–median (Q1Q3) | 29 (21-53) |
| GGT, IU/L–median (Q1Q3) | 188 (111-323) |
| ALP, IU/L–median (Q1Q3) | 162 (120-253) |
| Total bilirubin, μmol/L–median (Q1Q3) | 15.9 (11.1-25.6) |
| Albumin, g/L–median (Q1Q3) | 33.5 (27.9-39.2) |
| Creatinine, μmol/L–median (Q1Q3) | 82 (56-91) |
| Prothrombin time, %–median (Q1Q3) | 81 (70-92) |

PS: Performance status; BCLC: Barcelona Clinic Liver Cancer; AFP: Alpha-fetoprotein; CRP: C-reactive protein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-Glutamyl transpeptidase; ALP: Alkaline phosphatase.

line. Notably, the PFS of patients treated with CBZ as second- or third-line treatment was similar, which suggests comparable efficacy in these two situations, as observed in the CELESTIAL study.

**Table 5 Adverse events associated with regorafenib or cabometyx as second-line therapy**

| Adverse event | Cabozantinib (*n* = 28) | Regorafenib (*n* = 58) | *P* value |
|---|---|---|---|
| **Fatigue and/or decreased appetite and/or weight loss, *n* (%)** | 22 (79) | 46 (79) | 1.0000 |
| Grade 1-2/3-4 | 20 (89)/2 (11) | 40 (85)/7 (15) | 1.0000 |
| **Hand-foot skin, *n* (%)** | 9 (32) | 16 (28) | 0.8005 |
| Grade 1-2/3-4 | 8 (89)/1 (11) | 11 (69)/5 (31) | 0.3644 |
| **Diarrhea, *n* (%)** | 11 (39) | 13 (22) | 0.1021 |
| Grade 1-2/3-4 | 11 (100)/0 | 13 (100)/0 | 1.0000 |
| **Increased blood Bilirubin and/or AST and/or ALT, *n* (%)** | 9 (32) | 17 (29) | 0.8063 |
| Grade 1-2/3-4 | 4 (44)/5 (56) | 11 (65)/6 (35) | 0.4185 |
| **Hypertension, *n* (%)** | 6 (21) | 12 (21) | 1.0000 |
| Grade 1-2/3-4 | 5 (83)/1 (17) | 11 (92)/1 (8) | 1.0000 |
| **Other disorders[1], *n* (%)** | 14 (50) | 19 (33) | 0.1234 |
| Grade 1-2/3-4 | 12 (86)/2 (14) | 18 (95)/1 (5) | 0.5612 |
| **Interruptions, *n* (%)** | 12 (43) | 31 (53) | 0.3573 |
| **Dose reduction, *n* (%)** | 23 (82) | 49 (84) | 0.7646 |

[1]Other disorders: Oral mucositis, dysphonia, decrease in platelet count, muscular pain, ascites.
AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

**Table 6 Univariate and multivariate analyses of risk factors for tumor progression over time in matched-pair groups**

| Variables | Univariate analysis, *P* value | Multivariate analysis, *P* value |
|---|---|---|
| Treatment with CBZ *vs* REG | 0.8851 | - |
| NLR ≤ 3 *vs* > 3 | 0.0006[1] | 0.0006 |
| CRP (mg/L) > 10 *vs* ≤ 10 | 0.0364[1] | 0.0624 |
| ALP (IU) > 200 *vs* ≤ 200 | 0.5545 | - |
| Bilirubin total (μmol/L) > 17 *vs* ≤ 17 | 0.0270[1] | 0.3262 |
| Albumin (g/L) > 36 *vs* ≤ 36 | 0.3026 | - |
| PT (%) > 70 *vs* ≤ 70 | 0.0534 | - |
| AST (IU) > 45 *vs* ≤ 45 | 0.0048[1] | 0.0132 |
| AFP (ng/mL) > 400 *vs* ≤ 400 | 0.0634 | - |

[1]Included in multivariate analysis.
CBZ: Cabozantinib; REG: Regorafenib; NLR: Neutrophil-to-lymphocyte ratio; CRP: C-reactive protein; ALP: Alkaline phosphatase; PT: Prothrombin time; AST: Aspartate aminotransferase; AFP: Alpha-fetoprotein.

Most patients were out of control at the end of the follow-up period (nearly two-thirds). The therapeutic landscape in advanced HCC has profoundly changed since 2020, with the success of combination therapies in first-line (anti-PDL1 antibody + bevacizumab)[10] and second-line (anti-CTLA-4 + anti-PD1 antibodies)[9] treatments, which exhibit increased response rates and prolonged survival, despite the lack of available biomarkers. Beyond a simple association, a synergistic action exists between these molecules. Interfering with VEGF-VEGFR signaling improves the anti-tumor immune response by enhancing T-cell recruitment and functionality and reducing immunosuppressive cells, such as MDSCs and regulatory T cells[11]. The tumor microenvironment composed of endothelial cells, pericytes, fibroblasts and various immune cells adopting a pro-tumor phenotype plays a key role in the inhibition of the lymphocyte effective response. Some of the various mechanisms include the expression of PD-L1 co-inhibitory molecules, the presence of PD-1 and CTLA-4 inhibitory immune checkpoint molecules, and others, such as T-cell immunoglobulin and mucin-domain containing-3 and lymphocyte activation gene 3, on T cells and a decrease in functional dendritic cells and the presence of

immunosuppressive populations. Therefore, it is likely that other alternatives will be available in the near future for second-line treatment. Based on this interaction between neoangiogenesis and anti-tumor immunity, the combination of TKIs, such as cabometyx or REG, with immunotherapies targeting PD-1 and CTLA-4 is relevant. Trials evaluating various combinations are underway.

Overall, we found the most common side effects observed with TKIs in phase III trials[5,6] and real-life studies[21,22], namely fatigue, diarrhea, hand-foot skin reaction, loss of appetite, weight loss and hypertension. The general signs were associated with most patients in both groups. Differences were observed in the severity of hand-foot syndrome with REG and in the frequency of diarrhea with CBZ, but without significance. The phase III trials[5,6] and real-life studies[21] found dose reductions and interruptions in most patients (RESORCE: 68%, CELESTIAL: 62%). The frequency and magnitude of side effects associated with TKIs may be a limitation to the long-term use of this therapy, especially when used at full doses[33], despite improved clinician experience[34].

The present study also highlights the relevance of inflammation-related serum factors in the setting of advanced HCC, such as CRP and the NLR, which was shown in other studies[35,36]. These factors were independent prognostic variables that correlated with disease progression over time in our study. We focused on inflammatory markers to assess their role in predicting clinical outcome in this multicenter HCC cohort treated with TKI as second-line therapy because no markers, other than AFP, are currently used. Systemic inflammation is associated with tumor progression[16], the promotion of genomic instability[37], angiogenesis, and cell proliferation[38]. This systemic inflammation, as measured by a high CRP serum level or increased NLR, was reported as a worse prognostic marker in various types of cancer[15]. Tumor necrosis and inflammation are closely linked[39] and enable a hypoxic environment prone to mutations, which is driven by the release of reactive oxygen species[40]. Some of the mediators of this cancer-related inflammatory process involve transcription factors, such as nuclear factor-kappaB, the pro-angiogenic factor HIF-1 alpha, and their effects on interleukin (IL)-6 production, a multifunctional pro-inflammatory cytokine, and the IL-6/Janus kinase/signal transducer and activator of transcription 3 pathway, which promotes cell proliferation, survival and migration[40]. High levels of IL-6 are associated with tumor growth, and it contributes to angiogenesis[41] and the inhibition of apoptosis[42]. The over-expression of IL-6 also affects the immune response *via* the functional impairment of lymphocytes and the recruitment of immunosuppressive cells[43]. IL-6 is found in the epithelium and tumor stroma of various solid tumors[42]. IL-6 plays an important role in the hepatic overproduction of CRP, and some studies found a positive correlation between increased blood levels of IL-6 and CRP[44,45]. High CRP serum levels are also associated with hypoalbuminemia in cancer[46]. High neutrophil counts and low lymphocyte counts, which mirror this inflammatory process, are also prognostic markers in various cancers at different stages[47]. The prognostic value of NLR is now strongly suggested in the setting of immunotherapy[48], especially during the course of treatment[49]. Therefore, inflammatory scores, such as the Glasgow Prognostic Score or the NLR, demonstrated their prognostic value regardless of cancer type and stage[14], including in controlled studies[15,50]. In summary, these scores reflect this cancer-related inflammatory response.

We investigated baseline variables associated with 2-mo tumor progression risk, considering the short action time of TKI treatment. Our study suggests that higher inflammatory markers and increased AST, which may reflect deterioration of liver function and/or liver tumor growth, are associated with a higher risk of early progression under TKI, *i.e.*, nonresponse. Therefore, careful tumor assessment using imaging and a safety evaluation are required in these patients due to the high adverse event rate related to TKI. Because these parameters are easily available on a routine blood test, we developed a progression risk score based on these variables.

Limitations of the present study include the limited sample size, the retrospective design of the study and the lack of a control group, which prevent definitive conclusions of our model. However, our results are consistent with prior publications, and other studies after sorafenib included comparable population sizes[21,26]. Given the limited response rate to first-line TKIs and the time to control is frequently less than six months[1], few patients will complete a second-line regimen[29]. A previous study of first-line sorafenib therapy[34] had 188 patients in one center compared to 86 patients in three centers for the present study. The retrospective character necessarily leads to various biases, but we considered only patients with all data, and because the data were biological or radiological data, the risk of error was limited, especially because these data were collected in a recent period. Obviously, this model must be evaluated in an independent cohort, but it is based on robust variables.

## CONCLUSION

This indirect comparison from a real-life multicenter cohort found no difference in PFS with the use of REG or CBZ as second-line therapy for advanced HCC. Most patients did not achieve controlled disease at the end of follow-up, particularly patients with vascular invasion. Our results also show that TKIs are not indicated for CP-B patients. Inflammation-related factors (CRP and NLR ratio) and AST increased over time were associated with a higher risk of TKI failure. We propose an online score to assess progression risk based on these variables after two months of treatment.

## ARTICLE HIGHLIGHTS

### *Research background*

Switching to a second line of systemic therapy will theoretically concern most patients with advanced hepatocellular carcinoma (HCC), especially after sorafenib. The strict selection criteria in phase III trials result in a lack of data for many patients from current practice. Inflammation acts as a powerful tumor promoter.

### *Research motivation*

Two multi-targeted tyrosine kinase inhibitors (TKIs) [Regorafenib (REG), Cabozantinib (CBZ)] are currently the only available therapeutic options in France in this situation based on phase III trials after sorafenib. There are also no direct comparative studies between the "approved" second-line molecules or any predictive biomarker correlated with treatment activity.

### *Research objectives*

To assess both efficacy and safety of REG and CBZ as second-line systemic treatment after sorafenib in a "real-life" study. To investigate the relevance of serum inflammation-related markers as predictive factors for tumor progression over time in this setting. The current lack of treatment-guiding biomarkers and the safety profile of TKIs are limiting factors for this sequencing.

### *Research methods*

This is an indirect propensity score-matched comparative study based on recent retrospective data recorded in three French centers. We focused on progression-free survival and disease control rates of patients treated with REG or CBZ, and on factors associated with tumor progression over time.

### *Research results*

Both efficacy and safety of REG and CBZ are comparable in this real-life study, and CBZ is still a third-line therapeutic option. Elevated levels of pretherapeutic inflammation-related markers [C-reactive protein (CRP) serum level, neutrophil-to-lymphocyte ratio (NLR)] are associated with poorer survival by using TKIs as second-line treatment for HCC.

### *Research conclusions*

In light of the limited tumor control rate with TKIs and the positive results of first- (anti-programmed death ligand-1 + anti-vascular endothelial growth factor) and second-line (anti-human cytotoxic T-lymphocyte antigen-4 + anti-programmed death receptor-1) combination therapies, the therapeutic "landscape" of advanced HCC will be changed in the second-line setting. We propose a 2-mo online progression risk calculation based on CRP serum level, NLR, and aspartate aminotransferase level to estimate the disease course under ITKs treatment.

### *Research perspectives*

The tumor microenvironment plays a key role in the suppression of an effective lymphocyte response. TKIs exhibit anti-angiogenic and immunomodulatory properties. Combinations of TKIs and immune checkpoint inhibitors are currently being evaluated as second-line systemic therapy for HCC.

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**Institutional review board statement:** The study was reviewed and approved by the Fondation Hopital Saint Joseph Institutional Review Board.

**Informed consent statement:** This study was based on a retrospective analysis of clinical data. Therefore, the Institutional Review Board waived the requirement for informed patient consent. The ethics committee of our institution (Hôpital Saint-Joseph) authorized this work.

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**Data sharing statement:** Attached is the registration number of the Public Directory of Data Projects (No. F20210311142439). This is the website's link: https://www.health-data-hub.fr/projets/traitement-systemique-du-carcinome-hepatocellulaire-apres-sorafenib-etude-de-cohorte.

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ORIGINAL ARTICLE

Retrospective Study

# Profiling of gene fusion involving targetable genes in Chinese gastric cancer

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## Abstract

*BACKGROUND*

Approximately half of all new cases of gastric cancer (GC) and related deaths occur in China. More than 80% of patients with GC are diagnosed at an advanced stage, which results in poor prognosis. Although *HER2*-directed therapy and immune checkpoint inhibitors have been somewhat successful, new drugs are still needed for the treatment of GC. Notably, several gene fusion-targeted drugs have been approved by the United States Food and Drug Administration for solid tumors, including GC, such as larotrectinib for *NTRK* fusion-positive cancers and zenocutuzumab for *NRG1* fusion-positive cancers. However, gene fusions involving targetable genes have not been well characterized in Chinese patients

with GC.

***AIM***

To identify the profile of fusions involving targetable genes in Chinese patients with GC using clinical specimens and determine the distribution of patients with gene fusion variants among the molecular subtypes of GC.

***METHODS***

We retrospectively analyzed gene fusion events in tumor tissue samples from 954 Chinese patients with GC. Clinicopathological characteristics were obtained from their medical records. Genetic alterations, such as single nucleotide variants, indels, amplifications, and gene fusions, were identified using a targeted sequencing panel containing 825 genes. Fusions were validated by fluorescence in situ hybridization (FISH) using break-apart probes. The microsatellite instability (MSI) status was evaluated using MSIsensor from the targeted sequencing panel data. Tumor mutational burden (TMB) was calculated using the total number of nonsynonymous mutations divided by the total genomic targeted region. Chi-square analysis was used to determine the enrichment of gene fusions associated with the molecular subtypes of GC.

***RESULTS***

We found that 1.68% (16/954) of patients harbored 20 fusion events involving targetable genes. *RARA* fusions ($n$ = 5) were the most common, followed by *FGFR2*, *BRAF*, *MET*, *FGFR3*, *RET*, *ALK*, *EGFR*, *NTRK2*, and *NRG1* fusions. Two of the *RARA* fusions, *EML4-ALK* (E6:E20) and *EGFR-SEPTIN14* (E7:E10), have been identified in other tumors but not in GC. Surprisingly, 18 gene fusion events were previously not reported in any cancer types. Twelve of the eighteen novel gene fusions included complete exons encoding functional domains of targetable genes, such as the tyrosine kinase domain of receptor tyrosine kinases and the DNA- and ligand-binding domains of *RARA*. Consistent with the results of detection using the targeted sequencing fusion panel, the results of FISH (fluorescence in situ hybridization) confirmed the rearrangement of *FGFR2* and *BRAF* in tumors from patients 04 and 09, respectively. Genetic analysis indicated that the fusion genes were significantly enriched in patients with *ERBB2* amplification ($P$ = 0.02); however, there were no significant differences between fusion-positive and fusion-negative patients in age, sex, MSI status, and TMB.

***CONCLUSION***

We characterized the landscape of fusions involving targetable genes in a Chinese GC cohort and found that 1.68% of patients with GC harbor potential targetable gene fusions, which were enriched in patients with *ERBB2* amplification. Gene fusion detection may provide a potential treatment strategy for patients with GC with disease progression following standard therapy.

**Key Words:** Gene fusion; Targetable genes; Gastric cancer; Chinese population; *ERBB2* amplification



**Core Tip:** The proportion of patients with gene fusions in Chinese patients with gastric cancer (GC) has not yet been characterized. In our analysis, we found that 1.68% of such patients harbor fusions involving targetable genes. Moreover, these fusion genes were enriched in patients with *ERBB2* amplification. Our study indicates that gene fusion detection may provide a novel approach for GC therapy.

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## INTRODUCTION

Gastric cancer (GC) is the fifth most frequent cancer and the third leading cause of cancer deaths worldwide, with more than one million new cases and approximately 769000 deaths in 2020[1]. The overall survival rate of patients with early stage disease is around 90% after surgical resection[2];

however, more than 80% of patients with GC are diagnosed at an advanced stage in China, which limits the effectiveness of the treatment[3]. Although chemotherapy has improved the survival of advanced-stage patients with GC, the objective response rate remains less than 40%, and the median overall survival is less than 12 mo[4]. Nevertheless, new targeted therapies are capable of improving the objective response rate and overall survival of patients with GC expressing certain targets[5].

Approximately 13%-22% of GCs exhibit *HER2* overexpression or amplification[6-8]. The College of American Pathologists, the American Society for Clinical Pathology, and the American Society of Clinical Oncology recommend that all patients with advanced gastric adenocarcinoma should be tested for *HER2* overexpression[9]. Trastuzumab was approved by the United States Food and Drug Administration (FDA) in 2010 as first-line treatment in combination with chemotherapy for patients with *HER2*-positive GC. Microsatellite instability-high (MSI-H) tumors are considered a molecular subtype of gastric adenocarcinoma by The Cancer Genome Atlas (TCGA)[10]. The incidence of MSI-H GC is 10%-20%[11]. The NCCN guidelines recommend MSI testing as a standard test for all patients with GC. Regarding targeted therapy, the FDA has approved pembrolizumab (*PD1* monoclonal antibody) for the treatment of all unresectable or metastatic solid tumors with MSI-H/dMMR (deficient DNA mismatch repair), including GC. Although drug treatments have shown success to some extent, the development of more targeted drugs is required.

With rapid advancements in the field of oncogenomics, gene fusions in cancer have received increasing attention. The FDA has approved larotrectinib (Vitrakvi) and entrectinib (Rozlytrek) for the first- or subsequent-line treatment of solid tumors with *NTRK* fusions, including GC[12,13]. In 2021, the FDA accelerated the approval of the *NRG1* inhibitor, zenocutuzumab (MCLA-128), in patients with pan-cancer harboring an *NRG1* fusion. Apart from these fusion genes with approved drugs in pan-cancer, *ALK* fusions, such as *EML4-ALK*, *TFG-ALK*, and *STRN-ALK*, have been identified in the majority of tumors, including lung adenocarcinoma and colorectal cancer[14-16]. For lung cancer and mesenchymal tumors, patients harboring an *ALK* fusion are highly responsive to crizotinib and ceritinib[17,18]. Recently, a *RAB10-ALK* fusion was identified in a patient with GC[19], which indicates the possibility of future applications of *ALK*-TKIs (tyrosine kinase inhibitors) in these patients. Recent advances in next-generation sequencing (NGS) have contributed to a surge in the discovery of fusion genes, including *BRAF*; *EGFR*; *FGFR1*, *2*, and *3*; *RET*; and *ROS1*[20]. Gene fusion detection can guide the development of targeted therapeutic strategies for patients with GC with disease progression after standard therapy. Notably, there is a lack of comprehensive data characterizing gene fusions involving targetable genes in GC, particularly in the Chinese population.

## MATERIALS AND METHODS

### Patients

This multicenter retrospective study included 1341 patients with GC admitted to Fujian Provincial Hospital (Fuzhou, China) and Zhejiang Provincial People's Hospital (Hangzhou, China) between October 2015 and December 2021. The clinicopathological characteristics of the patients were retrieved from their medical records. Additionally, MSI status and tumor mutational burden (TMB) scores were extracted for statistical analysis. This study was approved by the Ethics Committee of the Fujian Provincial Hospital.

### Mutational profiling

Mutational profiling of the Onco PanScan panel was performed by Genetron Health (Beijing) Co., Ltd. The coding regions of 825 cancer-related genes were analyzed. Genomic DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue specimens with a minimum of 20% viable tumor nuclei. For sequencing, paired tumor and white blood cell DNA libraries were prepared using KAPA HyperPrep Kits (Roche, Germany). Libraries were quantified using Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, United States), and their quality was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). High-throughput sequencing was performed on Novaseq6000 platform (Illumina, United States). Paired-end reads from Illumina sequencing were processed using script bcl2fastq (v. 2.17.1.14) and aligned against the human genome reference build, GRCh37, using Burrows-Wheeler Aligner (BWA, version 0.7.13). Duplicate removal, local realignment, and base quality recalibration were performed using PICARD (http://broadinstitute.github.io/picard/) and the Genome Analysis Toolkit. Variant calling was performed using an in-house developed pipeline. Variants identified as germline variants were excluded, while single nucleotide variants (SNVs) and indels with allelic fractions of more than 5% and supported by more than 4 unique reads, amplification with a fold-change greater than 2.5 in more than 25% of regions covered, and gene fusions supported by more than 3 unique reads were included.

TMB was calculated using the total number of nonsynonymous mutations divided by the total genomic target region (2.13 Mb). MSI status was determined using MSIsensor from paired tumor-normal targeted sequence data, and 309 MSI sites were included in the panel of 825 cancer-related genes. An MSIsensor score below 10 defines microsatellite stability (MSS) status, while that above 50

defines MSI status. The prevalence of gene fusions involving a targetable gene and driver mutations was compared with the OrigiMed2020 and TCGA cohorts[21]. Clinicopathological and genomic data were retrieved from the cBioPortal (https://www.cbioportal.org).

### Fluorescence in situ hybridization

FFPE tissue sections (5 μm) were prepared on positively charged slides. After deparaffinizing and rehydrating, the slides were incubated with prewarmed 8% sodium thiocyanate in $dH_2O$ at 80 °C and incubated for 30 min. *FGFR2* (10q26) or *BRAF* (7q34) break-apart probes were placed on the slide, covered with a glass coverslip, and sealed with rubber cement. Hybridization was performed overnight at 37 °C. The slides were washed twice in 50% formamide at 47 °C for 2 min and then twice in 2X standard saline citrate at room temperature for 2 min. Nuclei were stained with DAPI as a counterstain. The slides were scanned using a 90i Nikon fluorescent microscope. For each probe, 200 nuclei were evaluated. The 5′ (red) and 3′ (green) signals separated by ≥ 2 signal diameters were considered split as positive.

### Statistical analysis

All statistical analyses were performed using SPSS 24.0 software (IBM, Chicago, IL, United States). $\chi^2$ or Fisher's exact test was used to analyze the association between fusion alterations and driver mutations. A $P$ value of < 0.05 was considered statistically significant.

## RESULTS

### Clinical characteristics of patients

We retrospectively analyzed 1341 Chinese patients with GC who underwent genetic analysis from multiple centers in China. Of these, 387 patients were excluded because gene fusion detection was not performed with the Onco PanScan panel using tumor tissue samples (Figure 1). Gene fusion events were detected in 20 patients; however, 4 patients without any gene fusions involving targetable genes were excluded. Finally, 16 patients with 20 fusion events involving targetable genes were included for further analysis. The clinical characteristics of 954 patients with GC are shown in Table 1. Of these patients, 310 (32.56%) were women and 644 (67.44%) were men, with a median age of 57 and 62, respectively, at diagnosis. There was no significant difference between targetable gene fusion-positive and -negative patients in age ($P$ = 0.293), sex ($P$ = 0.463), MSI status ($P$ = 0.551), or TMB ($P$ = 0.217) (Table 1).

### The landscape of gene fusions involving targetable genes in Chinese patients with GC

To gain insight into fusion events in GC, we evaluated 954 patients with GC undergoing gene fusion analysis. In total, 20 patients harbored 24 gene fusions, 2 patients had double fusions (patient 01 and 02), and 1 patient (09) harbored triple fusions. *RARA* fusions (5/24, 17.8%) and *FGFR* family gene fusions (5/24, 17.8%) occurred most frequently in the cohort, followed by *BRAF* (3/24, 10.7%) and *MET* (2/24, 8.3%) (Figure 2A). *ALK*, *RET*, *NTRK2*, *NRG1*, and *EGFR* fusions were identified in one patient each. Remarkably, 20 of 24 (83.3%) fusions involved targetable genes (Table 2). *RARA* has been frequently reported as a 3′ fusion partner in acute promyelocytic leukemia[22]. *RARA* was identified as a 5′ fusion partner in 4 patients and as a 3′ fusion partner in 1 patient; however, only the *KRPAT9-RARA* fusion was detected in patient 01 as the 3′ fusion partner including exons 3-9, which encodes a DNA-binding and a ligand-binding domain required for *RARA* transcription factor activity[22]. Three *BRAF* fusions were identified in patient 09 as the 3′ fusion partner containing the complete tyrosine kinase domain, which was coded by exons 11-18. All *FGFR2* and *FGFR3* fusions were detected as 5′ fusion partners. Four *FGFR2* fusions were consistent with other known activating *FGFR2* fusions[23], which frequently occur with a breakpoint after exon 17 at the 3′ end of *FGFR2* with a 3′ fusion partner. The kinase domain was retained in these fusion genes. In patient 10, the *MET* fusion involved the 5′ end of *MET* exon 7, thus retaining an intact *MET* kinase domain.

The frequency of fusion events involving the abovementioned 10 targetable genes in the TCGA GC cohort and another Chinese GC cohort (OrigiMed2020 cohort) were analyzed and compared with our patient data (Figure 2B). Neither our cohort nor the OrigiMed2020 cohort showed significant differences in the incidence of these gene fusions in Chinese patients. In two Chinese cohorts, *EGFR* fusions occurred less frequently. Fusions in *MET*, *BRAF*, *RET*, *ALK*, and *NTRK2* were only identified in two Chinese cohorts; however, the differences in the incidence of these genes were not statistically significant.

### Novel fusions involving targetable genes in GC

In total, 2 of 20 fusions involving the targetable genes, *EML4-ALK* and *EGFR-SEPTIN14*, were reported in other cancers, including non-small-cell lung cancer[24-26]. The remaining 18 gene fusions were not reported in any cancer types. In total, 13 of 18 novel gene fusions contained the exon encoding a tyrosine

**Table 1 Clinical characteristics in targetable gene fusion-positive and -negative patients**

| Variables | Total, *n* | Fusion involving targetable genes | | *P* value |
|---|---|---|---|---|
| | | Positive, *n* (%) | Negative, *n* (%) | |
| Sex | | | | 0.293 |
| Female | 310 | 3 (0.97) | 307 (99.03) | |
| Male | 644 | 13 (2.02) | 631 (97.98) | |
| Age, yr | | | | 0.463 |
| ≤ 60 | 451 | 6 (1.56) | 445 (98.44) | |
| > 60 | 503 | 10 (1.98) | 493 (98.01) | |
| MSI status | | | | 0.551 |
| MSI-H | 46 | 1 (2.17) | 45 (97.93) | |
| MSS | 908 | 15 (1.65) | 893 (98.35) | |
| TMB | | | | 0.217 |
| Median TMB score | 2.92 | 5.63 | 2.83 | |

Age, sex, microsatellite instability status, and tumor mutational burden between fusion-positive and -negative patients were compared. The one-tailed *P* value for Fisher's exact test was calculated. MSI-H: Microsatellite instability-high; MSS: Microsatellite stability; TMB: Tumor mutational burden.

kinase domain, such as exons 11-17 of *FGFR2*, exons 11-18 of *BRAF*, exons 12-19 of *MET*, and exons 16-21 of *NTRK2* (Figure 3). All fusions involving *FGFR2*, *BRAF*, *RET*, and *NTRK2* retained the kinase domain (Table 2). Furthermore, reads in Integrative Genomics Viewer plots supported these gene fusions. To verify these novel fusions, fluorescence in situ hybridization (FISH) was performed using break-apart probes. Because only two tumor tissue samples were available, only the *FGFR2* and *BRAF* arrangement in patient 04 and 09, respectively, were confirmed by FISH.

### *Gene fusions are enriched in patients with ERBB2 amplification but not in those with high MSI and TMB*

Because of the low frequency of gene fusions in patients with GC, we determined whether gene fusions are enriched in different molecular subtypes of GC, which may indicate the patients that could benefit from gene fusion detection. Fusions are mutually exclusive with other oncogenic mutations and are enriched in patients without driver mutations[27-29]. In our cohort, the frequency of genetic alterations in oncogenic driver genes of GC, such as *TP53*, *ARID1A*, *CDH1*, and *PIK3CA* mutations and *ERBB2* amplification, were comparable with those in the TCGA cohort (Supplementary Figure 1). There was no significant difference in the frequency of fusions involving targetable genes between patients with any alterations in all five driver genes and those without (Figure 4A). Notably, the fusion alteration frequency was significantly higher in patients with *ERBB2* amplification than in those without *ERBB2* amplification (Figure 4B, $P = 0.01$). To determine whether fusion alterations were enriched in other driver genes, *TP53*, *ARID1A*, *CDH1*, and *PIK3CA* were analyzed. There was no enrichment in fusion alterations for these genes (Supplementary Figure 2). Forty-six patients had the MSI-H phenotype. Of these, one patient with fusion genes exhibited MSI-H. There was no obvious difference in the incidence of gene fusions between patients with MSI-H and MSS (Figure 4C). Similarly, TMB scores were evaluated in targetable gene fusion-positive and -negative patients, but the results were not statistically significant (Figure 4D).

## DISCUSSION

Structural gene rearrangements leading to gene fusions are common events that occur in solid tumors. Gene fusions have been considered oncogenic drivers in neoplasia for more than 30 years[30]. Detection and characterization of gene fusions is important for clinical purposes[31]. As the first large-scale study focusing on gene fusion events in Chinese patients with GC, we retrospectively analyzed 954 tumor specimens to identify fusions involving targetable genes and confirmed the occurrence of these fusions in GC.

In this study, 16 of 954 patients harbored 20 fusions involving targetable genes, the majority of which had not been previously reported, including *FGFR2-PDE2A*, *STIM2-BRAF*, *OPALIN-RET*, and *ARHGAP10-NTRK2*. However, we did not find any significant differences between the Chinese GC

**Table 2 List of gene fusions involving targetable genes in Chinese patients with gastric cancer and drugs under clinical trial or approved by the Food and Drug Administration**

| Patients ID | Fusion gene | 5' partner gene | | | | 3' partner gene | | | | Variant frequency, % | Functional domain is included or not | Targeted drugs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Gene name | Chromosome | Last observed exon | Breakpoint | Gene name | Chromosome | First observed exon | Breakpoint | | | |
| Patient 01 | *RARA-PGAP3* | *RARA* | 17 | 3 | 38504951 | *PGAP3* | 17 | 8 | 37828020 | 24.1 | Partially include | Tamibarotene targeting RARA fusion[2] |
| Patient 01 | *KRTAP9-7-RARA* | *KRTAP9-7* | 17 | downstream | 39437039 | *RARA* | 17 | 3 | 38499547 | 56.9 | Completely include | Tamibarotene targeting RARA fusion[2] |
| Patient 02 | *RARA-KRT13* | *RARA* | 17 | 2 | 38491648 | *KRT13* | 17 | 8 | 39657269 | 29.2 | Partially include | Tamibarotene targeting RARA fusion[2] |
| Patient 02 | *RARA-ETV4* | *RARA* | 17 | 2 | 38499726 | *ETV4* | 17 | 5 | 41621243 | 76 | Partially include | Tamibarotene targeting RARA fusion[2] |
| Patient 03 | *RARA-IKZF3* | *RARA* | 17 | 2 | 38504120 | *IKZF3* | 17 | 2 | 38009555 | 18.4 | Partially include | Tamibarotene targeting RARA fusion[2] |
| Patient 04 | *FGFR2-PDE2A* | *FGFR2* | 10 | 17 | 123241248 | *PDE2A* | 11 | 7 | 72307251 | 1.4 | Completely include | Pemigatinib; Erdafitinib targeting FGFR fusion[1] |
| Patient 05 | *FGFR2-intergenic* | *FGFR2* | 10 | 17 | 123242196 | *intergenic* | 10 | - | 123394107 | 16.6 | Completely include | Pemigatinib; Erdafitinib targeting FGFR fusion[1] |
| Patient 06 | *FGFR2-intergenic* | *FGFR2* | 10 | 17 | 123240841 | *intergenic* | 10 | - | 122793842 | 4.2 | Completely include | Pemigatinib; Erdafitinib targeting FGFR fusion[1] |
| Patient 07 | *FGFR2-SHTN1* | *FGFR2* | 10 | 17 | 123242528 | *SHTN1* | 10 | 6 | 118709305 | 5.1 | Completely include | Pemigatinib; Erdafitinib targeting FGFR fusion[1] |
| Patient 08 | *FGFR3-PHTF2* | *FGFR3* | 4 | 18 | 1808927 | *PHTF2* | 7 | 11 | 77567982 | 3.3 | Completely include | Pemigatinib; Erdafitinib targeting FGFR fusion[1] |
| Patient 09 | *STIM2-BRAF* | *STIM2* | 4 | 11 | 27012641 | *BRAF* | 7 | 9 | 140487929 | 12.7 | Completely include | Selumetinib targeting BRAF fusion[1] |
| Patient 09 | *STIM2-BRAF* | *STIM2* | 4 | 11 | 27013243 | *BRAF* | 7 | 10 | 140486103 | 1.1 | Completely include | Selumetinib targeting BRAF fusion[1] |
| Patient 09 | *TBC1D19-BRAF* | *TBC1D19* | 4 | 4 | 26629603 | *BRAF* | 7 | 10 | 140486782 | 6.5 | Completely include | Selumetinib targeting BRAF fusion[1] |
| Patient 10 | *TES-MET* | *TES* | 7 | 1 | 115867013 | *MET* | 7 | 2 | 116332227 | 0.7 | Completely include | Crizotinib targeting MET fusion[1] |
| Patient 11 | *MET-TES* | *MET* | 7 | 21 | 116436166 | *TES* | 7 | 4 | 115889445 | 24.6 | Not include | Crizotinib targeting MET fusion[1] |

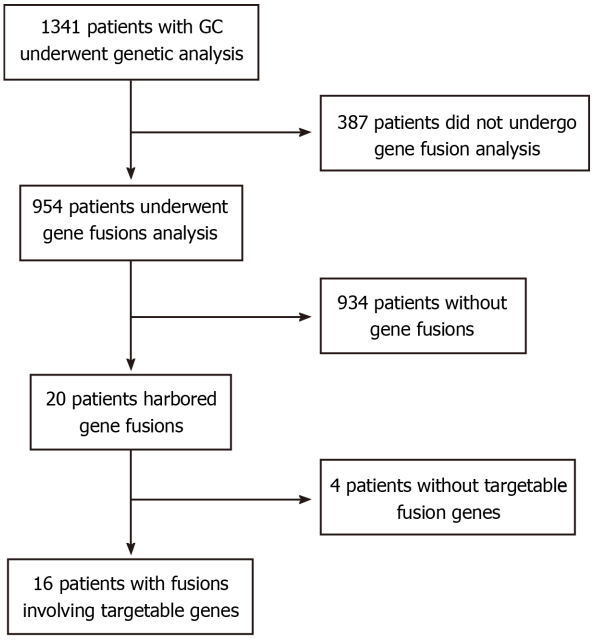
| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient 12 | *EML4-ALK* | *EML4* | 2 | 6 | 29447382 | *ALK* | 2 | 20 | 42498662 | 3.5 | Completely include | Crizotinib; ceritinib targeting ALK fusion[1] |
| Patient 13 | *OPALIN-RET* | *OPALIN* | 10 | 6 | 98104545 | *RET* | 10 | 11 | 43610099 | 5.46 | Completely include | Pralsetinib targeting RET fusion[1] |
| Patient 14 | *ARHGAP10-NTRK2* | *ARHGAP10* | 4 | 1 | 148716754 | *NTRK2* | 9 | 16 | 87476645 | 15.3 | Completely include | Larotrectinib targeting NTRK2 fusion[1] |
| Patient 15 | *NRG1-FDFT1* | *NRG1* | 8 | 12 | 32617907 | *FDFT1* | 8 | 8 | 11685375 | 8.9 | Partially include | MCLA-128 targeting NRG1 fusion[2] |
| Patient 16 | *EGFR-SEPTIN14* | *EGFR* | 7 | 25 | 55269173 | *SEPTIN14* | 7 | 10 | 55871179 | 9.6 | Completely include | Afatinib targeting EGFR fusion[2] |

[1]FDA-approved drugs targeting gene fusions.
[2]Drugs targeting gene fusions are under clinical trials.

cohort (our cohort and OrigiMed2020 cohort) and the TCGA cohort. Fusions in *BRAF*, *RET*, *ALK*, and *NTRK2* were detected in two Chinese cohorts but not in the TCGA cohort. This finding may have resulted from the small size of the TCGA cohort, which is prone to bias for gene fusions events because of the low occurrence rate in GC. A comparative study with a larger population is needed to identify differences in fusions involving targetable genes between races.

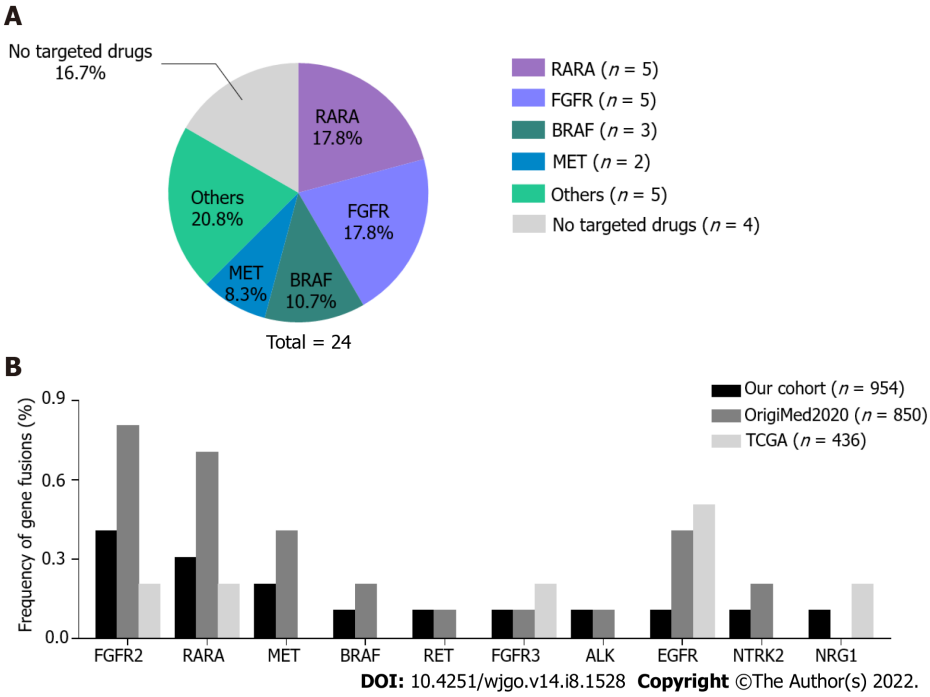
A major contribution of gene fusions to patients with tumor is the development of drugs that target fusion proteins encoded by these genes. The majority of advances in targeting gene fusions involve kinase domains that constitutively activate downstream signaling pathways[32]. In this study, except *RARA* and *NRG1* fusions, the 14 other fusions involving targetable genes included a receptor tyrosine kinase (RTK) gene, such as *FGFR2/3*, *BRAF*, *MET*, *ALK*, *RET*, *NTRK2*, and *EGFR*. Furthermore, most of all RTK gene fusions (13/14) completely retained the tyrosine kinase domain, which resulted in functional fusion proteins. We only verified the *BRAF* rearrangement in patient 09 and the *FGFR2* rearrangement in patient 04 using FISH because of insufficient tumor specimens. These fusions were consistent with previously observed fusions[23]; however, only 1 out of 5 *RARA* fusions contained exons 3-9, which encodes a DNA-binding and ligand-binding domain, which are required for *RARA* transcription factor activity. These results indicate that most patients with GC with fusions involving targetable genes may benefit from drugs that target fusions. However, patients in this retrospective study had not received targeted drug treatment; thus, we cannot determine whether they would have benefited from fusion-targeted drug therapy.

Interestingly, we also discovered 18 novel fusions with unreported partner genes or with an intergenic space. In other words, screening for known fusions in GC by FISH or polymerase chain reaction will likely miss most of the gene fusions that involve targetable genes. This is not conducive to patients with GC participating in clinical trials of fusion-targeted drugs in pan-cancer. Additionally, we found gene fusions enriched in patients with *ERBB2* amplification. We did not confirm all fusions using FISH because of limited tumor tissue samples, nor could we identify gene fusions enriched in distinct molecular subtypes of GC. Moreover, the efficacy of fusion-targeted drugs in GC remains to be further validated in clinical trials. Despite these limitations, for patients who fail standard therapy, NGS-based




**DOI:** 10.4251/wjgo.v14.i8.1528 

**Figure 1 Flowchart of patient selection.** GC: Gastric cancer.



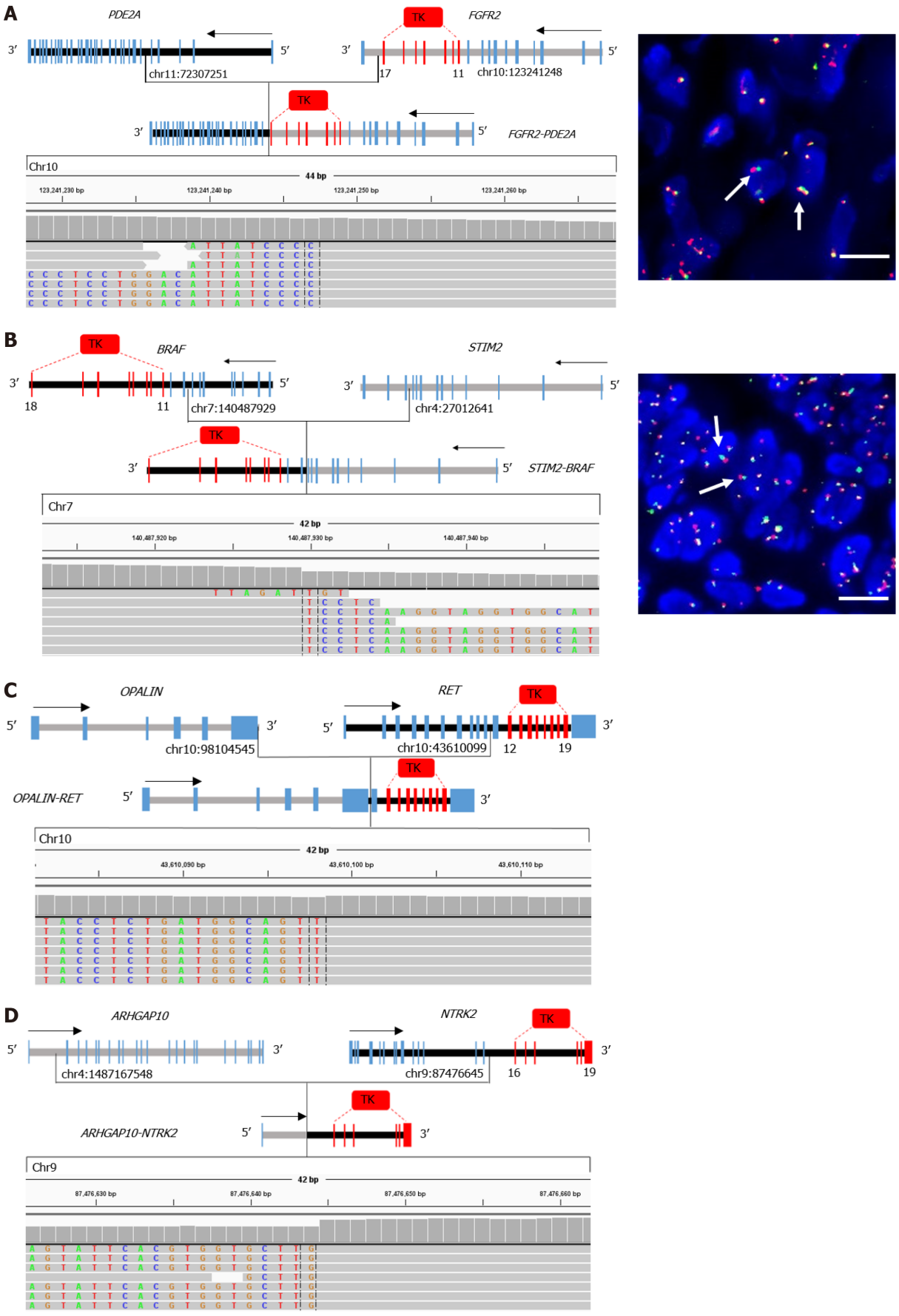

**DOI:** 10.4251/wjgo.v14.i8.1528 

**Figure 2 Profile of targetable gene fusions in gastric cancer.** A: The types and proportion of 24 gene fusions. Others included targetable *ALK*, *RET*, *NTRK2*, *NRG1*, and *EGFR* fusions. Four fusions without targetable genes were excluded from the analysis; B: Comparison of gene fusion frequencies in our cohort and the OrigiMed2020 and The Cancer Genome Atlas (TCGA) cohorts. No statistical differences were found among the cohorts.

novel gene fusion detection may provide a new treatment strategy and facilitate participation into clinical trials involving targeted therapy.

## CONCLUSION

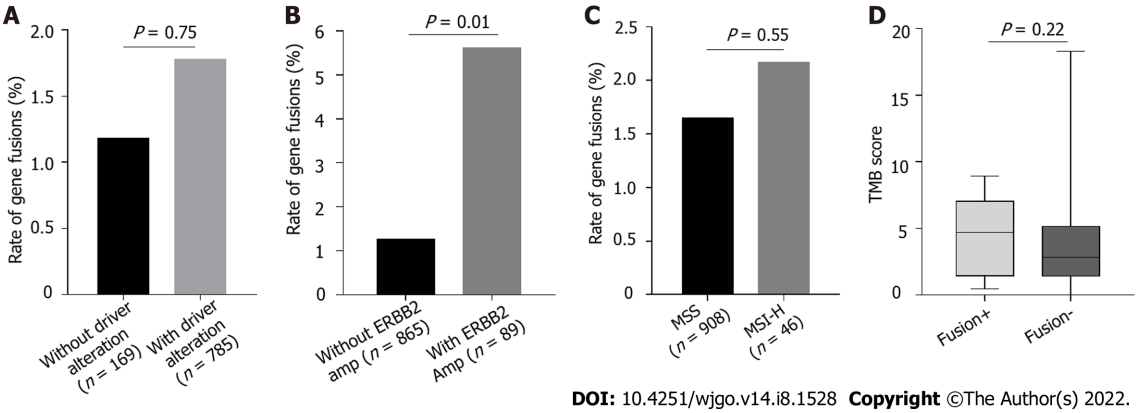
As the first large-scale study focusing on gene fusion events in Chinese patients with GC, we determined the frequency (16/954) of targetable gene fusions, and the majority of these fusions, including *TES-MET*, *FGFR2-PDE2A*, *OPALIN-RET*, *STIM-BRAF*, *ARHGAP10-NTRK2*, and *EGFR-*




**DOI:** 10.4251/wjgo.v14.i8.1528 

**Figure 3 Examples of novel gene fusions involving targetable genes in gastric cancer.** A-D: Schematic representation and Integrative Genomics Viewer screenshot of *FGFR2-PED2A* (A), *STIM-BRAF* (B), *OPALIN-RET* (C), and *NTRK2-ARHGAP10* (D) are shown; A and B: *FGFR2* and *BRAF* fusions were

confirmed by fluorescence in situ hybridization using *FGFR2* (10q26) or *BRAF* (7q34) break-apart probes. Red spot: 5' Probe signal; Green spot: 3' probe signal; Yellow spot: Target gene without rearrangement. Arrows indicate the cells with separate 5' (red) and 3' (green) signals. Bar: 100 μm. TK: Tyrosine kinase domain.




**DOI:** 10.4251/wjgo.v14.i8.1528 

**Figure 4 Enrichment of gene fusions in patients with gastric cancer with driver alterations.** A: The incidence of gene fusions in patients with and without driver alterations were analyzed, $P > 0.05$; B: The incidence of gene fusions in patients with and without *ERBB2* amplifications were analyzed, $P < 0.05$; C: The incidence of gene fusions in patients with microsatellite instability-high and microsatellite stability were analyzed, $P > 0.05$; D: Tumor mutational burden in targetable gene fusion-positive and -negative patients was compared, $P > 0.05$.

*SEPTIN14*, had not been previously described. These novel fusions completely retain a kinase domain. Additionally, we found gene fusions that were enriched in patients with *ERBB2* amplification. Gene fusion detection may aid in the development of novel treatment strategies for patients with GC.

## ARTICLE HIGHLIGHTS

### *Research background*

With rapid advancements in oncogenomics, increasing attention has been focused on gene fusions in cancer. The Food and Drug Administration has approved several fusion-targeted drugs for the treatment of solid tumors, such as larotrectinib for *NTRK* fusion-positive cancers and Zenocutuzumab for *NRG1* fusion-positive cancers. However, targetable gene fusions in Chinese patients with gastric cancer (GC) have not been well characterized.

### *Research motivation*

To investigate the incidence of gene fusions involving targetable genes in Chinese patients with GC and explore a potential treatment strategy for patients with GC.

### *Research objectives*

To explore the types and proportion of targetable gene fusions in Chinese patients with GC and determine the distribution of patients with gene fusions among the molecular subtypes of GC.

### *Research methods*

This was a multicenter retrospective study that evaluated patients with GC. A total of 954 tumor tissue samples from patients with GC who underwent gene fusion detection were included. Genetic alterations, including SNVs, indels, amplifications, and gene fusions, were analyzed. The enrichment of gene fusions in the molecular subtypes of GC was explored.

### *Research results*

Twenty fusions involving targetable genes were detected. Among them, 18 novel gene fusion events were previously not reported in other cancers. Owing to a limited number of tumor tissue samples, only *BRAF* and *FGFR2* fusions were identified by fluorescence in situ hybridization. Additionally, we found that gene fusions were enriched in patients with *ERBB2* amplification.

### *Research conclusions*

Gene fusions involving targetable genes were characterized in Chinese patients with GC. Testing gene fusions may provide insight for the treatment of GC.

### *Research perspectives*

A large study should be performed to further confirm the targetable gene fusions and identify whether gene fusions are enriched in distinct molecular subtypes of GC.

## FOOTNOTES

**Author contributions:** Liu ZH and Ma TH designed the study and reviewed the manuscript; Liu ZH, Zhu BW, Shi M analyzed the clinical and gene fusions data and wrote the manuscript; He XJ, Ma J, Liu ZC, and Tao HQ provided clinical advice; Yuan HL, Li W, Zhao DD, Wang BM, and Wang CY reviewed the manuscript and provided advice; All authors have read and approved the final manuscript.

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**Informed consent statement:** All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

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ORIGINAL ARTICLE

Retrospective Study

# Adjuvant chemoradiotherapy *vs* adjuvant chemotherapy in locally advanced Siewert type II/III adenocarcinoma of gastroesophageal junction after D2/R0 resection

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## Abstract

### *BACKGROUND*

For Siewert type II/III adenocarcinoma of gastroesophageal junction (AGE), the efficacy of adjuvant chemoradiotherapy (CRT) after D2/R0 resection remains uncertain.

### *AIM*

To determine whether CRT was superior to chemotherapy (CT) alone after D2/R0 resection for locally advanced Siewert type II/III AGE.

### *METHODS*

We identified 316 locally advanced Siewert type II/III AGE patients who were

treated with D2/R0 resection at National Cancer Center from 2011 to 2018. 57 patients received adjuvant CRT and 259 patients received adjuvant CT. We followed patients for overall survival (OS), relapse-free survival, and recurrence pattern.

***RESULTS***

Five-year OS rates of the CRT group and the CT group for all patients were 66.7% and 41.9% ($P$ = 0.010). Five-year OS rates of the CRT group and the CT group for Siewert type III AGE patients were 65.7% and 43.9% ($P$ = 0.006). Among the 195 patients whose recurrence information could be obtained, 18 cases (34.6%) and 61 cases (42.7%) were diagnosed as recurrence in the CRT group and CT group, respectively. The local and regional recurrence rates in the CRT group were lower than that in the CT group (22.2% *vs* 24.6%, 27.8% *vs* 39.3%). Multivariable cox regression analysis showed that vascular invasion, nerve invasion, and adjuvant CRT were important prognostic factors for Siewert type III AGE.

***CONCLUSION***

For locally advanced Siewert type III AGE, adjuvant CRT may prolong OS and reduce the regional recurrence rate.

**Key Words:** Siewert type II/III; Gastroesophageal junction; Adjuvant chemoradiotherapy; Adjuvant chemotherapy; Survival; Recurrence



**Core Tip:** This is a retrospective study to investigate the value of adjuvant chemoradiotherapy (CRT) in locally advanced Siewert type II/III adenocarcinoma of gastroesophageal junction. We identified 316 such patients and followed their overall survival (OS), relapse-free survival, and recurrence pattern. Our study found that for locally advanced Siewert type III gastroesophageal junction, adjuvant CRT may prolong OS and reduce the regional recurrence rate.

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## INTRODUCTION

In recent years, the incidence of adenocarcinoma of the gastroesophageal junction (AGE) has been increasing[1-3]. The Siewert type II/III type is the most common type of AGE in Asia[4-6]. At present, treatment for this type of AGE is based on the principle of gastric cancer. However, due to the special anatomical site of Siewert type II/III AGE, it differs from middle-distal gastric cancer in terms of its biological characteristics and prognosis. An increasing number of researchers have recognized AGE as an independent tumor entity[7,8]. For locally advanced AGE, the local and regional recurrence and distant metastasis rates remain high after D2/R0 resection, leading to poor prognosis[9-11]. As an important local treatment, radiotherapy can reduce the local recurrence rate and prolong survival time [12]. Phase III clinical studies of adjuvant chemoradiotherapy after D2/R0 resection. For resectable gastric cancer in the East and West have reached different conclusions[13,14]. Postoperative radiotherapy may be beneficial for patients who fail to achieve D2/R0 resection for various reasons, as well as in those with high-risk factors for local recurrence (high rate of lymph node metastasis, insufficient safe resection distance, *etc.*)[15]. Although the INT-0116 trial confirmed the survival benefit of postoperative radiotherapy in patients with resectable gastric cancer, the majority of patients in this study underwent D0 or D1 gastrectomy[16]. The efficacy of adjuvant radiotherapy in patients with gastric cancer undergoing D2/R0 resection is controversial[17]. Neoadjuvant CRT + D2/R0 resection + adjuvant chemotherapy (CT) has been successful in the study of AGE. Long-term follow-up results from the POET study showed that preoperative CRT had the advantage of reducing local and regional recurrence and was prone to improving overall survival (OS) compared to preoperative CT[18]. However, the significance of postoperative adjuvant radiotherapy for locally advanced Siewert type II/III AGE is unclear. In this study, we reviewed 316 patients with locally advanced Siewert type II/III AGE patients to determine whether CRT was superior to CT alone after D2/R0 resection, comparing the OS, relapse-free survival (RFS), and recurrence modes between the CRT and CT groups.

## MATERIALS AND METHODS

### *Patient selection and data collection*

We identified 316 patients with locally advanced Siewert type II/III AGE who were admitted to the Department of Pancreatic and Gastric Surgery, National Cancer Center, between January 2011 and May 2018. All patients underwent D2/R0 resection and did not receive neoadjuvant CT or radiotherapy. Patients were divided into a CT group and a CRT group according to whether they received postoperative adjuvant radiotherapy. Patients were followed-up by telephone, which was completed on April 30, 2020. The median follow-up time was 62.7 mo.

Pathologists determined the classification of the Siewert type. The pathological staging (pTNM) criteria were based on the 8th edition of the American Joint Committee on Cancer guidelines for esophageal adenocarcinoma (all Siewert type II adenocarcinomas invade the dentate line) and gastric cancer (Siewert type III). Patients who were lost to follow-up or were unwilling to cooperate were excluded. Patients with Siewert type I AGE were not included in this study. Patients younger than 18 years or older than 80 years, those who received neoadjuvant CT or radiotherapy, and those with less than 1 mo of postoperative survival were excluded from the study.

### *Adjuvant CRT and CT regimen*

Patients in the CRT group received a total dose of 45–50.4 Gy in 25–28 fractions. Intensity-modulated radiation therapy or volumetric modulated arc therapy were used. For tumor margins ≤ 3 cm, the anastomosis site was included in the clinical target volume (CTV). For the T4b stage, the tumor bed should also be included. Regional draining lymph nodes according to the Japanese Gastric Cancer Association (JGCA)[19] were included in the CTV. Based on the International Commission Radiological Units report No. 83[20], the planning target volume should consider not only the setup error but also the breath elements and tumor movement. Concurrent CT regimens included FU-based drugs such as 5-FU, capecitabine, or tegafur (S-1) on radiotherapy days. Patients in the CRT group received 4-6 cycles of CT, followed by CRT.

In the CT group, the main CT regimens included oxaliplatin + 5-FU (SOX, XELOX), cisplatin + 5-FU (PF, XP, SP), albumin-bound paclitaxel + S-1, and single-drug CT regimen (S-1, docetaxel, and irinotecan). Adjuvant CT is usually performed for 4-6 cycles.

### *Recurrence pattern*

Specific imaging or pathological diagnosis is needed when defining tumor recurrence. In this study, we divided recurrence into local recurrence, regional recurrence, and metastatic recurrence according to the first site of recurrence during the follow-up period.

Recurrence in the anastomotic stoma, tumor bed, and remnant stomach was defined as a local recurrence. Recurrence in the lymphatic drainage area, according to the JGCA guidelines[19] for gastric cancer and the Japan Esophageal Cancer Research Association guidelines[21] for the esophagus, was defined as a regional recurrence. The diagnosis of recurrent lymph nodes on imaging should satisfy one of the following criteria: (1) Short diameter of lymph nodes > 8 mm; (2) Ratio of the long diameter to the short diameter of lymph nodes is close to one; (3) Central lymph nodes are necrotic; (4) Multiple lymph nodes are aggregated; and (5) Lymph nodes are significantly enhanced on computed tomography. Distant recurrence was defined as distant lymph node, bone, peritoneal or pleural, and solid organ metastases such as liver, lung, and ovarian metastases.

## RESULTS

### *Clinicopathological characteristics*

Table 1 summarizes the clinicopathological characteristics of the patients enrolled in this study. There were 57 patients in the CRT group and 295 patients in the CT group. The number and proportion of patients with Siewert type II and III AGE were 148 (46.8%) and 168 (53.2%), respectively.

### *Survival results*

Figure 1A and B summarize the OS and RFS curves of the CRT and CT groups, respectively, for all patients. Figure 1C and D summarize the OS and RFS curves of the CRT and CT groups for patients with Siewert type II AGE, respectively. Figure 1E and F summarize the OS and RFS curves of the CRT and CT groups for patients with Siewert type III AGE, respectively.

Five-year OS rates of the CRT and CT groups for all patients were 66.7% and 41.9%, respectively ($P$ = 0.010). Five-year OS rates of the CRT and CT groups for Siewert type II AGE patients were 59.3% and 39.4%, respectively ($P$ = 0.063). Five-year OS rates of the CRT and CT groups for Siewert type III AGE patients were 65.7% and 43.9%, respectively ($P$ = 0.006).

Five-year recurrence-free survival rates of the CRT and CT groups for all patients were 64.5% and 55.3%, respectively ($P$ = 0.254). Five-year recurrence-free survival rates of the CRT and CT groups for

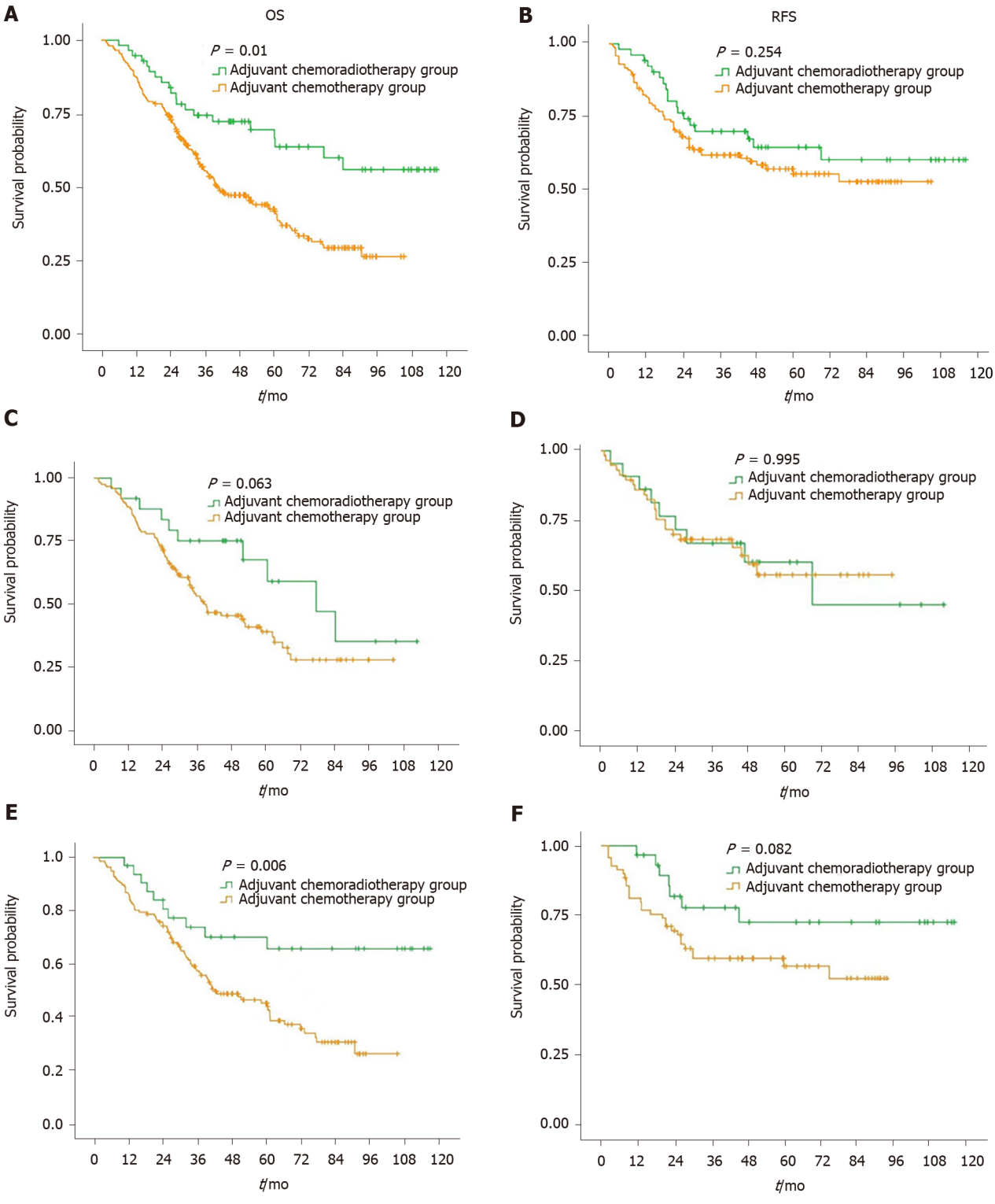


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**Figure 1 Kaplan-Meier curve for overall survival and relapse-free survival between patients in chemoradiotherapy group and chemotherapy group.** A and B: summarizes the overall survival (OS) and relapse-free survival (RFS) survival curves of the chemoradiotherapy (CRT) group and chemotherapy (CT) group for all patients; C and D: summarizes the OS and RFS survival curves of the CRT group and CT group for Siewert type II gastroesophageal junction (AGE) patients; E and F: summarizes the OS and RFS survival curves of the CRT group and CT group for Siewert type III AGE patients).

Siewert type II AGE patients were 60.5% and 55.9%, respectively ($P$ = 0.995). Five-year recurrence-free survival rates of the CRT and CT groups for Siewert type III AGE patients were 72.6% and 56.8%, respectively ($P$ = 0.082) (Table 2 and Figure 2).

### *Results of recurrence*

Among the 195 patients for whom recurrence information could be obtained, 18 (34.6%) and 61 (42.7%)

**Table 1 Clinicopathological features of all patients**

| Variable | Overall | Adjuvant chemoradiotherapy group | Adjuvant chemotherapy group |
|---|---|---|---|
| | 316 | 57 | 259 |
| **Age, *n* (%)** | | | |
| < 40 yr | 6 (1.9) | 2 (3.5) | 4 (1.5) |
| ≥ 40 yr | 310 (98.1) | 55 (96.5) | 255 (98.5) |
| **Sex, *n* (%)** | | | |
| Male | 268 (84.8) | 46 (80.7) | 222 (85.7) |
| Female | 48 (15.2) | 11 (19.3) | 37 (14.3) |
| **BMI, *n* (%)** | | | |
| < 18.5 or > 23.9 | 169 (53.5) | 27 (47.4) | 142 (54.8) |
| 18.5-23.9 | 147 (46.5) | 30 (52.6) | 117 (45.2) |
| **The degree of differentiation, *n* (%)** | | | |
| Poorly differentiated | 233 (73.7) | 45 (78.9) | 188 (72.6) |
| Moderately-highly differentiated | 83 (26.3) | 12 (21.1) | 71 (27.4) |
| **Nerve invasion, *n* (%)** | | | |
| Yes | 269 (85.1) | 43 (75.4) | 226 (87.3) |
| No | 47 (14.9) | 14 (24.6) | 33 (12.7) |
| **Vascular invasion, *n* (%)** | | | |
| Yes | 263 (83.2) | 40 (70.2) | 223 (86.1) |
| No | 53 (16.8) | 17 (29.8) | 36 (13.9) |
| **Pathologic T stage, *n* (%)** | | | |
| pT1-3 | 179 (56.6) | 34 (59.6) | 145 (56.0) |
| pT4a-4b | 137 (43.4) | 23 (40.4) | 114 (44.0) |
| **Pathologic N stage, *n* (%)** | | | |
| pN1-2 | 73 (23.1) | 10 (17.5) | 63 (24.3) |
| PN3 | 243 (76.9) | 47 (82.5) | 196 (75.7) |
| Pathological stage, Siewert type II | 148 (46.8) | 25 (43.9) | 123 (47.5) |
| IIIB stage | 30 (9.5) | 6 (10.5) | 24 (9.3) |
| IVA stage | 118 (37.3) | 19 (33.3) | 99 (38.2) |
| Pathological stage, Siewert type III | 168 (53.2) | 32 (56.1) | 136 (52.5) |
| IIIA | 32 (10.1) | 1 (1.8) | 31 (12.0) |
| IIIB | 80 (25.3) | 22 (38.6) | 58 (22.4) |
| IIIC | 56 (17.7) | 9 (15.8) | 47 (18.1) |

BMI: Body mass index.

were diagnosed with recurrence in the CRT and CT groups, respectively. The local and regional recurrence rates were lower in the CRT group than in the CT group (22.2% *vs* 24.6% and 27.8% *vs* 39.3%, respectively). The distant recurrence rate was higher in the CRT group than in the CT group (100% *vs* 68.9%) (Table 3 and Figure 3).

### *Multivariable Cox regression analysis*

For Siewert type II AGE, multivariate Cox regression analysis showed that vascular invasion was an important prognostic factor (Table 4). For Siewert type III AGE, multivariate Cox regression analysis showed that vascular invasion, nerve invasion, and adjuvant CRT were important prognostic factors (Table 5).

**Table 2 5-year survival rate in adjuvant chemoradiotherapy group and adjuvant chemotherapy group**

| | Group | *N* | 5-yr survival rate (%) | Log rank test |
|---|---|---|---|---|
| OS | All patietnts | | | $P = 0.010$ |
| | Adjuvant chemoradiotherapy group | 57 | 66.7 | |
| | Adjuvant chemotherapy group | 295 | 41.9 | |
| RFS | All patietnts | | | $P = 0.254$ |
| | Adjuvant chemoradiotherapy group | 52 | 64.5 | |
| | Adjuvant chemotherapy group | 128 | 55.3 | |
| OS | Siewert type II | | | $P = 0.063$ |
| | Adjuvant chemoradiotherapy group | 25 | 59.3 | |
| | Adjuvant chemotherapy group | 123 | 39.4 | |
| RFS | Siewert type II | | | $P = 0.995$ |
| | Adjuvant chemoradiotherapy group | 22 | 60.5 | |
| | Adjuvant chemotherapy group | 58 | 55.9 | |
| OS | Siewert type III | | | $P = 0.006$ |
| | Adjuvant chemoradiotherapy group | 32 | 65.7 | |
| | Adjuvant chemotherapy group | 136 | 43.9 | |
| RFS | Siewert type III | | | $P = 0.082$ |
| | Adjuvant chemoradiotherapy group | 30 | 72.6 | |
| | Adjuvant chemotherapy group | 70 | 56.8 | |

OS: Overall survival; RFS: Relapse-free survival.

**Table 3 Distributions of recurrence in Adjuvant chemoradiotherapy group and adjuvant chemotherapy group**

| Recurrence site | Adjuvant chemoradiotherapy group | | Adjuvant chemotherapy group | |
|---|---|---|---|---|
| | No. of patients | % of recurrence patients ($n = 18$) | No. of patients | % of recurrence patients ($n = 61$) |
| **Local recurrence, *n* (%)** | | | | |
| Remnant stomach | 1 | 5.6 | 1 | 1.6 |
| Anastomosis site | 3 | 16.7 | 14 | 23.0 |
| Regional recurrence | 5 | 27.8 | 24 | 39.3 |
| **Distant metastasis, *n* (%)** | | | | |
| **One site** | | | | |
| Peritoneum | 5 | 27.8 | 8 | 13.1 |
| Pleura | 2 | 11.1 | 4 | 6.6 |
| Solid organ | 4 | 22.2 | 16 | 26.2 |
| Distant LNs | 2 | 11.1 | 5 | 8.2 |
| Bone metastases | 2 | 11.1 | 3 | 4.9 |
| **≥ 2 sites** | | | | |
| Peritoneum + solid organ | 2 | 11.1 | 3 | 4.9 |
| Solid organs | 1 | 5.6 | 2 | 3.3 |
| Peritoneum + distant LNs | 0 | 0 | 1 | 1.6 |
| Solid organs + distant LNs | 0 | 0 | 1 | 1.6 |

LNs: Lymph nodes.

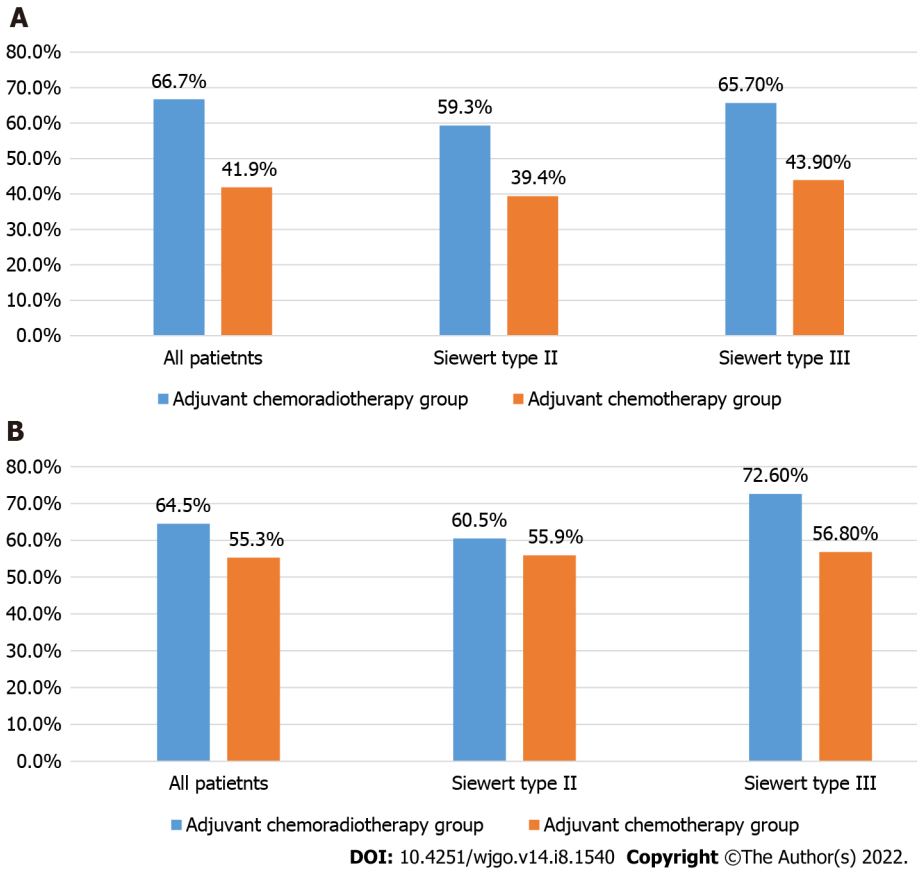


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**Figure 2 Five-year overall survival rate and recurrence free survival rate of all patients, Siewert type II gastroesophageal junction patients, and Siewert type III gastroesophageal junction patients in chemoradiotherapy group and chemotherapy group.** A: Five-year overall survival rate in chemoradiotherapy (CRT) group and chemotherapy (CT) group; B: Five-year recurrence free survival rate in CRT group and CT group.

## DISCUSSION

In the past, adjuvant treatment for AGE was based on the experience of esophageal adenocarcinoma or gastric adenocarcinoma. However, a growing number of researchers believe that AGE is a separate tumor[22]. There have been very few randomized controlled studies on the significance of adjuvant CRT for AGE[23]. In this study, we found that postoperative adjuvant CT combined with regional radiotherapy could prolong the OS of patients with locally advanced Siewert type III AGE. The local and regional recurrence rates in the CRT group were lower than those in the CT group. These results provide evidence supporting the use of postoperative adjuvant radiotherapy for AGE.

The role of adjuvant radiotherapy after D2/R0 resection in gastric cancer remains controversial. The Korean ARTIST 1 study[17] suggested that patients with lymph node metastases may benefit from postoperative irradiation. However, the ARTIST 2 study[24] showed that SOX CT combined with radiotherapy did not improve outcomes in patients with lymph node metastasis after D2/R0 resection. In addition, the majority of patients enrolled in these clinical trials had distal gastric cancer, while fewer had proximal gastric cancer. Owing to the specificity of the anatomic site, the benefits of adjuvant radiotherapy in AGE may not be consistent with those in gastric cancer. Therefore, we designed this retrospective clinical study to evaluate the value of adjuvant CRT in locally advanced AGE.

Because of the special location of AGE, cancer cells can metastasize to the mediastinum and abdominal cavity along the lymphatic vessels. However, for Siewert type II/III AGE, abdominal lymph node metastasis is the main direction of metastasis[25]. The JCOG9502 study showed that for Siewert type II/III AGE that underwent D2/R0 lymph node dissection, the positive rate of lymph nodes at station 16 was 15.2%, and the postoperative lymph node recurrence rate was 17.4%[26]. These results provide evidence for the delineation of radiotherapy targets for locally advanced AGE.

Our study found that adjuvant radiotherapy did not significantly improve RFS in all patients or OS in patients with Siewert type II AGE. However, we observed a significant extension of OS in patients with Siewert type III AGE after adjuvant CRT. A possible explanation is that adjuvant radiotherapy reduces

**Table 4 Univariate and multivariable cox proportional hazards modeling for overall survival in Siewert type II gastroesophageal junction**

| Clinicopathological features | Univariate analysis | | Multivariable analysis | |
|---|---|---|---|---|
| | HR (95%CI) | *P* value | HR (95%CI) | *P* value |
| **Sex** | | | | |
| Male | Ref. | | | |
| Female | 0.683 (0.389-1.199) | *P* = 0.185 | | |
| **BMI** | | | | |
| 18.5-23.9 | Ref. | | | |
| < 18.5 or > 23.9 | 1.019 (0.657-1.581) | *P* = 0.931 | | |
| **The degree of differentiation** | | | | |
| Poorly differentiated | Ref. | | | |
| Moderately-highly differentiated | 0.733 (0.437-1.227) | *P* = 0.237 | | |
| **Nerve invasion** | | | | |
| Yes | Ref. | | Ref. | |
| No | 0.083 (0.020-0.339) | *P* = 0.001 | 0.117 (0.028-0.500) | *P* = 0.004 |
| **Vascular invasion** | | | | |
| Yes | Ref. | | Ref. | |
| No | 0.215 (0.093-0.496) | *P* < 0.001 | 0.425 (0.178-1.014) | *P* = 0.054 |
| **Pathologic T stage** | | | | |
| Group 1-3 | Ref. | | Ref. | |
| Group 4a-4b | 1.404 (0.899-2.194) | *P* = 0.136 | 1.271 (0.777-2.080) | *P* = 0.340 |
| **Pathologic N stage** | | | | |
| Group 1-2 | Ref. | | Ref. | |
| Group 3a-b | 2.089 (1.137-3.721) | *P* = 0.012 | 1.027 (0.385-2.739) | *P* = 0.958 |
| **Pathologic TNM stage** | | | | |
| IIIB stage | Ref. | | Ref. | |
| IVA stage | 2.540 (1.267-5.091) | *P* = 0.009 | 2.081 (0.629-6.885) | *P* = 0.230 |
| **Adjuvant chemoradiotherapy** | | | | |
| Yes | Ref. | | Ref. | |
| No | 1.859 (0.956-3.614) | *P* = 0.068 | 1.877 (0.943-3.738) | *P* = 0.073 |

BMI: Body mass index; HR: Hazard ratio; CI: Confidence interval.

local and regional recurrence rates. The overall recurrence rate in the CRT group was lower than that in the CT group. Compared with the CT group, more attention should be paid to distant metastasis during postoperative reexamination in the CRT group. We found that patients with Siewert type III AGE were more sensitive to adjuvant radiotherapy. The difference between Siewert type II and III AGE in adjuvant CRT requires further study. Multivariable Cox regression analysis showed that adjuvant CRT was an important factor affecting the prognosis of patients with Siewert type III AGE, further verifying the necessity of adjuvant CRT.

Our study has several limitations. First, this was a single-center retrospective study, and thus inherently has a lower level of evidence than a multicenter prospective clinical trial. Furthermore, did not compare surgical approaches for different types of AGE. In addition, the toxic effects of radiotherapy were not investigated in this study.

## CONCLUSION

For locally advanced Siewert type III AGE, adjuvant CRT following D2/R0 resection may prolong OS

**Table 5 Univariate and multivariable cox proportional hazards modeling for overall survival in Siewert type III gastroesophageal junction**

| Clinicopathological features | Univariate analysis | | Multivariable analysis | |
|---|---|---|---|---|
| | HR (95%CI) | *P* value | HR (95%CI) | *P* value |
| **Sex** | | | | |
| Male | Ref. | | | |
| Female | 0.573 (0.277-1.186) | *P* = 0.134 | | |
| **BMI** | | | | |
| 18.5-23.9 | Ref. | | | |
| < 18.5 or > 23.9 | 1.142 (0.755-1.728) | *P* = 0.530 | | |
| **The degree of differentiation** | | | | |
| Poorly differentiated | Ref. | | | |
| Moderately-highly differentiated | 0.945 (0.592-1.509) | *P* = 0.813 | | |
| **Nerve invasion** | | | | |
| Yes | Ref. | | Ref. | |
| No | 0.086 (0.021-0.349) | *P* = 0.001 | 0.169 (0.037-0.774) | *P* = 0.022 |
| **Vascular invasion** | | | | |
| Yes | Ref. | | Ref. | |
| No | 0.041 (0.006-0.295) | *P* = 0.002 | 0.092 (0.012-0.689) | *P* = 0.020 |
| **Pathologic T stage** | | | | |
| Group 1-3 | Ref. | | Ref. | |
| Group 4a-4b | 1.479 (0.975-2.242) | *P* = 0.065 | 1.151 (0.751-1.763) | *P* = 0.518 |
| **Pathologic N stage** | | | | |
| Group 1-2 | Ref. | | Ref. | |
| Group 3 | 1.983 (1.099-3.580) | *P* = 0.023 | 3.621 (0.380-34.469) | *P* = 0.263 |
| **Pathologic TNM stage** | | | | |
| IIIA stage | Ref. | | Ref. | |
| IIIB stage | 1.420 (0.759-2.656) | *P* = 0.273 | 1.801 (0.180-18.037) | *P* = 0.617 |
| IIIC stage | 2.783 (1.472-5.261) | *P* = 0.002 | 0.681 (0.434-1.069) | *P* = 0.095 |
| **Adjuvant chemoradiotherapy** | | | | |
| Yes | Ref. | | Ref. | |
| No | 2.465 (1.270-4.782) | *P* = 0.008 | 2.258 (1.145-4.453) | *P* = 0.019 |

BMI: Body mass index; HR: Hazard ratio; CI: Confidence interval.

and reduce the local and regional recurrence rate. Postoperative radiotherapy may be feasible for Siewert type III AGE patients. Multicenter prospective clinical trials should be conducted to investigate the significance of adjuvant CRT in AGE.

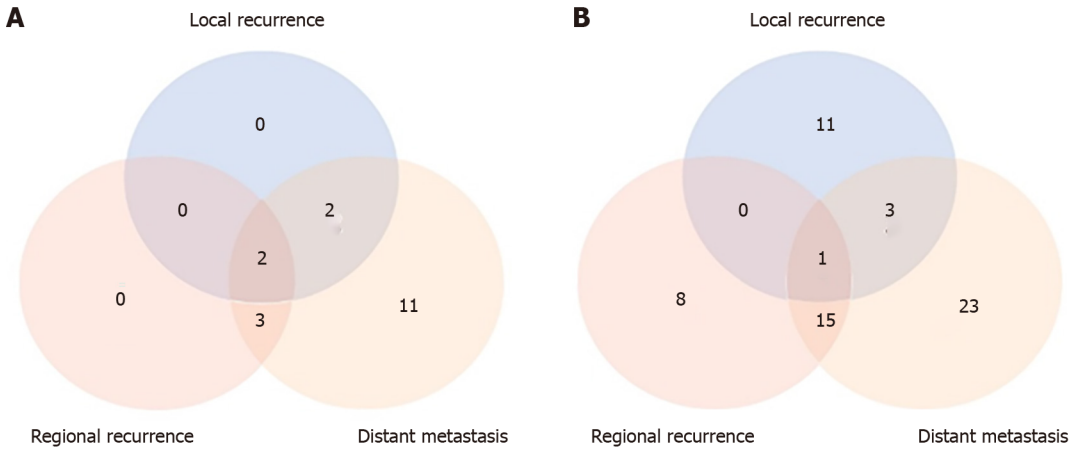


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**Figure 3 Distributions of recurrence in chemoradiotherapy group and chemotherapy group.** A: Chemoradiotherapy group; B: Chemotherapy group.

## ARTICLE HIGHLIGHTS

### *Research background*

The role of adjuvant chemoradiotherapy (CRT) in adenocarcinoma of gastroesophageal junction (AGE) is unclear.

### *Research motivation*

Radiotherapy may reduce the local recurrence rate and prolong survival time for locally advanced Siewert II/III type adenocarcinoma of AGE.

### *Research objectives*

To evaluate the effect of adjuvant CRT *vs* adjuvant chemotherapy (CT) on overall survival (OS), relapse-free survival (RFS), and recurrence pattern in locally advanced Siewert II/III type adenocarcinoma of AGE patients undergoing D2/R0 resection.

### *Research methods*

We compared the OS, RFS, and recurrence modes between the adjuvant CRT and adjuvant CT groups.

### *Research results*

Adjuvant CRT improves the 5-year survival rate of adenocarcinoma of AGE, especially Siewert type III adenocarcinoma of AGE, and reduces local and regional recurrence rates.

### *Research conclusions*

Adjuvant CRT may be appropriate for adenocarcinoma of AGE, especially for Siewert type III adenocarcinoma of AGE.

### *Research perspectives*

Multicenter prospective clinical trials should be conducted to investigate the significance of adjuvant CRT in adenocarcinoma of AGE.

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## FOOTNOTES

**Author contributions:** Tian YT and Jin J designed the research; Kang WZ, Shi JM, Wang BZ, Xiong JP and Shao XX analyzed the data and wrote the paper; Hu HT collected the patient's clinical data; Kang WZ and Shi JM contributed equally to this work.

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**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous data that were obtained after each patient agreed to treatment by written consent.

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ORIGINAL ARTICLE

Observational Study

# Duodenal-type follicular lymphoma more than 10 years after treatment intervention: A retrospective single-center analysis

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## Abstract

### *BACKGROUND*

Duodenal-type follicular lymphoma (D-FL) has been recognized as a rare entity that accounts for approximately 4% of primary gastrointestinal lymphomas. D-FL follows an indolent clinical course compared with common nodal FL and is generally considered to have a better prognosis. Therefore, the "watch and wait" approach is frequently adopted as the treatment method. Alternatively, there is an option to actively intervene in D-FL. However, the long-term outcomes of such cases are poorly understood.

### *AIM*

To clarify the clinical outcomes after long-term follow-up in cases of D-FL with treatment intervention.

### *METHODS*

We retrospectively analyzed patients who met the following criteria: the lesion was confirmed by endoscopy, the diagnosis of D-FL was confirmed histopathologically, and the patient was followed-up for more than 10 years after the intervention at our center.

### *RESULTS*

We identified 5 cases of D-FL. Two patients showed a small amount of bone marrow involvement (Stage IV). Rituximab was used as a treatment for remission in all 5 patients. It was also used in combination with chemotherapy in 2 Stage IV patients as well as for maintenance treatment. Radiation therapy was performed in 2 cases, which was followed by complete remission (CR). Eventually, all 5 patients achieved CR and survived for more than 10 years. However, 3 patients

experienced recurrence. One patient achieved a second CR by retreatment, and in another case, the lesion showed spontaneous disappearance. The remaining patient had systemic widespread recurrence 13 years after the first CR. Biopsy results suggested that the FL lesions were transformed into diffuse large B-cell lymphoma. The patient died 4 years later despite receiving various chemotherapies.

*CONCLUSION*
In this study, the treatment for patients of D-FL in Stage IV was successful. In the future, criteria for how to treat "advanced" D-FL should be established based on additional cases. This study of patients with D-FL indicates that whole-body follow-up examinations should continue for a long time due to a fatal recurrence 13 years after reaching CR.

**Key Words:** Duodenal-type follicular lymphoma; Treatment; Long-term follow-up; Radiation; Rituximab; Chemotherapy



**Core Tip:** Since duodenal-type follicular lymphoma (D-FL) progresses more indolently than common nodal FL, the "watch and wait" approach is frequently used without intervention. To elucidate the clinical assessments of long-term follow-up in cases of D-FL with treatment intervention, we retrospectively examined 5 D-FL patients for more than 10 years after treatment at our center. All 5 patients eventually achieved complete remission and survived for a long period. However, 3 patients experienced recurrence, and 1 patient died of the primary disease 21 years after first onset. In the future, it will be necessary to establish criteria for how to treat Stage IV "advanced" D-FL.

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## INTRODUCTION

Duodenal-type follicular lymphoma (D-FL) is an entity that was newly classified as a variant of FL in the 2017 World Health Organization classification[1]. Most D-FLs are asymptomatic and are often incidentally found by esophagogastroduodenoscopy (EGD). Many large-scale clinical analyses of D-FL have been conducted in Japan[2,3], where endoscopic screening for gastric cancer is frequently performed. This is because the incidence of gastric cancer is higher in Japan than in Western countries due to genetic and dietary factors[4]. The most common endoscopic findings are "white granular or multiple nodular, polypoid lesions" in the descending portion of the duodenum[5]. In addition, 85% of D-FL cases have been shown to have jejunal or ileal lesions, which were detected by capsule or double-balloon enteroscopy[6].

D-FL follows an indolent progression compared with common nodal FL and has a generally better prognosis. Gene expression profiling of D-FL has yielded results similar to those obtained for mucosa-associated lymphoid tissue (MALT) lymphoma[7,8]. Sufficient consensus has not yet been reached as to whether therapeutic treatment should be administered to patients with D-FL, and the "watch and wait" strategy is currently frequently performed[9,10]. Long-term observations after intervention for D-FL have not been reported, and the long-term outcome of therapeutic treatment is not well understood. To clarify the clinical outcomes after long-term follow-up in cases of D-FL with treatment, we analyzed patients with D-FL who were followed-up for more than 10 years after intervention at our center.

## MATERIALS AND METHODS

### *Study design*

This was a retrospective, observational study at our center.

### Patients

We included D-FL patients who were diagnosed endoscopically and histopathologically and followed clinically for more than 10 years after starting treatment intervention at our center between January 1998 and December 2009.

FL was predominantly diagnosed by a pathological diagnosis, although the detection of *IgH-BCL2* by fluorescence in situ hybridization (FISH) was also respected to the same extent. In addition to EGD, patients were examined by colonoscopy, contrast computed tomography (CT), positron emission tomography (PET)-CT, bone marrow aspiration/biopsy, and wherever possible enteroscopy of the distal small intestine. Patients with a predominant systemic spread of nodal lesions were excluded. However, not all patients with swelling of the lymph nodes were excluded, as mentioned below in the "Results" section. Characteristics of the patients were assessed by performance status, histological grading, and follicular lymphoma international prognostic index. Staging followed the Lugano classification for gastrointestinal lymphoma.

### Treatment

This study was carried out as part of standard care in daily clinical practice under Japanese health insurance, and no treatments specific to this study were performed. Because bendamustine had not been approved by health insurance in Japan by 2010, we did not use it at the time of initial onset for applicable patients. Since 2008, for patients with newly developed nodal FL that progressed to Ann Arbor Stage III or higher, our center has provided maintenance treatment with rituximab bimonthly after reaching complete remission (CR).

## RESULTS

Five patients were included in this study. Of these patients, 4 were referred by Hokkaido University Hospital (Cases 1-4), including 1 recurrent patient (Case 1), and we were requested to continuously follow these patients. Table 1 shows the clinical features of these patients. Three patients were males in their 40 s, and two patients were females in their 60 s. None of the 5 patients had subjective symptoms, and the trigger for the diagnosis was discovered incidentally by EGD. In all patients, performance status was 0, and the histopathological grade was 1. Enteroscopy of the small bowel was performed in 3 patients, and distal intestinal lesions were observed in 2 patients (Figure 1A and B). Two patients showed a small amount of bone marrow involvement and were evaluated as Stage IV. Based on the follicular lymphoma international prognostic index, 4 patients were low risk, and 1 patient was intermediate risk.

In all 5 patients, therapeutic intervention was performed with the goal of remission, and the treatment details and outcomes are shown in Table 2. Two patients were treated because of an advanced stage (Stage IV), and the other 2 patients requested treatment.

Rituximab was used as a treatment for remission in all 5 patients. In 2 patients, it was used as a single agent, and in 2 Stage IV patients it was used in combination with chemotherapy. Additionally, it was used for maintenance treatment. Radiation therapy was performed in 2 cases, followed by CR. Eventually, all 5 patients achieved CR and survived for more than 10 years. However, 3 patients experienced recurrence but not within 10 years. One patient achieved a second CR by retreatment, and for another patient, the lymphoma lesion disappeared spontaneously. The remaining patient had systemic widespread recurrence 13 years after the first CR. Later, the biopsy results suggested that the FL lesions had transformed into diffuse large B-cell lymphoma (DLBCL). The patient died 4 years later despite receiving various anticancer drugs.

### Treatment summary of 5 cases

**Case 1:** Rituximab monotherapy (375 mg/m$^2$ × 4 times) resulted in a first CR at Hokkaido University Hospital. However, 1 year and 3 mo later, D-FL recurred not only locally in the duodenum but also in the cervical lymph nodes (Lugano Stage IV). Six cycles of rituximab, cyclophosphamide, pirarubicin, vincristine, and prednisone chemotherapy [a regimen in which doxorubicin in cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) was changed to pirarubicin, which is less cardiotoxic; rituximab 375 mg/m$^2$ + cyclophosphamide 750 mg/m$^2$, pirarubicin 50 mg/m$^2$, and vincristine 1.4 mg/m$^2$, all on day 1, and prednisone 60 mg/body on days 1-5, every 3 wk] were administered, and this patient achieved a second CR.

**Case 2:** In addition to the duodenum, FL lesions spread to the jejunum and a slight extent to the bone marrow. *IgH-BCL2* was detected in barely 1% of the nucleated cells by FISH. The lesion was in Stage IV and presented an intermediate risk in the follicular lymphoma international prognostic index. The patient's first CR, including bone marrow findings (*IgH-BCL2*, 0.0%), was reached after administration of 3 cycles of rituximab + cyclophosphamide, vincristine, and prednisone (rituximab + cyclophosphamide 750 mg/m$^2$ and vincristine 1.4 mg/m$^2$, all on day 1, and prednisone 60 mg/body on days 1-5 every 3 wk) and 5 cycles of rituximab + oral fludarabine (40 mg/m$^2$ on days 1-5 every 4 wk).

**Table 1 Clinical features of five patients**

| Case | Age | Sex | Trigger to be found | PS | Grade | Distal intestinal lesion | Extra-duodenal lesion | Initial stage | FLIPI |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 65 | F | Screening EGD | 0 | 1 | Jejunum | (-) | I | Low |
| 2 | 63 | F | Follow-up for GERD | 0 | 1 | Jejunum | Bone marrow | IV | Int |
| 3 | 40 | M | Screening EGD | 0 | 1 | Not tested | Bone marrow, mesenteric LN | IV | Low |
| 4 | 42 | M | Screening EGD | 0 | 1 | (-) | (-) | I | Low |
| 5 | 42 | M | Screening EGD | 0 | 1 | Not tested | (-) | I | Low |

PS: Performance status; FLIPI: Follicular lymphoma international prognostic index; F: Female; M: Male; EGD: Esophagogastroduodenoscopy; GERD: Gastroesophageal reflux disease; LN: Lymph nodes; Int: Intermediate.

**Table 2 Treatment and outcome of 5 patients**

| Case | Treatment motive | Initial treatment | Effect | Rituximab maintenance | Relapse lesion/re-Stage (from the end of treatment) | 2nd treatment | Outcome (from the 1st onset) |
|---|---|---|---|---|---|---|---|
| 1 | Previously followed doctor's judgment | RTX | 1st CR | (-) | Duodenum + cervical LN/Stage IV (1 yr and 3 mo) | R-THP-COP | 2nd CR (18 years) |
| 2 | In stage IV | R-CVP + R-F | 1st CR | (+) | Colon + mesenteric LN/Stage II1 (1 yr and 7 mo) | Watch | 2nd CR (12 yr) |
| 3 | In stage IV | R-CHOP | 1st CR | (+) | (-) | - | 1st CR (13 yr) |
| 4 | Patient's request | Radiation + RTX | 1st CR | (-) | (-) | - | 1st CR (16 yr) |
| 5 | Patient's request | CHOP/RTX/radiation | 1st CR | (-) | Lung + systemic LN/Stage IV (13 yr) | B-R/R-BAC/CHOEP/ONTZ | Death due to primary disease (21 yr) |

RTX: Rituximab; R-CVP: Rituximab + cyclophosphamide, vincristine, and prednisone; R-F: Rituximab + oral fludarabine; R-CHOP: Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; R-THP-COP: Rituximab, cyclophosphamide, pirarubicin, vincristine, and prednisone; B-R: Rituximab + bendamustine; R-BAC: Rituximab, bendamustine, and cytarabine; CHOEP: Cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisone; ONTZ: Obinutuzumab; LN: Lymph node; CR: Complete remission.

Maintenance treatment with rituximab monotherapy was performed; however, 1 year and 7 mo later, PET-CT indicated lesions of the intestinal tract and nearby mesenteric lymph nodes in the ileocecal region (Figure 1C). Although no abnormalities were found in the duodenum or jejunum by endoscopy, multiple lymphomatous polyposis-like lesions were found in the ascending colon to the cecum (Figure 1D) and the rectum. *IgH-BCL2* positivity was found in 78.0% of the cells in biopsy tissue by FISH, suggesting recurrence (Lugano Stage II1). After follow-up with no treatment, the lesion disappeared spontaneously approximately 1 year later (Figure 1E).

**Case 3:** FL lesions were observed not only in the duodenum (Figure 1F) but also in the mesenteric small lymph nodes (Figure 1G, diagnosed by biopsy) and bone marrow. *IgH-BCL2* positivity was observed in 5.8% of the nucleated cells by FISH (Stage IV). The distal small intestine was not searched by enteroscopy, and it cannot be ruled out completely that an extraduodenal primary lesion was present. The first CR was achieved after 8 cycles of R-CHOP (rituximab + cyclophosphamide 750 mg/m$^2$, doxorubicin 50 mg/m$^2$, and vincristine 1.4 mg/m$^2$, all on day 1, and prednisone 60 mg/m$^2$ on days 1-5 every 3 wk), and maintenance treatment with rituximab was administered. The patient has not relapsed and still maintains the first CR.

**Case 4:** Double-balloon enteroscopy revealed no abnormal lesions in the distal small intestine. Radiation therapy (30 Gy) was performed, and rituximab (375 mg/m$^2$) was administered twice during this

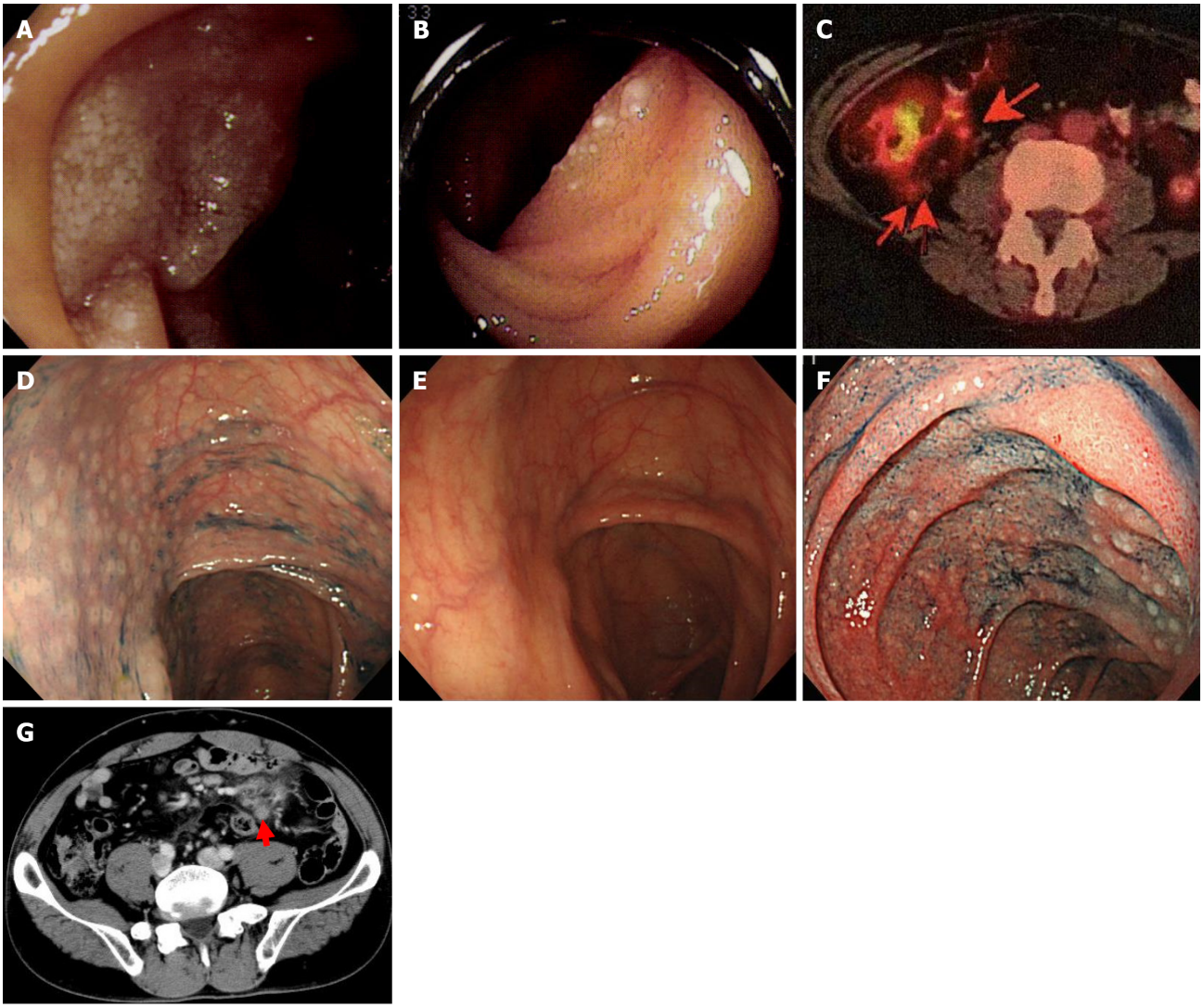


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**Figure 1 Videography findings of cases 1-3.** A and B: Endoscopic findings of case 1, lesions at (A) the descending portion of the duodenum and (B) the jejunum; C-E: Positron emission tomography findings of case 2; the arrow indicates a mesenteric nodal lesion in the ileocecal region (C); colonoscopy findings showed multiple lymphomatous polyposis-like lesions in the ascending colon (D); 1 year later, the lesion spontaneously disappeared (E); F and G: Esophagogastroduodenoscopy findings of case 3; lymphoma lesions were revealed in the descending portion of the duodenum (F); abdominal computed tomography findings. Mesenteric lymph nodes were swollen (arrowhead) (G).

treatment. This treatment strategy resulted in CR. The patient's D-FL did not recur without additional treatment.

**Case 5:** A 42-year-old male was incidentally found to have an FL lesion in the descending portion of the duodenum by EGD screening at our hospital, and he was pathologically diagnosed with D-FL (Figure 2A-C). Although small bowel enteroscopy had not been performed, the tumor indicated Lugano Stage I. Thus, we continued the 'watch and wait' approach for a year. However, the patient and his family requested treatment. He underwent chemotherapy with CHOP × 2 cycles, followed by oral therapy with etoposide (50 mg) for 2 mo. The extent of the duodenal lesion was slightly decreased (minimal response), and the patient was followed-up without treatment. Rituximab, which had at that time just been approved for use in Japan, was then administered as a single agent (once weekly, 4 times), and the lesion regressed steadily (partial response). Seven months later, radiation (40 Gy) was administered, and the patient's first CR was finally achieved 3 years after the intervention.

Treatment-free follow-up continued nearly 13 years after achieving the first CR, and then the patient noted swelling in his neck. Despite having a lymph node biopsy, a pathological diagnosis could not be made. PET-CT showed clear uptake in the lungs and lymph nodes throughout the whole body. The maximum standardized uptake value ranged from 3 to 15, which is consistent with the recurrence of FL (Stage IV) (Figure 3A-C). The duodenal lesion had maintained CR. After R-CHOP × 1 (stable disease), the patient underwent six cycles of the rituximab + bendamustine (B-R) regimen (90 mg/m$^2$ days 1-2, every 4 wk) and achieved metabolic CR according to PET-CT.

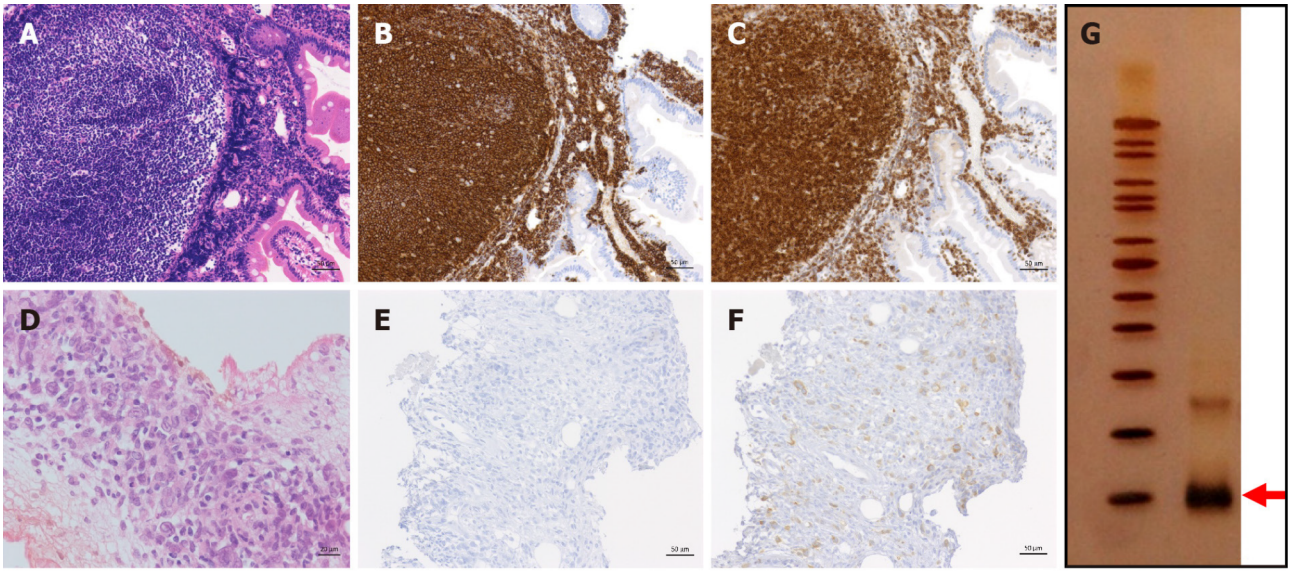


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**Figure 2 Pathological findings of case 5.** The upper row shows the histology at the time of onset; the lower row shows images obtained 21 years later and at the final stage of treatment; A and D: Hematoxylin and eosin staining; B and E: CD20 staining; C and F: BCL-2 staining; G: The PCR-single strand conformation polymorphism method. The arrow indicates bands that represent B-cell clones.

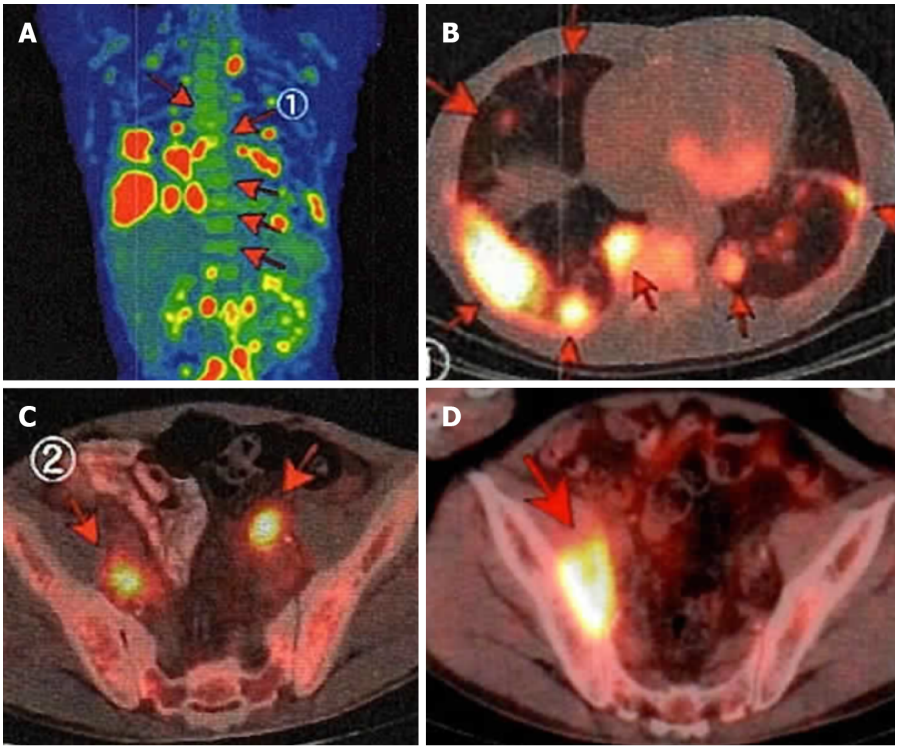


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**Figure 3 Positron emission tomography-computed tomography imaging of case 5.** Arrows point to lymphoma lesions at recurrence; A: Longitudinal image; B: Chest; C: Pelvic cavity; D: After treatment with various anticancer drugs, the nodal lesions in the pelvic cavity progressed further.

He then received maintenance treatment with rituximab every 2-3 mo. However, the lesions of the lung and pelvic lymph nodes recurred. After 3 cycles of chemotherapy with rituximab + bendamustine 70 mg/m$^2$ days 1-2, cytarabine 800 mg/m$^2$ days 1-3, every 4 wk, the lung lesions disappeared. However, the nodal lesions in the pelvic cavity had progressed on PET-CT (Figure 3D). He was then treated with 2 cycles of cyclophosphamide 750 mg/m$^2$, doxorubicin 50 mg/m$^2$, vincristine 1.4 mg/m$^2$ all day 1, etoposide 100 mg/body days 1-3, and prednisone 60 mg/m$^2$ days 1-5, every 3 wk and with various other drugs, including obinutuzumab. He remained non-CR.

Biopsy of the pelvic lesion showed that the tumor cells were CD20-negative (Figure 2D and E). However, they were positive for BCL2 (Figure 2F), and B-cell clonality was demonstrated by the PCR-single strand conformation polymorphism method (Figure 2G). The pathological diagnosis was DLBCL transformed from FL. The patient died approximately 4 years after recurrence and 21 years from the first onset.

## DISCUSSION

D-FL is a unique subtype of FL that is classified in the WHO 2017 classification of FLs[1]. It is usually localized to the intestinal tract and does not spread to the lymph nodes[11]. Two clinical studies conducted by Takata *et al*[6] and Schmatz *et al*[11] reported that of a total of 162 patients with D-FL, no patients had a Lugano stage higher than Stage II. Epidemiologically, D-FL has been recognized as a rare entity that accounts for approximately 4% of primary gastrointestinal lymphomas[9]. Bende *et al*[12] identified the expression of surface IgA, which is not found in nodal FL, in the mucosal immune system as a feature of D-FL cells in the intestinal mucosa and the expression of α4β7 integrin, which is thought to mediate "mucosal homing." In addition, the gene expression profile of D-FL has been shown to be similar to that of MALT lymphoma[8]. D-FL is almost asymptomatic and has an indolent clinical course, suggesting that it is biologically more similar to MALT lymphoma than to nodal FL[7]. Therefore, follow-up with a "watch and wait" approach without immediate intervention after diagnosis is frequently applied in cases of D-FL[9,10]. In Case 2, FL lesions recurred in the ileocecal region and the rectum but spontaneously regressed. It was previously reported that D-FL disappeared spontaneously in 3%-30% of patients[6,11]; as a result, it may have been possible to address this patient even if "watch and wait" was initiated.

Radiation is a representative treatment for D-FL, and there are several reports on its effectiveness[11,13,14]. However, Takata *et al*[6] reported that 46 of 54 patients (85%) with D-FL in the descending portion of the duodenum also had lesions in the distal small intestine, primarily the jejunum; thus, it is necessary to reliably determine the extent of the lesion. In Case 4, enteroscopy was performed, and the lesion did not extend to the distal small intestine. Because the lesion was localized to the duodenum, local irradiation was considered to be the most reasonable treatment.

Anticancer drug treatments for D-FL are based on the administration of rituximab ± chemotherapy, such as R-CHOP/rituximab + cyclophosphamide, vincristine, and prednisone, and B-R. D-FL is a low-grade malignancy and rarely requires chemotherapy, except in cases that exhibit histological transformation[11,15]. In indolent non-Hodgkin B-cell lymphoma, it has been reported that B-R was significantly better in progression-free survival than R-CHOP[16], and there is also a case report of D-FL for which B-R was effective[17]. However, as mentioned above, bendamustine was not yet available in Japan until 2010. In this case series, two patients had Lugano Stage IV disease (Case 2 and Case 3), and therapeutic intervention was performed using R-CHOP/Rituximab + cyclophosphamide, vincristine, and prednisone (change to fludarabine during the treatment course in Case 2). CR was reached in both cases, and maintenance treatment with rituximab monotherapy was performed. Rituximab monotherapy is effective for patients with high tumor-burden nodal FL[18] and has been used as a treatment at our center. The efficacy and safety of chemotherapy in Stage IV "advanced" D-FL cases without histological transformation and the importance of subsequent rituximab maintenance therapy should be investigated in a large number of cases in the future.

In Case 5, this patient was treated with a long nontreatment interval and reached his first CR over 3 years after the intervention. Considering this result, it might have been possible to achieve CR earlier by radiation-centered treatment from the beginning, as in Case 4. Unfortunately, the patient relapsed nearly 13 years after reaching his first CR. Although he was treated with various anticancer drugs, he died of the primary disease approximately 4 years after recurrence. The PET-CT findings at the time of recurrence were consistent with FL. Lesions showing maximum standardized uptake value of 15 (> 13.55, mean value of FL-grade 3b/DLBCL[19]) were also detected, suggesting that they contained partial DLBCL component. Therefore, we clinically speculated that the initial D-FL in this patient underwent clonal evolution to become the final DLBCL.

The incidence of histological transformation of D-FL into DLBCL was found to be 3.8%[20] in 5 retrospective studies[2,14,15,21,22] and 1 prospective study[3]. This incidence is lower than the incidence of nodal FL, which has an incidence of histological transformation of 10.7% over 5 years at a rate of 2% per year[23]. This transformation incidence is close to that of gastric MALT lymphoma, which is almost 3%[24]. In past cases, D-FL patients with histological transformation to DLBCL did not receive systemic chemotherapy at the time of onset but instead underwent the "watch and wait" approach[15,25-27]. Even in that situation, since the lymphoma was in remission due to R-CHOP chemotherapy, D-FL did not require aggressive treatment in the absence of histological transformation. Thus, the "watch and wait" follow-up approach was approved. However, in recent years, several patients with D-FL with histological transformation refractory to R-CHOP chemotherapy have been reported[20,28].

The reason our patient became refractory was likely histological transformation in addition to a change of the immunophenotype of the tumor to CD20-negative[29]. Furthermore, the therapeutic

response to the anticancer drugs was not good at the first onset. Fatal histological transformation occurred even after a long period of more than 17 years from the first onset; thus, patients with D-FL require lifelong follow-up.

## CONCLUSION

In this study, 5 patients with D-FL who received treatment intervention regardless of clinical stage were evaluated with respect to the therapeutic effects. The treatment of 3 Stage IV cases was successful, and in the future, criteria for how to treat "advanced" D-FL should be established based on additional cases. This study indicates that it is necessary to continue to follow-up with whole body examinations while paying careful attention to the possibility of recurrence in D-FL because fatal recurrence can occur even 13 years after a patient achieves CR.

## ARTICLE HIGHLIGHTS

### *Research background*

Duodenal-type follicular lymphoma (D-FL) has been recognized as a rare primary gastrointestinal lymphoma. Because D-FL follows an indolent clinical course compared to nodal FL, the "watch and wait" approach is currently the general follow-up policy.

### *Research motivation*

There is still insufficient consensus regarding the appropriate treatment of D-FL, and an option to actively treat D-FL is available. The long-term outcomes following the active treatment of D-FL are poorly understood.

### *Research objectives*

This study aimed to clarify the clinical outcomes through long-term follow-up in cases of D-FL with treatment intervention.

### *Research methods*

We retrospectively examined 5 D-FL patients who underwent therapeutic intervention at our center from January 1998 to December 2009 and followed the clinical course of these patients for more than 10 years.

### *Research results*

As a result of therapeutic intervention, all 5 cases reached complete remission (CR) and survived for more than 10 years. However, 3 of these cases experienced recurrence. One patient achieved a second CR after retreatment, and in the other case, the lesion spontaneously disappeared. The remaining patient experienced widespread systemic recurrence 13 years after the first CR. This patient died 4 years later despite treatment with various anticancer chemotherapies.

### *Research conclusions*

Five patients with D-FL who received treatment interventions regardless of clinical stage were evaluated with respect to the therapeutic effects of the treatment. Because fatal recurrence was found to occur even 13 years after the first CR, it is necessary to continue whole-body follow-up examinations for individuals diagnosed with D-FL.

### *Research perspectives*

Only 5 cases were examined in this study. By including more D-FL patients and evaluating their treatment, criteria for how to treat Stage IV "advanced" cases can be explored.

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## FOOTNOTES

**Author contributions:** Saito M designed this study; Mori A, Tsukamoto S, Ishio T, Yokoyama E, Izumiyama K, Morioka M, and Kondo T took part in drafting the article; Sugino H was involved in the pathological procedure; all authors collected and analyzed the patient's clinical data and agreed to be accountable for all aspects of the work.

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ORIGINAL ARTICLE

Observational Study

# Evaluation of the diagnostic value of serum-based proteomics for colorectal cancer

Hui-Juan Wang, Yi-Bin Xie, Peng-Jun Zhang, Tao Jiang

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**Peer-review model:** Single blind

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## Abstract

### *BACKGROUND*

Colorectal cancer (CRC) is a highly malignant cancer with a high incidence and mortality in China. It is urgent to find a diagnostic marker with higher sensitivity and specificity than the traditional approaches for CRC diagnosis.

### *AIM*

To provide new ideas for the diagnosis of CRC based on serum proteomics.

### *METHODS*

Specimens from 83 healthy people, 62 colon polyp (CRP) patients, and 101 CRC patients were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The diagnostic value of the profiles of differentially expressed proteins was then analyzed.

### *RESULTS*

Compared with the healthy control group, CRC patients had elevated expression of 5 proteins and reduced expression of 14 proteins. The area under the curve (AUC) for a differentially expressed protein with a mass-to-charge ratio of 2022.34

was the largest; the AUC was 0.843, which was higher than the AUC of 0.717 observed with carcinoembryonic antigen (CEA), and the sensitivity and specificity of this identified marker were 75.3% and 79.5%, respectively. After cross-validation, the accuracy of diagnosis using levels of this differentially expressed protein was 82.37%. Compared with the CRP group, the expression of 3 proteins in the serum of CRC patients was elevated and 11 proteins were expressed at reduced levels. Proteins possessing mass-to-charge ratio values of 2899.38 and 877.3 were selected to establish a classification tree model. The results showed that the accuracy of CRC diagnosis was 89.5%, the accuracy of CRP diagnosis was 81.6%, and the overall accuracy of this approach was 86.3%. The overall sensitivity and specificity of diagnosis using the proteomics approach were 81.8% and 66.75%, respectively. The sensitivities and specificities of diagnoses based on CEA and carbohydrate antigen 19-9 expression were 55.6% and 91.3% and 65.4% and 65.2%, respectively.

***CONCLUSION***

We demonstrated that serum proteomics may be helpful for the detection of CRC, and it may assist clinical practice for CRC diagnosis.

**Key Words:** Colorectal cancer; Colorectal polyps; Serum; Proteomics; Diagnostic value



**Core Tip:** At present, the main techniques for proteomic research are mass spectrometry and two-dimensional gel electrophoresis. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry can analyze not only cells and tissues but also powders, solutions, and membranes. This technology is an ideal platform for the identification of tumor markers to be used in clinical practice. In this study, by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, we analyzed the serum protein expression profiles of healthy controls, colorectal polyp patients, and colorectal cancer patients to find differentially expressed protein peaks, and aimed to evaluate the diagnostic value of serum proteomics for colorectal cancer.

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## INTRODUCTION

Colorectal cancer (CRC) is a highly malignant cancer with a high incidence and mortality in China[1]. Although many scholars in China and abroad have performed many studies on the pathogenesis and clinical manifestations of CRC, the CRC etiology is still not fully understood, and its pathogenesis has not been substantially elucidated. The commonly used serum tumor markers for CRC are carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA-199)[2]. Due to the low sensitivity and specificity of these tumor markers, it is urgent to find a diagnostic marker with higher sensitivity and specificity[3].

Due to the advent of the post-gene era, by analyzing the proteins and peptides of normal and cancerous cells, we can search for disease-specific markers and provide a new technical platform for theoretical and clinical research on CRC[4]. The study of altered protein and peptide expression with CRC could provide a reliable molecular theoretical basis for its early diagnosis, postoperative detection, postoperative recurrence prediction and prognosis[5]. At present, the main techniques for proteomic research are mass spectrometry and two-dimensional gel electrophoresis (2D-PAGE). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a technology that was developed in the 1980s. At present, MALDI-TOF mass spectrometry technology has been widely used in the detection of sugars, nucleic acids, proteins, *etc.* The structural analysis and molecular weight determination of biological macromolecules and synthetic polymers have become some of the core objectives of current proteomics research[6]. This technique has many advantages, such as accurate mass-to-charge ratio calculation, low cost, large affinity surface, and good reproducibility, and MALDI-TOF-MS can analyze not only cells and tissues but also powders, solutions, and membranes. This technology was demonstrated to be a potential screening method for various diseases[7-9]. It is also a label-free detection technology, which reduces the cost of detection, and has high sensitivity and high-throughput detection capabilities[10,11]. Therefore, this technology is an ideal platform for the identi-

fication of tumor markers to be used in clinical practice. This approach to tumor biomarker discovery will play an important role in tumor screening, early diagnosis, individualized treatment and other aspects of cancer management[12].

In this study, by using MALDI-TOF mass spectrometry, we analyzed the serum protein expression profiles of healthy controls, colorectal polyp (CRP) patients, and CRC patients to find differentially expressed protein peaks. After specific marker proteins were identified, a diagnostic model based on their profiles was built to verify the clinical value of this proteomic approach, and the model's performance was compared with that of the conventional tumor markers CEA and CA-199. Thus, we aimed to utilize a new strategy for the diagnosis of CRC and to provide evidence that this approach yields high-quality and efficient diagnostic models for clinical use.

## MATERIALS AND METHODS

### *Study subjects*

Signed informed consent forms were obtained, and this study was approved by the Ethics Committee of the First Center of Chinese PLA General Hospital. Serum specimens in our study were all taken from the First Center of Chinese PLA General Hospital. This study included patients with precancerous lesions and colorectal tumors. The total number of specimens was 246, including samples from 83 healthy people, 62 CRP patients, and 101 CRC cancer patients. The inclusion criteria of the control group were no obvious organic lesions after physical examination, no diseases involved in this study and no other major clinically diagnosed diseases. CRC and CRP patients were diagnosed by pathologists after surgical resection of tumor tissue and endoscopic biopsy. The exclusion criteria were patients lacking confirmation by medical examination, patients undergoing chemotherapy or current acute infection [13], and sample coagulation for more than 12 h. Table 1 shows the clinical characteristics of the participants. After the whole blood samples were collected, they were centrifuged at 3500 r/min for 7 min at room temperature and immediately stored at -80 °C.

### *Preparation of serum samples*

Blood was collected from the study subjects in the morning after fasting, and the blood was anticoagulated with EDTA and centrifuged with a clinical centrifuge at 3500 r/min for 7 min within 30 min. The serum was aliquoted at 50 μL per tube and stored in a -80 °C freezer according to the universal method. Sample freezing and thawing for more than 2 times was avoided. For the analysis of the serum samples, a 1:50 dilution of the sample in assay buffer was performed.

### *Precautions for specimen collection*

A maximum of 25 μL of serum or plasma was required per well. The samples used were stored in polypropylene tubes, and sample storage in glass tubes was avoided. The processing of samples with obvious hemolysis or lipemia was also avoided. It should be noted that heparin concentrations > 10 IU/mL in blood were not used as an anticoagulant because excessive heparin leads to an abnormal increase in measured values. Repeated freezing and thawing of serum samples can easily cause peptide precipitation, which will result in the loss of some peptides in the peptide MALDI-TOF MS spectrum. For this reason, repeated freezing and thawing was avoided.

### *Generation of protein profiles*

The magnetic bead kit was removed from the 4 °C refrigerator, a tube of weak cation magnetic bead suspension was removed, and the tube was shaken up and down manually for 1 min to suspend the magnetic beads completely and uniformly in the liquid phase. Then, 5 μL of SPE-CM magnetic bead suspension was pipetted into a 200-μL sample tube, and 10 μL of magnetic beads was added to the sample tube and mixed by pipetting up and down to avoid foaming. Next, 5 μL of serum was added to the sample tube and mixed by pipetting up and down at least 5 times with the pipette to avoid foaming. The mixture was incubated at room temperature for 5 min, and then the sample tube was placed into the magnetic bead separator. After the magnetic beads adhered to the wall of the separator for 1 min, the magnetic beads were separated from the suspended liquid, and the color of the separated liquid was confirmed to be clear. The suspended liquid was absorbed with the sample addition gun. Care was taken to avoid the pipette tip touching the magnetic beads to prevent the magnetic beads from being aspirated. Subsequently, 100 μL of Magnetic Bead Wash Buffer was added to the sample tube. The sample tube was moved 10 times between two adjacent openings before and after being placed into the magnetic bead separator. The sample tube was then placed on the magnetic bead separator so that the magnetic beads adhered to the wall, and then the suspended liquid was absorbed with the sample addition gun. During this step, touching the magnetic beads with the pipette tip was avoided to prevent the magnetic beads from being aspirated. The suspended liquid was then completely aspirated during a final pipetting step. The sample tube was removed from the magnetic bead separator, 5 μL of magnetic bead elution buffer was added to the sample tube, and the process was repeated 10 times while

**Table 1 Clinical characteristic of individuals in our study**

| Group | *n* | Ratio of gender (Male/female) | mean age |
|---|---|---|---|
| CRP | 62 | 1.3:1 | 58.3 |
| CRC | 101 | 1.1:1 | 59.7 |
| HC | 83 | 1:1 | 51.1 |

CRP: Colorectal polys; CRC: Colorectal cancer; HC: Healthy control.

avoiding foaming. The sample tube was placed into the magnetic bead separator, and the magnetic beads adhered to the wall for 2 min. After the magnetic beads were completely separated from the suspended liquid, the supernatant was transferred to a clean 0.5-mL sample tube. Five microliters of magnetic bead stabilization buffer were added to a 0.5-mL sample tube, carefully pipetted, and mixed with the sample pipette. Then, the sample was collected in a 0.5-mL tube, and the eluate with magnetic bead stabilization buffer was used immediately for mass spectrometry analysis or frozen at -20 °C for analysis within 24 h.

### *Preparing the matrix and standards*

α-Cyano-4-hydroxycinnamic acid (1 g/L) was prepared and dissolved in acetone. The final matrix solution of 0.3 g/L, with ethanol/acetone = 2:1, was prepared. Fresh matrix solution was prepared on the same day of analysis. At room temperature, Peptide Calibration Standard (#206195) was dissolved in 125 μL of 0.1% TFA water, mixed for 1 min, and allowed to stand for 5 min. Protein Calibration Standard I (#206355) was dissolved in 125 μL of 0.1% TFA water, mixed for 1 min, and allowed to stand for 5 min. A total of 77 mg of ammonium acetate was dissolved in 100 mL of Milli-Q water to prepare 10 mmol/L ammonium acetate. A solution of 5 μL (25 μL) of Peptide Calibration Standard, 25 μL (125 μL) of Protein Calibration Standard I, and 20 μl (100 μL) of 10 mmol/L ammonium acetate were mixed well for 1 min. Solutions were aliquoted at 5 μL and stored at -20 °C for several weeks. For the list of standard products, see Table 2.

### *Cleaning the AnchorChip Target and MALDI-TOR detection*

The target surface was first rinsed with hot water. The target surface was then cleaned with dust-free paper and acetone, followed by Milli-Q water, and then with methanol and dried at room temperature. A tube of aliquoted standard and several aliquoted samples were thawed at room temperature. One microliter of standard was then mixed with 10 μL of matrix. One microliter of this solution was placed on a region of the 600-μm AnchorChip standard and dried at room temperature for several min. One microliter of the magnetic bead-treated specimen was mixed with 10 μL of matrix. The magnetic bead eluent was placed onto the AnchorChip sample position. The collection range and adjustment of laser energy were essentially identical across the collection of standard products. The same crystallization point of each sample was collected at 8 points and accumulated to 500 shots. Then, the accumulated maps were saved to a specified folder and labelled.

### *Spectrum generation and statistical analysis*

The acquisition range was 1-13 kDa. The laser energy was determined according to the laboratory mass spectrometer. High laser energy can be used to bombard the sample to the crystallization point, and then the energy spectrum that is 10%-20% lower than the high laser energy can be used to collect the spectrum. Data taken at the crystallization target point and different points were used for multipoint analysis, which resulted in a total of 8 crystallization point data values used to obtain the accumulated spectrum, and the average molecular weight deviation of the standard product was less than 100 ppm. The data were statistically processed by ClinProTools software[14,15]. When comparing the protein peak intensities between the two groups, $P < 0.05$ was considered statistically significant.

## RESULTS

### *Comparison of serum protein profiles between the CRC group and the healthy control group*

Spectra of CRC patient and healthy control (HC) samples were imported into ClinProTools software for processing and generation of serum differential protein profiles. Processes such as normalization, baseline extraction, peak definition, recalibration, and comparison of multiplex spectra were automated. The ClinProTools software in MALDI-TOF-MS was used to analyze the serum protein profiles of the CRC group and the HC group. According to the changes in the peak intensities in the two groups of data, a *T* test was used to calculate the *P* value. Nineteen of these differentially expressed protein peaks

**Table 2 List of standards of matrix-assisted laser desorption/ionization time-of-flight**

| Substance | Average mass $(M + H)^+$ | Resolution | StDeV |
|---|---|---|---|
| Angiotensin II | 1047.18 | 360 | 10 |
| Angiotensin I | 1297.48 | 365 | 10 |
| Substance P | 1348.64 | 380 | 17 |
| Bombesin | 1620.86 | 420 | 23 |
| ACTH clip 1-17 | 2094.42 | 475 | 22 |
| ACTH clip 18-39 | 2466.68 | 540 | 17 |
| Somatostatin 28 | 3149.57 | 580 | 25 |
| Ubiquitin | 4283.45 | 800 | 40 |
| Insulin | 5734.56 | 580 | 25 |
| Cytochrome c | 6181.05 | 560 | 85 |
| Ubiquitin | 8565.89 | 345 | 25 |

formed the basis of the auxiliary diagnostic protein profiles (Table 3). Compared with the HC group, CRC patients had elevated expression of 5 proteins with mass-to-charge ratios of 2022.34, 1866.09, 2899.72, 1778.9, and 1897.01 and reduced expression of 14 proteins with mass-to-charge ratios of 4210.57, 2932.56, 3192.08, 3883.65, 877.4, 7772.42, 3158.36, 2272.09, 4645.79, 4092.12, 4268.05, 2952.92, 3262.98, and 2660.37.

### *Comparison of the diagnostic value of differential proteins and validation*

A receiver operating curve was drawn for the 19 differentially expressed proteins, and their respective area under the curves (AUCs) are shown in Table 4. The AUC of the differentially expressed protein P5 (mass-to-charge ratio of 2022.34) was the largest; the AUC was 0.843, the sensitivity was 75.3%, and the specificity was 79.5%, and thus, all of these metrics indicated high performance of protein P5-based diagnosis. The AUC obtained using the profile of the marker possessing a mass-to-charge ratio of 2022.34 was higher than the AUC of CEA-based diagnosis, which was 0.717. The comparison of the mean expression levels of the differentially expressed protein with a mass-to-charge ratio of 2022.34 in the serum of colon cancer patients and the serum of healthy groups is shown in Figure 1. Using the difference peaks obtained by the *T* test in ClinProTools software, the built-in Genetic Algorithm was used to calculate the cross-validation rate and identification ability. The diagnostic value of the two differentially expressed proteins possessing molecular weights of 4210.57 Da and 2932.56 Da was analyzed, as shown in Figure 2, and the cross-validation accuracy was 82.37%.

### *Comparison of serum protein profiles of the CRC and CRP groups*

According to the change in the peak intensity across the two groups of data, the *P* value was calculated by the *T* test, and 14 differentially expressed protein profiles were obtained (Table 5). Compared with the CRP group, in the serum of CRC patients, 3 proteins were highly expressed, with mass-to-charge ratios of 2863.23, 2022.52, and 2899.88 m/z. Eleven proteins were expressed at reduced levels, with mass-to-charge ratios of 861.19, 4475.16, 845.16, 4210.83, 866.41, 1072.04, 2106.07, 4645.62, 2953.55, 2661.25, and 877.3.

### *Analysis of the serum protein profiles of CRC and CRP by principal component analysis*

The peaks of differentially expressed proteins obtained by the *T* test in ClinProTools software were analyzed with the principal component analysis algorithm built in the software. As shown in Figure 3, 7 protein profile peaks (1866.45, 1945.58, 2022.52, 2082.82, 4210.83, 5906.31, and 7767.55 m/z) with relatively large relative dispersion are shown in the first three loading (Loading 1, Loading 2, and Loading 3) models. The total contribution of each peak is the cumulative sum of the three loading values of the peak multiplied by the contribution rate of the main factor. Other peaks that are located near the main axis with loading values close to 0 were ignored. The coordinate values and total contribution values of the seven peaks are shown in Table 6.

### *Using differential proteins to build a classification tree model for discriminating the CRC and CRP groups*

The spectra of 14 differentially expressed proteins were analyzed by CLINPROT software in MALDI-TOF-MS, and a classification tree model was established. Complete random classification of the 163 collected specimens (62 colon polyp specimens, 101 colon cancer specimens) into the training group or

**Table 3 Comparison of serum protein profiles between colorectal cancer group and healthy control group**

| Mass (m/z) | DAve | PTTA | PWKW | PAD | Ave1 | Ave2 |
|---|---|---|---|---|---|---|
| 4210.57 | 536.3 | 0.00000308 | 0.00000149 | 0.066 | 700.27 | 1236.57 |
| 2932.56 | 22.98 | 0.00000635 | < 0.000001 | 0.00721 | 28.61 | 51.6 |
| 2022.34 | 282.69 | 0.0000161 | < 0.000001 | < 0.000001 | 309.73 | 27.04 |
| 3192.08 | 26.53 | 0.0000333 | 0.0000105 | 0.000326 | 30.96 | 57.49 |
| 3883.65 | 31.52 | 0.000476 | 0.000129 | 0.00122 | 46.6 | 78.12 |
| 877.4 | 3.23 | 0.000645 | 0.000129 | 0.000683 | 5.93 | 9.16 |
| 1866.09 | 62.56 | 0.000826 | 0.000407 | < 0.000001 | 83.77 | 21.21 |
| 2899.72 | 13.03 | 0.000997 | 0.000189 | < 0.000001 | 38.31 | 25.28 |
| 7772.42 | 338.93 | 0.00141 | 0.00147 | 0.0503 | 594.88 | 933.81 |
| 3158.36 | 53.74 | 0.00284 | 0.000264 | < 0.000001 | 62.18 | 115.92 |
| 2272.09 | 20.34 | 0.00284 | 0.0000451 | < 0.000001 | 22.18 | 42.51 |
| 4645.79 | 22.93 | 0.00432 | 0.00483 | 0.231 | 57.18 | 80.11 |
| 4092.12 | 22.14 | 0.00577 | 0.0321 | 0.324 | 113.17 | 135.31 |
| 1778.9 | 7.03 | 0.00739 | 0.00672 | < 0.000001 | 18.66 | 11.64 |
| 4268.05 | 27.62 | 0.00795 | 0.000333 | < 0.000001 | 69.05 | 96.67 |
| 2952.92 | 27.19 | 0.0104 | 0.0265 | 0.232 | 70.78 | 97.97 |
| 3262.98 | 50 | 0.0116 | 0.00564 | < 0.000001 | 69.12 | 119.11 |
| 1897.01 | 14.98 | 0.0183 | 0.0122 | < 0.000001 | 41.59 | 26.61 |
| 2660.37 | 35.86 | 0.0197 | 0.0367 | 0.286 | 115.58 | 151.44 |

PTTA: *P* value of *t*-test; PWKW: *P* value of Wilcoxon; PAD: *P* value of Anderson-Darling test.

the test group was performed. The numbers of CRC patients and CRP patients in the test and validation groups were 57 and 38 and 44 and 24, respectively. A classification tree diagnostic model was established using the 95 samples in the training group. Through this approach, it was revealed that the differentially expressed proteins with mass-to-charge ratios of 2899.38 and 877.3 were automatically selected to establish a classification tree model. The classification tree model established by these two differentially expressed proteins was then used to classify patient diagnosis. The results showed that 6 of 57 patients with CRC were missed, resulting in an accuracy of 89.5%, and 7 of 38 patients with CRP were misdiagnosed with CRC, resulting in an accuracy of 81.6% and an overall accuracy of 86.3%.

### *Validation of the classification tree model*

The established model was validated with the remaining samples (44 CRC and 24 CRP specimens). The levels of the differentially expressed proteins with mass-to-charge ratios of 2899.38 and 877.3 were used to establish a classification tree model, and the levels of CEA and CA-199 in the same samples (44 CRC specimens and 44 CRP specimens) were also measured. The accuracy of the proteomics-based diagnostic model was evaluated according to the sensitivity and specificity of the validation results, and these values were compared with the sensitivities and specificities of diagnosis with CEA and CA-199 Levels. The sensitivity and specificity of diagnosis using the classification tree were 81.8% and 66.75, respectively. The sensitivity and specificity values for diagnosis using CEA and CA-199 Levels were 55.6% and 91.3% and 65.4% and 65.2%, respectively, as shown in Table 7.

## DISCUSSION

MALDI-TOF mass spectrometry has been widely used in the structural analysis and molecular weight determination of sugars, nucleic acids, proteins and other biological macromolecules and synthetic polymers and has become one of the core technologies in current proteomics research[16]. This technique has many advantages, such as a precise mass-to-charge ratio, low cost, large affinity surface, and good repeatability[9,17].

**Table 4 Area under curve values of 19 differential protein profile and carcinoembryonic antigen**

| DPP | m/z | AUC |
|---|---|---|
| P1 | 877.4 | 0.714 |
| P2 | 1778.9 | 0.595 |
| P3 | 1866.09 | 0.687 |
| P4 | 1897.01 | 0.640 |
| P5 | 2022.34 | 0.843 |
| P6 | 2272.09 | 0.705 |
| P7 | 2899.72 | 0.746 |
| P8 | 2932.56 | 0.783 |
| P9 | 2952.92 | 0.589 |
| P10 | 3158.36 | 0.759 |
| P11 | 3192.08 | 0.811 |
| P12 | 3262.98 | 0.751 |
| P13 | 3883.65 | 0.642 |
| P14 | 4092.12 | 0.655 |
| P15 | 4210.57 | 0.805 |
| P16 | 4268.05 | 0.744 |
| P17 | 4645.79 | 0.617 |
| P18 | 7772.42 | 0.714 |
| P19 | 2660.37 | 0.666 |

DPP: Differential protein profile; AUC: Area under curve.

MALDI-TOF mass spectrometry was used to analyze the serum protein expression profile of patients with CRC to identify differentially expressed protein peaks. At the same time, the serum proteins of patients with CRC and CRP were analyzed. Using these biological data, specific protein markers were selected, and then a CRC classification tree diagnostic model was established. The model was used to predict diagnosis with the analyzed serum protein markers of the patients to verify its clinical value and to compare the result of this approach with that of diagnoses achieved with the conventional tumor markers CEA and CA-199. This approach may thus improve diagnostic evaluation of CRC. Using MALDI-TOF mass spectrometry data from colon cancer patient serum and healthy human serum, 19 protein profiles with significant differences across the two groups were obtained. Compared with the healthy control group, there were 5 protein peaks with increased expression and 14 protein peaks with reduced expression in the serum of CRC patients. The receiver operating curves of the 19 differentially expressed proteins were drawn, and it was found that the AUC of diagnosis obtained using data from a protein with a mass-to-charge ratio of 2022.34 was the largest at 0.843, and the sensitivity and specificity were 75.3% and 79.5%, respectively. Therefore, this differentially expressed protein exhibited high diagnostic value for CRC patients. Based on these metrics, the diagnostic value of this approach was higher than that of either CEA or CA-199 alone, and this approach could be further optimized.

To this end, we used MALDI-TOF mass spectrometry technology to analyze the peripheral blood protein auxiliary diagnostic spectrum of patients with colorectal disease to find the differentially expressed proteins with high diagnostic sensitivity and specificity to verify whether it can be used as a new diagnostic biomarker of CRC. Using MALDI-TOF mass spectrometry technology to analyze the differences in protein expression in the serum of patients with CRC and patients with CRP, 14 protein peaks with significant differences were obtained. Compared with the serum of patients with CRP, the serum of patients with CRC had elevated expression of 3 proteins and reduced expression of 11 proteins. Fourteen differential peak proteins were calculated using CLINPROT software in MALDI-TOF-MS, and a classification tree model was established. Differential proteins with mass-to-charge ratios of 2899.38 and 877.3 were automatically selected to build a classification tree model. Using this classification tree model to classify patients, the results showed that 6 of 57 colon cancer patients were missed with an accuracy of 89.5%, and 7 of 38 colon polyp patients were misdiagnosed with colon cancer for an accuracy of 81.6%. The overall accuracy rate was 86.3%. Compared with diagnoses made using CEA and CA-199 Levels, this proteomics model has high sensitivity and specificity and can more

**Table 5 Comparison of serum protein profiles between colorectal cancer group and colorectal polys group**

| Mass | DAve | PTTA | PWKW | PAD | Ave1 | Ave2 |
|---|---|---|---|---|---|---|
| 861.19 | 33.51 | 0.000077 | 0.00000468 | < 0.000001 | 36.09 | 69.6 |
| 4475.16 | 87.57 | 0.000186 | 0.00000468 | 0.00000185 | 96.92 | 184.49 |
| 2863.23 | 37.14 | 0.000332 | 0.00073 | < 0.000001 | 88.71 | 51.57 |
| 845.16 | 4.89 | 0.000376 | 0.0000351 | 0.00000115 | 7.69 | 12.59 |
| 4210.83 | 278.83 | 0.000672 | 0.00073 | 0.135 | 702.87 | 981.7 |
| 2022.52 | 251.03 | 0.000672 | 0.0167 | < 0.000001 | 353.51 | 102.48 |
| 866.41 | 9.71 | 0.00209 | 0.000277 | < 0.000001 | 15.5 | 25.21 |
| 2899.88 | 12.32 | 0.00232 | 0.0000748 | < 0.000001 | 38.41 | 26.09 |
| 1072.04 | 5.17 | 0.0032 | 0.000277 | < 0.000001 | 8.66 | 13.83 |
| 2106.07 | 12.84 | 0.00374 | 0.0000351 | < 0.000001 | 35.74 | 48.59 |
| 4645.62 | 24.65 | 0.00634 | 0.00872 | 0.0751 | 65.03 | 89.68 |
| 2953.55 | 28.69 | 0.0112 | 0.0167 | 0.114 | 77.95 | 106.64 |
| 2661.25 | 41.77 | 0.0134 | 0.0137 | 0.0322 | 121.96 | 163.73 |
| 877.3 | 6.11 | 0.0278 | 0.0000078 | < 0.000001 | 5.81 | 11.92 |

PTTA: *P* value of *t*-test; PWKW: *P* value of Wilcoxon; PAD: *P* value of Anderson-Darling test.

**Table 6 Coordinate values and total contribution values of seven peaks in principal component analysis**

| m/z | 1866.45 | 1945.58 | 2022.52 | 2082.82 | 4210.83 | 5906.31 | 7767.55 |
|---|---|---|---|---|---|---|---|
| Loading 1 | -0.05 | -0.35 | -0.2 | -0.225 | 0.32 | 0.76 | 0.25 |
| Loading 2 | 0.17 | -0.4 | 0.81 | -0.17 | -0.22 | 0.11 | -0.12 |
| Loading 3 | -0.015 | 0.14 | -0.1 | 0.11 | 0.02 | 0.36 | -0.9 |
| TC | 5.45 | 25.93 | 24.36 | 15.52 | 20.08 | 44.84 | 23.07 |

TC: Total contribution values.

**Table 7 The level comparison between classification tree diagnostic model validation and carcinoembryonic antigen, carbohydrate antigen 19-9**

| Index | Sensitivity (%) | Specificity (%) |
|---|---|---|
| Classification tree | 81.8 | 66.7 |
| CEA | 55.6 | 91.3 |
| CA-199 | 65.4 | 65.2 |

CEA: Carcinoembryonic antigen; CA-199: Carbohydrate antigen 19-9.

effectively distinguish CRC from CRP. The high sensitivity and specificity of MALDI-TOF-based diagnostics indicated that the combination of MALDI-TOF and bioinformatics could help to improve the early diagnosis of CRC and could be used as an auxiliary diagnostic method for the treatment and prognosis of patients.

MALDI-TOF mass spectrometry technology has been widely used in clinical tumor diagnosis, treatment, and prognosis monitoring and can be used as a powerful tumor marker discovery research tool[18]. In the next few years, with the development of genomics and proteomics, an increasing number of new markers with high diagnostic sensitivity and specificity will be discovered, identified, and applied in the clinic, which will significantly improve the clinical detection rate of cancer, allow for its early diagnosis and treatment[19], and provide new methods and ideas for the study of tumorigenesis

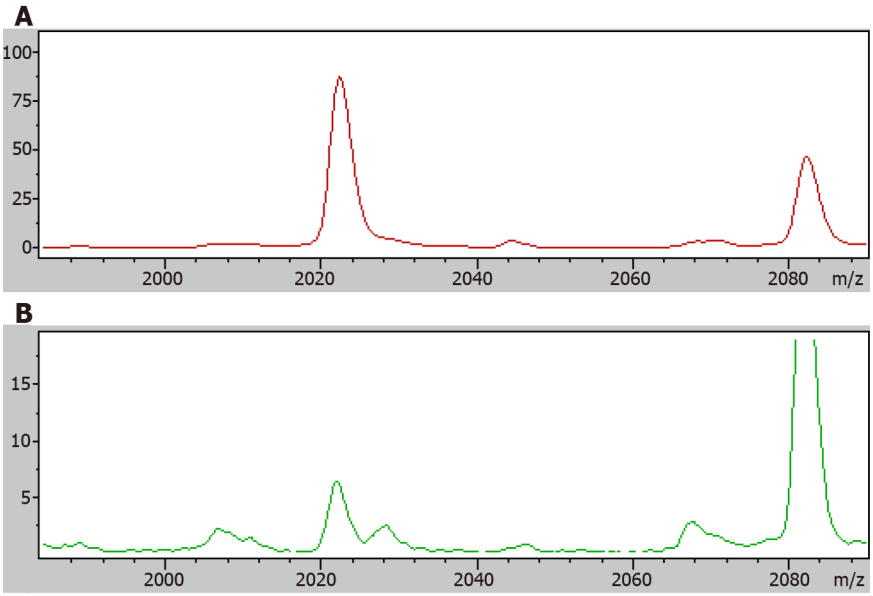


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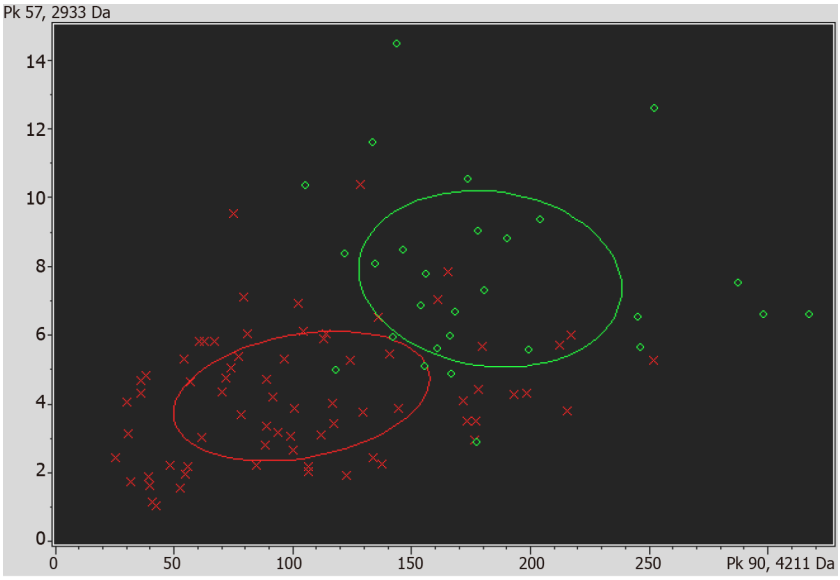
**Figure 1 The average expression level of differential protein 2022.34 in the serum of colorectal cancer patients and healthy control group.**
A: Colorectal cancer patients; B: Healthy control.




**DOI:** 10.4251/wjgo.v14.i8.1562 

**Figure 2 The diagnostic value of differential protein profile with mass-to-charge ratios of 4210.57Da and 2932.56.**

mechanisms[20].

However, there are still some limitations to this study. First, since MALDI-TOF-MS is a relatively novel protein detection method for serum samples, a standard operation protocol was not determined. This limits the utility of this approach to clinical practice. Second, the diagnostic performance of specific protein profiles in our study was assessed, but we have not confirmed these specific protein profiles in our study. Third, the relatively small sample size may somewhat bias the results.

## CONCLUSION

In conclusion, we demonstrated that serum proteomics may be helpful for the detection of CRC, and it may provide a potential tool for CRC clinical management. For discriminating HC and CRC subjects using this approach, the sensitivity was 75.3%, and the specificity was 79.5%. After cross-validation, the diagnostic accuracy was 82.37%. For discriminating CRP and CRC patients, the overall accuracy of the

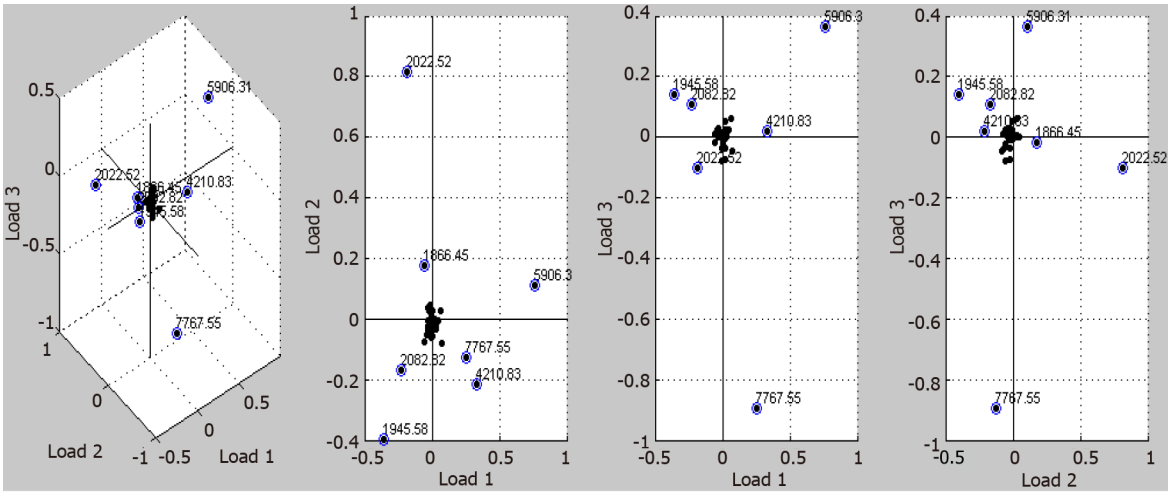



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**Figure 3 Seven protein profile peaks (1866.45, 1945.58, 2022.52m/z, 2082.82, 4210.83, 5906.31, 7767.55) with relatively large relative dispersion in the first three loading (Loading 1, Loading 2, Loading 3) model.**

classification tree model based on the 2899.38 m/z and 877.3 m/z protein profiles was 86.3%. The overall sensitivity and specificity of this approach were 81.8% and 66.75%, respectively.

## ARTICLE HIGHLIGHTS

### *Research background*

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) plays an important role in tumor research and in clinical applications and provides new ideas and approaches for tumor prevention, early diagnosis, and individualized treatment.

### *Research motivation*

The clinical use of MALDI-TOF-MS serum-based biomarkers for colorectal cancer detection should be investigated.

### *Research objectives*

We aimed to evaluate the diagnostic value of serum proteomics for colorectal cancer detection.

### *Research methods*

Eighty-three healthy controls, 62 colon polyp patients and 101 colorectal cancer patients were enrolled, and their serum samples were analyzed by serum proteomics. The diagnostic value of differential protein profiles was evaluated and compared with that of conventional biomarkers.

### *Research results*

The area under the curve resulting from a diagnostic based on the levels of a differentially expressed protein with a mass-to-charge ratio of 2022.34 for discriminating healthy controls and colorectal cancer patients was 0.843, while the sensitivity was 75.3% and the specificity was 79.5%. After cross-validation, the diagnostic accuracy of this approach was 82.37%. For classification of the colorectal polyp group, proteins with mass-to-charge ratios of 2899.38 and 877.3 were automatically selected to establish a classification tree model. The sensitivity and specificity were 81.8% and 66.75%, respectively. The sensitivities and specificities of diagnostics based on the levels of carcinoembryonic antigen and carbohydrate antigen 19-9 were 55.6% and 91.3% and 65.4% and 65.2%, respectively.

### *Research conclusions*

We have built an assistant diagnostic method for the detection of colorectal cancer based on serum proteomics. It may be helpful for colorectal cancer clinical management.

### *Research perspectives*

Studies with standard detection protocols and larger sample sizes should be performed in the future, and the protein profiles should also be confirmed.

## FOOTNOTES

**Author contributions:** Wang HJ and Jiang T designed the study; Wang HJ and Zhang PJ performed the research; Wang HJ and Xie YB analyzed the date; Wang HJ wrote the paper; Jiang T and Zhang PJ revised the manuscript for final submission; Wang HJ and Xie YB contributed equally to this study; Zhang PJ and Jiang T are the co-corresponding authors; and all authors have read and approve the final manuscript.

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**Informed consent statement:** All study participants or their legal guardian provided written informed consent prior to study enrollment.

**Conflict-of-interest statement:** We declare that we have no financial or personal relationships with other individuals or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature in any product, service and/or company that could be construed as influencing the position presented in or the review of the manuscript.

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ORIGINAL ARTICLE

Observational Study

# RASSF1A methylation as a biomarker for detection of colorectal cancer and hepatocellular carcinoma

Jian Li, Huan Li, Zeng-Ci Run, Zhen-Lei Wang, Tao Jiang, Yang An, Zhi Li

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## Abstract

***BACKGROUND***

Studies have validated the potential of methylated cell-free DNA as a biomarker in various tumors, and methylated DNA in plasma may be a potential biomarker for cancer.

***AIM***

To evaluate the diagnostic value of RASSF1A methylation in plasma for colorectal cancer (CRC) and hepatocellular carcinoma (HCC).

***METHODS***

A total of 92 CRC patients, 67 colorectal polyp (CRP) patients, 63 HCC patients, and 66 liver cirrhosis (LC) patients were enrolled. The plasma DNA was subjected to DNA extraction, double-strand DNA concentration determination, bisulfite conversion, purification, single-strand DNA concentration determination, and digital polymerase chain reaction (PCR) detection. The methylation rate was calculated. The diagnostic value was evaluated by the area under the curve (AUC).

***RESULTS***

The age and sex in the CRC and CRP groups and the HCC and LC groups were

also matched. The DNA methylation rate of RASSF1A in plasma in the CRC group was 2.87 ± 1.80, and that in the CRP group was 1.50 ± 0.64. DNA methylation of RASSF1A in plasma showed a significant difference between the CRC and CRP groups. The AUC of RASSF1A methylation for discriminating the CRC and CRP groups was 0.82 (0.76-0.88). The AUCs of T1, T2, T3 and T4 CRC and CRP were 0.83 (0.72-0.95), 0.87 (0.78-0.95), 0.86 (0.77-0.95), and 0.75 (0.64-0.85), respectively. The DNA methylation rate of RASSF1A in plasma in the HCC group was 4.45 ± 2.93, and that in the LC group was 2.46 ± 2.07. DNA methylation of RASSF1A in plasma for the HCC and LC groups showed a significant difference. The AUC of RASSF1A methylation for discriminating the HCC and LC groups was 0.70 (0.60-0.79). The AUCs of T1, T2, T3 and T4 HCC and LC were 0.80 (0.61, 1.00), 0.74 (0.59-0.88), 0.60 (0.42-0.79), and 0.68 (0.53-0.82), respectively.

***CONCLUSION***

RASSF1A methylation in plasma detected by digital PCR may be a potential biomarker for CRC and HCC.

**Key Words:** RASSF1A; Methylation; Digital polymerase chain reaction; Colorectal cancer; Hepatocellular carcinoma



**Core Tip:** Accurate quantitative polymerase chain reaction (qPCR) quantification relies on a standard curve and good amplification efficiency and is sensitive to factors affecting amplification efficiency. Digital PCR technology is an emerging PCR technology. In this study, we evaluated the diagnostic value of RASSF1A methylation in plasma by digital polymerase chain reaction.

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## INTRODUCTION

As a very important epigenetic modification, DNA methylation is closely related to the occurrence and development of tumors[1]. Most of the DNA methylation studies of cancer currently focus on tumor tissue; however, due to its invasive characteristics, it is difficult to use for cancer screening and early diagnosis. Noninvasive biological samples (such as blood) have the advantages of being minimally invasive or noninvasive, having a simple operation and being suitable for multiple collections. Blood DNA mainly includes plasma or serum DNA and blood cell DNA. It is generally believed that DNA in plasma or serum mainly comes from tumor cell necrosis or apoptosis[2-5]. The concentration of free DNA in normal human plasma is ng/mL, and the concentration of DNA in the plasma of benign and malignant lesions can be increased by 5-15 times. Cell-free DNA (cfDNA) in plasma can provide a new method for tumor diagnosis and prognosis[6].

The abnormal methylation changes of different cancers are specific, and the cfDNA of different stages of cancer is also different[7]. By detecting the level and methylation of cfDNA, tumor diagnosis and staging can be achieved. Studies have validated the potential of methylated cfDNA as biomarkers in various tumors, and genes such as DCLK1 were found in the plasma of lung cancer patients[8]. Abnormal hypermethylation occurred in cfDNA; SOX17 promoter hypermethylation was found in cfDNA of patients with early breast cancer, primary breast cancer, and metastatic breast cancer[9]; *SEPT9* gene promoter methylation in plasma cfDNA was found to have good sensitivity and specificity in the diagnosis of early colorectal cancer (CRC)[10-12]; quantitative methylation detection of the *NEUROG1* gene in serum has also been proven to be an early screening method for CRC[13]; and there are also studies that simultaneously detect multiple genetic loci of cfDNA to establish a combined methylation diagnostic model. At present, liquid biopsy technology to detect cfDNA methylation is gradually becoming a new type of cancer screening and diagnosis.

Currently, real-time quantitative PCR (qPCR) is the main quantitative detection technology for the detection of methylation in nucleic acid samples. However, it is a relatively quantitative technique[14]. Accurate qPCR quantification relies on a standard curve and good amplification efficiency and is sensitive to factors affecting amplification efficiency (such as method design and PCR inhibitors). Digital PCR (dPCR) technology is an emerging PCR technology. Compared with qPCR, dPCR does not require

a standard curve, can achieve absolute quantification, has higher sensitivity and specificity and is resistant to background sequences and reaction inhibitors. dPCR has obvious advantages in the detection of rare mutations and rare methylated alleles[15], its lower limit of detection and improved detection accuracy, and the absolute quantification of the nucleic acid to be detected[16].

In this study, using a dPCR detection method, we aimed to evaluate the diagnostic value of RASSF1A methylation in plasma for CRC and hepatocellular carcinoma (HCC).

## MATERIALS AND METHODS

### Study samples

After approval by the Ethics Committee of Chinese PLA General Hospital, the research subjects signed informed consent forms. CRC staging was performed according to the American Joint Committee on Cancer tumor node metastasis staging. All patients received no treatment when peripheral blood samples were taken, including surgical resection, radiotherapy, chemotherapy, and targeted therapy; all patients underwent colonoscopy and biopsy and were pathologically diagnosed with colorectal polyps (CRP), CRC, HCC, and liver cirrhosis (LC). All patients needed to undergo follow-up in the later period. If the tumor tissue was obtained by surgery for biopsy and the pathological result was inconsistent with the biopsy under endoscopy, the biopsy result of the tumor tissue would prevail. The healthy control samples were from the physical examination population in the same time period with healthy physical examination results and normal blood biochemical test results.

### DNA extraction

The samples in this study were peripheral blood collected on an empty stomach in the morning, and EDTA was an anticoagulant. The collected whole blood samples were directly aliquoted into 1.5 mL Eppendorf tubes in 200 μL. The whole blood was centrifuged at 1500 ×*g* for 10 min to obtain plasma samples. If hemolysis or lipid blood appeared, the samples were discarded. Finally, 1000 μL of plasma was dispensed into Eppendorf tubes for subsequent experiments. Extraction of plasma DNA Extract DNA from 1 mL of plasma samples was performed according to the QIAamp MinElute ccfDNA Mini Kit instructions, and finally 24 μL of ultra-purified water was added to elute the DNA.

### Double strand DNA concentration determination

The QubitTM double strand DNA (dsDNA) HS assay kit was removed from 4 °C and placed at room temperature for 30 min; 199 μL dsDNA buffer and 1 μL dsDNA reagent were added to specially matched QubitTM assay tubes and mixed by vortexing; 10 μL of the mixture in the two tubes corresponding to the standard were discarded with a pipette, and 1 μL of the mixture in the tube that aspirated the sample was discarded; 10 μL of dsDNA Standard #1 and dsDNA Standard #2 was pipetted into the corresponding tubes, 1 μL was pipetted from each sample to be tested and added to the corresponding tubes, and vortexing was used to mix them well; the Qubit 3.0 instrument was turned on, and the dsDNA high-sensitivity program was selected; the tubes containing standard 1 and standard 2 were inserted into the instrument in turn, the standard curve was drawn, and the samples were placed into the measurement concentration in turn.

### Bisulfite conversion

Twenty microliters of DNA samples were transformed according to the instructions of the EZ DNA Methylation-Gold Kit, and finally 22 μL of M-Elution Buffer was added to the column matrix to elute the DNA. When the PCR tube was placed in the thermal cycler, the cover temperature of the PCR instrument was set to 105 °C, and the program was changed to (1) 98 °C for 10 min; (2) 64°C with 20 min as a node to set the temperature gradient: 90 min, 110 min, 130 min, and 150 min; and (3) storing at 4 °C.

### Purification

The sample was then purified again according to the instructions of the Cycle-Pure Kit. After transformation, the DNA was purified again, and 7 μL of elution buffer was added for elution.

### Single-strand DNA concentration determination

The operation steps were the same as those in 2.6.3, except that the dsDNA assay kit reagents were correspondingly replaced with the reagents in the ssDNA assay kit. ssDNA was selected as the assay type to measure.

### dPCR detection

The dPCR reader QX200 Droplet Reader was turned on and warmed up for 30 min, and the computer and QuantaSoft software were turned on; a 20 μL probe-based quantitative reaction system ddPCR Supermix for Probes, 10 μL, was prepared with methylated upstream primer (10 μM), 1.6 μL; methylation downstream primer (10 μM), 1.6 μL; and methylation probe (10 μM), 0.5 μL. The DNA

**Table 1 General clinical characteristics of study subjects**

| | CRC | CRP | HC | LC |
|---|---|---|---|---|
| *n* | 92 | 67 | 63 | 66 |
| Age (yr) | | | | |
| mean | 57 | 55 | 55 | 53 |
| Range | 42-66 | 41-62 | 35-68 | 34-65 |
| Sex | | | | |
| Male | 51 | 38 | 32 | 29 |
| Female | 41 | 29 | 23 | 24 |
| TNM stage | | | | |
| T1 | 15 | | 9 | |
| T2 | 24 | | 18 | |
| T3 | 22 | | 14 | |
| T4 | 31 | | 22 | |

CRC: Colorectal cancer; CRP: Colorectal polyps; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; TNM: Tumor node metastasis.

volume was based on the concentration, and dd water was added according to the reaction system, totaling 20 μL. The RASSF1A methylation primer was synthesized according to previous research[17]. The above reaction system was shaken and mixed and centrifuged briefly to remove air bubbles. The droplet generating card was placed into the metal holder in the direction of the notch. Then, 20 μL of the sample reaction system was added to the 8 wells in the middle row of the droplet generating card, and 70 μL of the droplet generating oil was added to the 8 wells in the bottom row of the droplet generating card. The samples were added slowly to avoid generating air bubbles, as air bubbles in the system would seriously affect the generation of droplets. The disposable rubber pad was hooked to the small holes on both sides of the metal holder, and the droplet-generating card was added. The middle part of the metal holder was held and placed in the droplet generator stably, until droplets started to generate, and whether the droplet generation was completed was judged according to the status of the indicator light. The liquid in the top row of holes of the droplet generation card, generally 40 μL, was aspirated, transferred to the corresponding 96-well plate, and covered with tin foil to prevent the oil from volatilizing. When it was completely transferred, the side marked with the red line was placed on the tin foil film on the 96-well plate. After fixing, the samples were placed in a heat sealer to seal the film. The running program was as follows: 180 °C, 10 s; the sealed film was placed on the C1000 TouchTM Thermal Cycler, and the program was set (95 °C for 10 min, 1 cycle; 94 °C for 30 s and 56 °C for 1 min, 45 cycles; 98 °C for 10 min, 1 cycle; and holding at 12 °C). After amplification, the 96-well plate was placed into the corresponding holder, the button plate was pressed with both hands at the same time, and it was assembled and smoothly placed into the droplet reader. QuantaSoft software was opened, "Flush System" was selected to clean the system, the sample information in the reaction well was set, the program was run after completion, and the data were analyzed after reading.

### *Statistical analysis*

The number of droplets, copy number, concentration, and copy number ratio of the two channels in each reaction well were obtained in QuantaSoft software for analysis. The area under the curve (AUC) was used to evaluate the diagnostic value, and specificity and sensitivity were listed as the evaluation indicators.

## RESULTS

### *General clinical characteristics of the study subjects*

As shown in Table 1, 92 CRC patients, 67 CRP patients, 63 HCC patients, and 66 LC patients in the training group were enrolled. The age and sex in the CRC and CRP groups of the training and validation groups were matched, and the age and sex in the HCC and LC groups were also matched. The CRC at T1, T2, T3 and T4 were 15, 24, 22, and 31, and the HCC at T1, T2, T3, and T4 were 9, 18, 14, and 22.

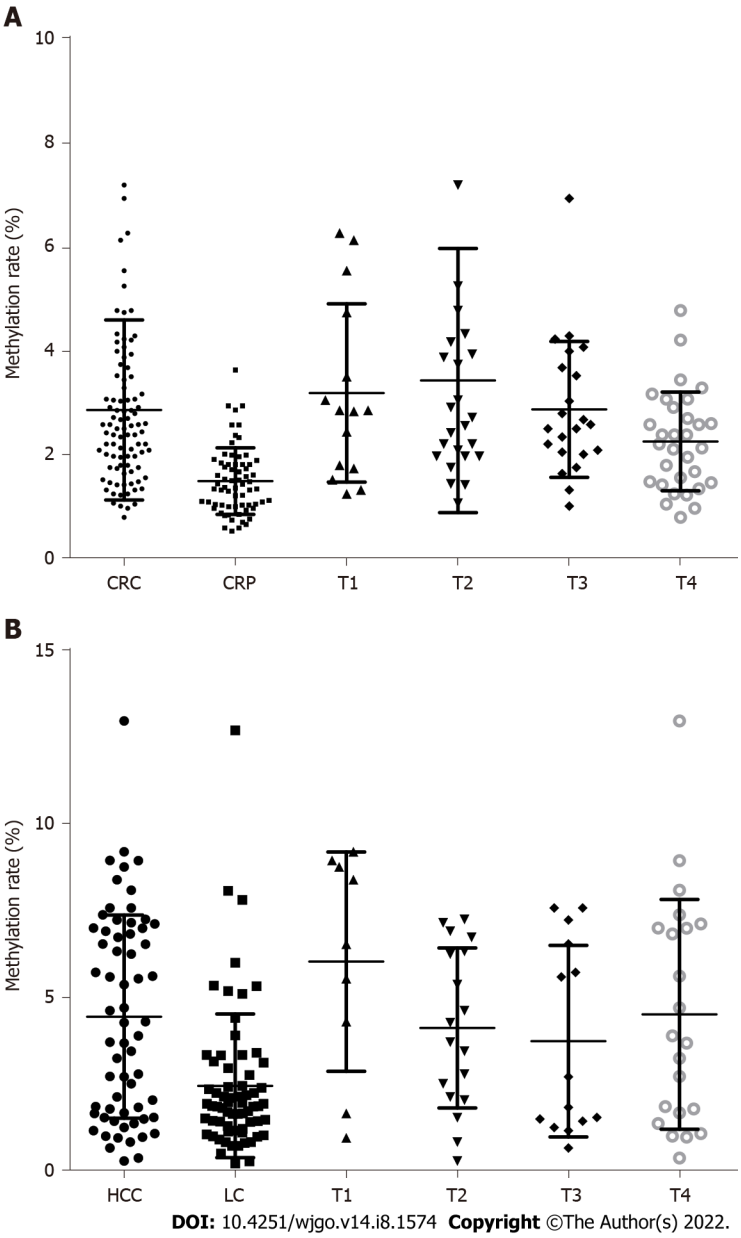


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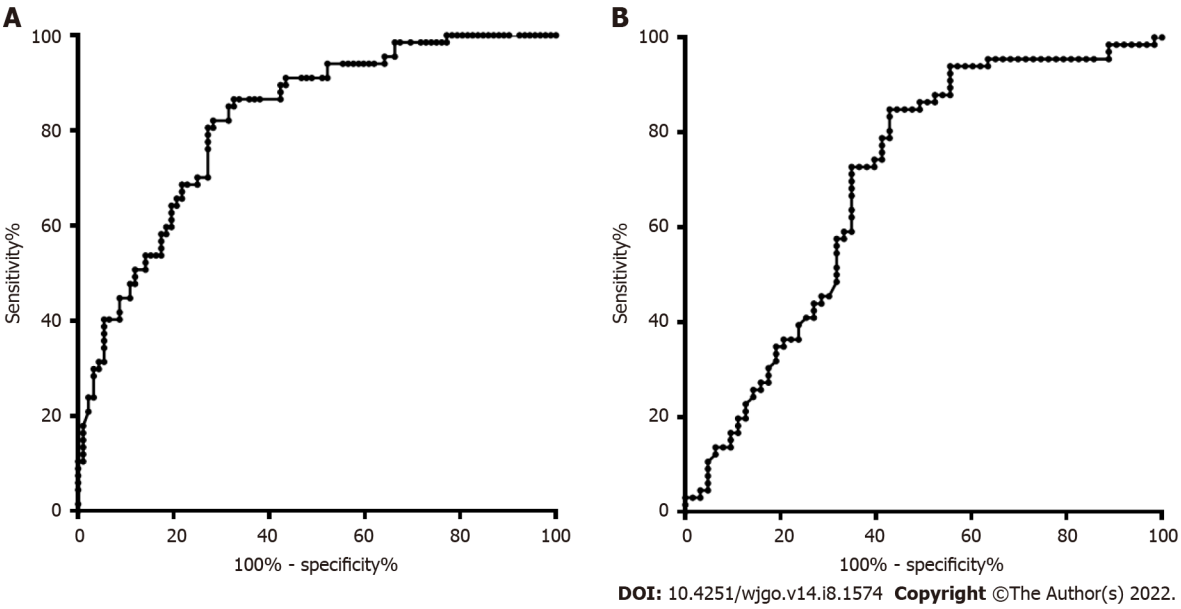
**Figure 1 RASSF1A methylation rate in plasma.** A: RASSF1A methylation rate in plasma of colorectal cancer (CRC), colorectal polyps and T1-T4 stage of CRC; B: RASSF1A methylation rate in plasma of hepatocellular carcinoma (HCC), liver cirrhosis and T1-T4 stage of HCC. CRC: Colorectal cancer; CRP: Colorectal polyps; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis.

### *Comparison of DNA methylation of RASSF1A in plasma for the CRC and CRP groups*

The DNA methylation rate of RASSF1A in plasma in the CRC group was 2.87 ± 1.80, and that in the CRP group was 1.50 ± 0.64 (Figure 1A). DNA methylation of RASSF1A in plasma showed a significant difference between the CRC and CRP groups. The methylation rates of RASSF1A in plasma at T1, T2, T3 and T4 in CRC were 3.20 ± 1.71, 3.45 ± 2.54, 2.88 ± 1.31, and 2.27 ± 0.95, respectively. When CRC at T1, T2, T3 and T4 were compared with the CRP group, all four stages showed significant differences. The AUC of RASSF1A methylation for discriminating the CRC and CRP groups was 0.82 (0.76-0.88) (Figure 2A). The AUCs of T1, T2, T3 and T4 CRC and CRP were 0.83 (0.72-0.95), 0.87 (0.78-0.95), 0.86 (0.77-0.95), and 0.75 (0.64-0.85), respectively (Figure 3A-D).

### *Comparison of DNA methylation of RASSF1A in plasma for the HCC and LC groups*

The DNA methylation rate of RASSF1A in plasma was 4.45 ± 2.93 in the HCC group and 2.46 ± 2.07 in the LC group (Figure 1B). DNA methylation of RASSF1A in plasma for the HCC and LC groups showed a significant difference. The methylation rates of RASSF1A in plasma at T1, T2, T3 and T4 in HCCs were 6.04 ± 3.16, 4.13 ± 2.31, 3.75 ± 2.76, and 4.52 ± 3.31, respectively. When T1, T2, T3 and T4 in the HCC group were compared with those in the LC group, T1, T2 and T4 showed significant differences. T3 showed no significant difference ($P$ = 0.061). The AUC of RASSF1A methylation for discriminating the HCC and LC groups was 0.70 (0.60-0.79) (Figure 2B). The AUCs of T1, T2, T3 and T4 HCC and LC were




**DOI:** 10.4251/wjgo.v14.i8.1574 

**Figure 2 Diagnostic value evaluation of RASSF1A methylation rate in plasma for discriminating colorectal cancer, colorectal polyps, hepatocellular carcinoma, and liver cirrhosis.** A: Colorectal cancer and colorectal polyps; B: Hepatocellular carcinoma and liver cirrhosis.

0.80 (0.61-1.00), 0.74 (0.59-0.88), 0.60 (0.42-0.79), and 0.68 (0.53-0.82), respectively (Figure 4A-D).

## DISCUSSION

By detecting the methylation level of the PCDH10 promoter region in the tissue and plasma of colorectal cancer patients, the PCDH10 methylation level in the tissue of early colorectal cancer patients was found to be highly correlated with the DNA methylation level in the plasma, suggesting that the PCDH10 promoter in the plasma is highly correlated[18]. The level of regional methylation can be used as a biomarker for the early diagnosis of colorectal cancer. With the advancement of technology, other specific gene methylation levels in plasma, such as *RASSF2*, *sFPR1*, *SDC2* and other gene promoter methylation, have been confirmed for use as biomarkers for the early diagnosis of colorectal cancer[19-21]. In addition, the determination of Septin 9 methylation in plasma is considered a sensitive and specific biomarker for the early diagnosis of colorectal cancer; however, when it is used for screening in general risk populations of colorectal cancer, it can only be detected. It produces approximately 50% of asymptomatic colorectal cancer patients, with a specificity comparable with the fecal occult blood test. Studies have confirmed that changes in methylation sites in plasma can be used as biomarkers for the early diagnosis of colorectal cancer. However, the methylation sites in plasma for the early diagnosis of colorectal cancer are poorly studied. RASSF1A was the most widely investigated gene in serum or plasma, and it was also demonstrated to be more frequently methylated in cancer patients. Global hypomethylation of RASSF1A was related to increased breast cancer risk[22]. RASSF1A methylation is an attractive biomarker for early cancer detection, which, for most cancers, results in improved clinical outcome. RASSF1A methylation may be used as a diagnostic and prognostic marker in cancer management[23]. RASSF1A hypermethylation is a promising biomarker for the diagnosis of HCC in tissue and blood and is an emerging biomarker for HCC[24-26]. In addition, RASSF1A hypermethylation is an early and potential prognostic biomarker in CRC[21,27,28].

There are many detection methods for DNA methylation sites, mainly including the following methods: (1) Methylation-specific PCR: The basic principle is that after bisulfite treatment, two pairs of primers are designed: one pair amplifies the bisulfite-treated DNA template, and the other pair amplifies the unmethylated fragment. Then, according to whether it can be amplified, it is judged whether methylation has occurred. The disadvantage is that the sequence of the gene to be tested needs to be known in advance, and primers with relatively high specificity are designed[29]; (2) The bisulfite sequencing method: The basic principle is that after bisulfite treatment, PCR amplification is performed, the amplified product is sequenced, and methylation is determined by comparison with the untreated sequence. The disadvantage is that it needs to undergo much cloning, and the process is cumbersome and expensive[30]; (3) Restriction endonuclease analysis method: The basic principle is that after bisulfite treatment and PCR amplification, the amplification product is purified and then digested with restriction enzymes. Then, according to whether it can be cut, it is judged whether methylation has occurred. Its disadvantage is that it can only obtain the methylation status of special enzyme cleavage

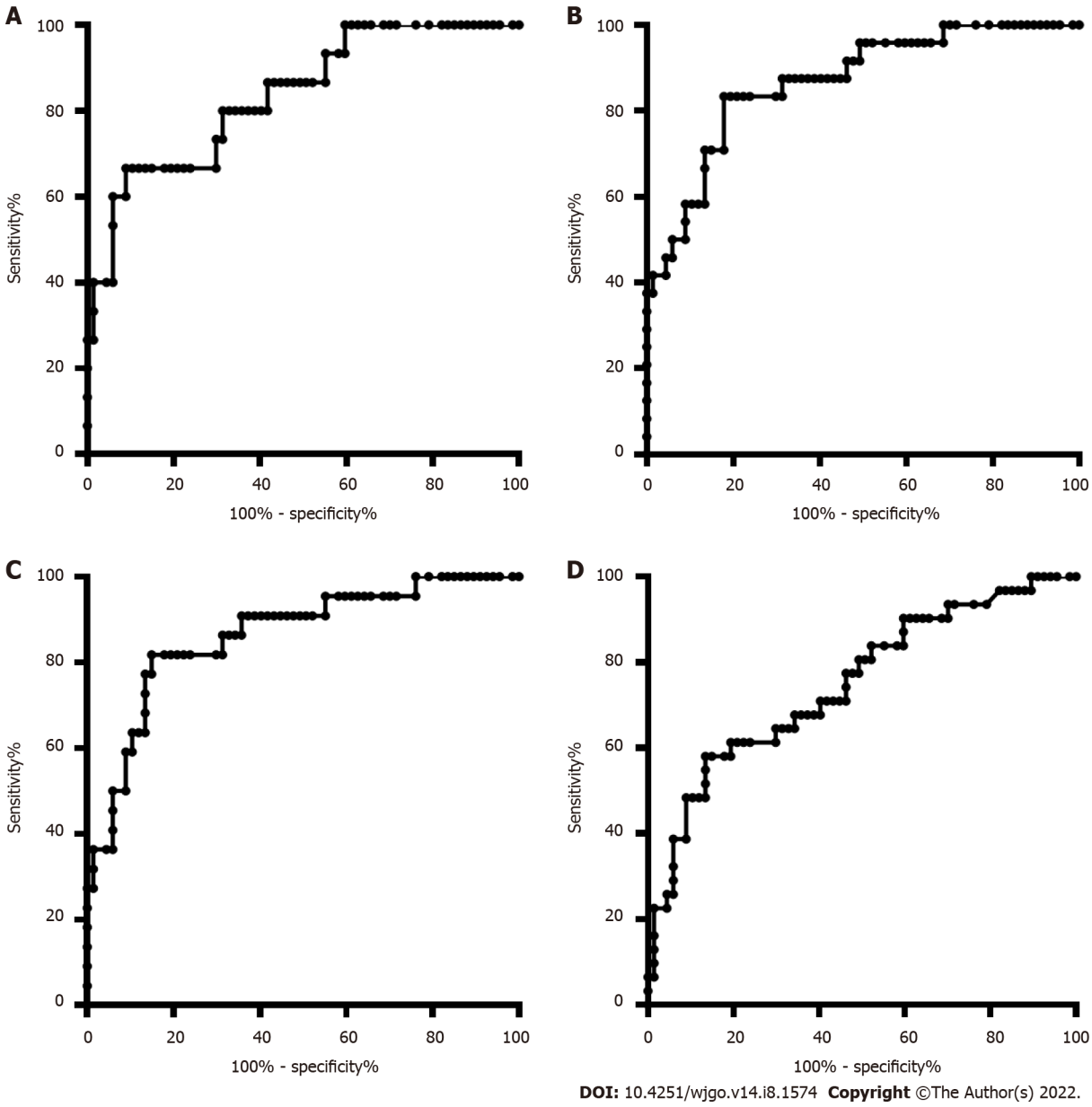



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**Figure 3 Diagnostic value evaluation of RASSF1A methylation rate in plasma for discriminating T1-T4 stage colorectal cancer and colorectal polyps.** A: T1 stage colorectal cancer (CRC) and colorectal polyps (CRP); B: T2 stage CRC and CRP; C: T3 stage CRC and CRP; D: T4 stage CRC and CRP.

sites[31]; (4) Methylation-sensitive high-resolution melting curve analysis: The basic principle is that after bisulfite treatment, the difference between methylated sites and unmethylated DNA can be found by melting curve analysis due to the presence of more GCs. The disadvantage is that it is greatly affected by primer design and cannot achieve quantitative detection[32]; (5) Pyrosequencing: The basic principle is that after bisulfite treatment, by accurately quantifying the methylation frequency on a single continuous site, the methylation frequency can be quickly detected, and the methylation sites in the sample can be qualitatively and quantitatively detected. The disadvantage is that there are many steps, so it is difficult to use as a conventional methylation detection method and more often used as a verification method of methylation sites[33]; (6) Sequenom MassArray platform: The basic principle is that after bisulfite treatment, primers are designed for PCR amplification, and the product is subjected to a single-base extension reaction after outpatient substance abuse program treatment. Flight mass spectrometry can detect the molecular weight difference between methylated and unmethylated sites to be detected. The disadvantage is that the experimental operation requirements are high, its detection sensitivity is low, and it is difficult to achieve quantitative detection of methylation sites[34]; and (7) Fluorescence quantitative method: The basic principle is to use TaqMan probes and PCR primers to distinguish methylated and unmethylated DNA after bisulfite treatment. As a highly sensitive relative quantitative detection method for DNA methylation, it is widely used. The disadvantage is that it is difficult to achieve absolute quantitative detection of methylated sites[35]. In addition to the

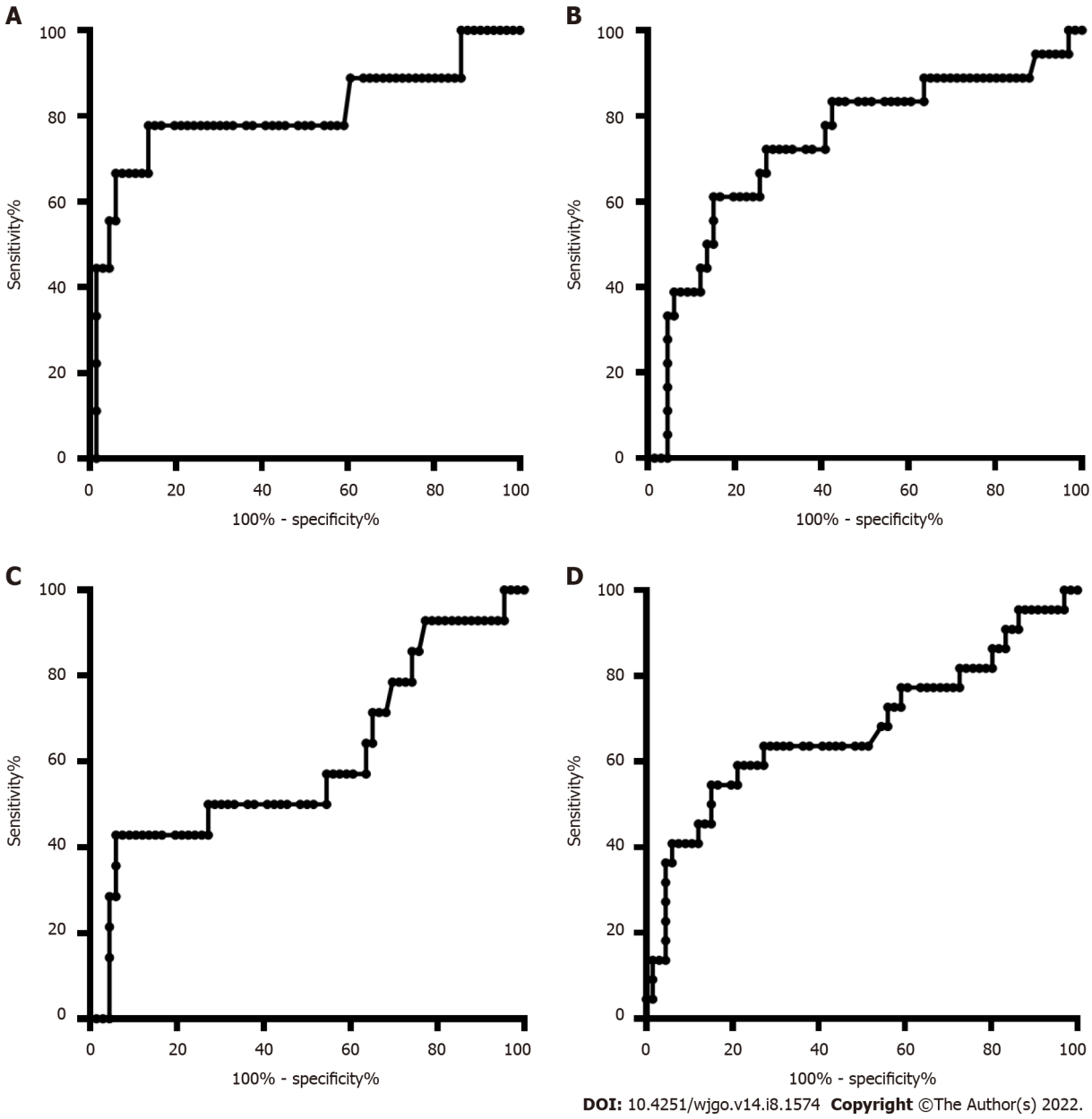


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**Figure 4 Diagnostic value evaluation of RASSF1A methylation rate in plasma for discriminating T1-T4 stage hepatocellular carcinoma and liver cirrhosis.** A: T1 stage hepatocellular carcinoma (HCC) and liver cirrhosis (LC); B: T2 stage HCC and LC; C: T3 stage HCC and LC; D: T4 stage HCC and LC.

fluorescence quantitative method, the above methylation detection methods have difficulty achieving high-sensitivity detection for trace DNA, the detection result of the fluorescence quantitative method is relatively quantitative, and it is difficult to achieve high-precision and absolute quantitative detection of plasma DNA methylation.

dPCR technology evenly distributes the PCR system into tens of thousands of reaction units. Each reaction unit does not contain or only contains one nucleic acid sequence to be tested. After the number of nucleic acids to be tested conformed to the Poisson distribution, PCR amplification was independently performed in each reaction unit. Finally, the fluorescence signal of each reaction unit was detected, and the copy number of the nucleic acid sequence to be tested was calculated according to the Poisson distribution and the proportion of reaction units with positive fluorescence signals to all reaction units. Compared with other methylation detection methods, ddPCR has the following advantages: (1) High sensitivity: ddPCR turns PCRs into tens of thousands of PCRs that independently detect nucleic acids. Compared with traditional detection methods, the detection sensitivity is greatly improved; (2) High accuracy: ddPCR can accurately detect small changes in the nucleic acid to be detected by calculating the number and proportion of positive reaction units in tens of thousands or even tens of millions of reaction units; (3) High tolerance; and (4) absolute quantification: ddPCR technology can achieve the absolute quantitative detection of the nucleic acid to be detected without relying on the Ct value and the standard curve.

There are some limitations in our study. First, the digital PCR methylation of RASSF1A was not compared with the conventional real-time PCR method. Second, the healthy control group was not detected in our study, and the methylation rate of RASSF1A was not evaluated. Third, the sample size was small, and the results may be affected.

## CONCLUSION

In conclusion, we demonstrate that RASSF1A methylation in plasma detected by digital PCR may be a potential biomarker for CRC and HCC.

## ARTICLE HIGHLIGHTS

### *Research background*

DNA methylation in serum or plasma was demonstrated to be a potential biomarker for cancer detection and prognosis.

### *Research motivation*

More sensitive and accurate methods for detecting methylation in plasma are urgently needed in clinical practice.

### *Research objectives*

In this study, we aimed to evaluate RASSF1A methylation in plasma by digital polymerase chain reaction (PCR) for colorectal cancer (CRC) and hepatocellular carcinoma (HCC).

### *Research methods*

A total of 92 CRC patients, 67 colorectal polyp (CRP) patients, 63 HCC patients, and 66 liver cirrhosis (LC) patients were enrolled. The plasma DNA was detected by digital PCR. The diagnostic value was evaluated by the area under the curve (AUC).

### *Research results*

The DNA methylation rate of RASSF1A in plasma in the CRC group was 2.87 ± 1.80, and that in the CRP group was 1.50 ± 0.64. The AUC of RASSF1A methylation for discriminating the CRC and CRP groups was 0.82 (0.76-0.88). The AUCs of T1, T2, T3 and T4 CRC and CRP were 0.83 (0.72-0.95), 0.87 (0.78-0.95), 0.86 (0.77-0.95), and 0.75 (0.64-0.85), respectively. The DNA methylation rate of RASSF1A in plasma in the HCC group was 4.45 ± 2.93, and that in the LC group was 2.46 ± 2.07. The AUC of RASSF1A methylation for discriminating the HCC and LC groups was 0.70 (0.60-0.79). The AUCs of T1, T2, T3 and T4 HCC and LC were 0.80 (0.61-1.00), 0.74 (0.59-0.88), 0.60 (0.42-0.79), and 0.68 (0.53-0.82), respectively.

### *Research conclusions*

We demonstrate that RASSF1A methylation in plasma detected by digital PCR may be a potential biomarker for CRC and HCC.

### *Research perspectives*

Different methylation detection methods should be compared, and more samples need to be detected.

## FOOTNOTES

**Author contributions:** Li J and Li Z designed the study; Li J and Li H contributed equally to this study; An Y and Li Z are the co-corresponding authors; Run ZC, Wang ZL, Jiang T, and An Y collected the samples; Li J, Li H, An Y performed the research; Li J, Li H and Li Z analyzed the date; Li J wrote the paper; Li J and Li Z revised the manuscript for final submission.

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**Data sharing statement:** No additional data are available.

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CASE REPORT

# Ewing sarcoma of the ileum with wide multiorgan metastases: A case report and review of literature

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## Abstract

*BACKGROUND*

Ewing sarcoma (ES) is an aggressive small round cell tumor that usually occurs in younger children and young adults but rarely in older patients. Its occurrence in elderly individuals is rare. ES of the ileum with wide multiorgan metastases is rarely reported and difficult to distinguish radiologically from other gastrointestinal tract tumors.

*CASE SUMMARY*

A 53-year-old man presented with right lower quadrant pain for 2 wk. Computed tomography results showed a heterogeneous mass within the ileum and widespread multiorgan metastases. This mass was biopsied, and pathological examination of the resected specimen revealed features consistent with an extraskeletal ES.

*CONCLUSION*

This case emphasizes the importance of recognizing this rare presentation in the small intestine to broaden the differential diagnosis of adult intraabdominal tumors.

**Key Words:** Ewing sarcoma; Intestinal neoplasms; Neoplasm metastasis; Oncology; Carcinoma; Case report



**Core Tip:** Ewing's sarcoma (EOES) originating in the ileum with wide multiorgan metastases is rare and easily misdiagnosed. When a small intestine mass accompanied by calcification and wide multiorgan metastases is seen on computed tomography, a suspicion of EOES should not be overlooked. Together with previous reports, this case expands knowledge regarding the spectrum of tumors in the small intestine.

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## INTRODUCTION

Ewing sarcoma (ES) of the bone represents the second most common primary malignant tumor of bone in children and adolescents, exceeded in prevalence only by osteosarcoma[1]. Osseous ES, together with extraosseous Ewing's sarcoma (EOES), primitive neuroectodermal tumor, and Askin's tumor are members of the Ewing sarcoma family of tumors[1,2]. The treatment of EOES patients includes chemotherapy, radiation therapy, and surgery. To date, the 5-year survival rate of EOES is relatively high (65%-75%)[3]. The outcome for metastatic patients is usually poor (< 30%), despite the use of surgery, chemo- and/or radiotherapy. EOES is rarer than ES of the bone. The prevalence of EOES is generally accepted to be between 15% and 20% of that of ES of the bone[2]. The most common sites of EOES are the paravertebral region, lower extremities, chest wall and retroperitoneum[4]. To our knowledge, EOES originating in the ileum is not common, with only nearly 30 cases reported worldwide. However, there were few reports regarding EOES of the ileum with multiorgan metastases at the time of diagnosis[5-7]. In this paper, we present a case with an initial diagnosis of gastrointestinal stromal tumor (GIST), but histopathology indicated EOES with widespread multiorgan metastases.

## CASE PRESENTATION

### *Chief complaints*

A 53-year-old man suffered from right lower quadrant abdominal pain for 2 wk.

### *History of present illness*

The patient experienced right lower quadrant abdominal pain for 2 wk, accompanied by acid reflux, belching, and emesis (an oral discharge without digested food and hematemesis), but denied having fevers, night sweats, unintentional weight loss, and blood in the stool.

### *History of past illness*

The patient had a medical history free of previous diseases.

### *Personal and family history*

The patient denied that the family had any genetic diseases. There was no similar disease in the family.

### *Physical examination*

On physical examination, the patient's abdomen was soft with tenderness on the right side abdominal without rebound tenderness or muscle guarding, and normal bowel sounds were present. In palpation, a mass with unclear boundary was identified in the right lower abdomen, measuring 4 cm × 6 cm approximately, and the mass can be mobile.

### *Laboratory examinations*

After admission, laboratory investigations showed slightly increased levels of monocytes (0.987 × $10^9$/L; normal range: 0.10–0.60 × $10^9$/L), decreased eosinophil rate (0.1%; normal range: 0.4%-8%), decreased hemoglobin levels (119 g/L; normal range: 130-175 g/L), and prealbumin levels (14.9 mg/dL; normal

range: 16-45 mg/dL), and increased platelet count (418 × $10^9$/L; normal range: 85-303 × $10^9$/L). All serum tumor marker levels were normal.

### *Imaging examinations*

Contrast-enhanced computed tomography (CT) of the abdomen showed an 8.1 cm × 4.0 cm mass in the right iliac fossa area, which interacted with the small intestinal lumen. The mass was heterogenetic, and areas of low attenuation and high attenuation were observed, likely corresponding to areas of necrosis and calcification (Figure 1). In addition, multiple metastatic lesions were observed on the bilateral adrenal gland, lung, liver and pancreas, and several enlarged lymph nodes were seen in the retroperitoneal and mediastinum areas, with the largest exhibiting a diameter of 2.3 cm (Figure 2).

## MULTIDISCIPLINARY EXPERT CONSULTATION

From the contrast-enhanced CT of the abdomen and chest, multidisciplinary consultation determined that malignant GISTs with widespread multiorgan metastases were first considered. The patient could not receive surgical treatment because of widespread multiorgan metastases. Therefore, adjuvant chemotherapy was recommended.

## FINAL DIAGNOSIS

To make the diagnosis, transabdominal ultrasound guided needle biopsy was performed with the consent of the patient. The biopsy was performed as an outpatient procedure under local anesthesia. Histopathology of the small intestinal tumor is composed of heteromorphic cells, and distributed in the shape of a sheet nest with round or oval cells and visible nucleoli (Figure 3). Tumor cells showed positive immunoreactivity for CD99, NKX2.2, S100, Syn and Ki-67, and the Ki-67 Level was greater than 60%. The results were negative for CK, CgA, CK5/6, P63, CK7, CAM5.2, CK20, CD56, CD117 and alpha-inhibin (Figure 4). Therefore, the histopathologic findings were consistent with EOES. Although without molecular biological examination, after multidisciplinary consultation, the clinician concluded the diagnosis was primary small intestinal ES because of the positive immunoreactivity for NKX2.2, FLI-1 and CD99 combined with morphological characteristics.

## TREATMENT

After multidisciplinary consultation, the physicians recommended 5 cycles of neoadjuvant chemotherapy with vincristine, ifosfamide, and doxorubicin for the patient, which could reduce the size of the primary tumor and metastases. However, the patient refused this treatment strategy. The patient was given fluid rehydration (0.9% sodium chloride solution, 5% glucose sodium chloride injection), nutritional support (Compound amino acid injection, 20% medium and long chain fat emulsion injection and ω-3 fish oil fat emulsion injection) and intravenous injection of parecoxib sodium 40 mg to relieve the pain.

## OUTCOME AND FOLLOW-UP

One month later, the patient could not eat and received symptomatic nutritional support (Compound amino acid injection, 20% medium and long chain fat emulsion injection and ω-3 fish oil fat emulsion injection) and analgesic treatment (intravenous injection of parecoxib sodium 40 mg). Despite the medical advice, the patient refused to receive any systemic treatment. The patient chose to be transferred to hospice care ward and died of multiple organ failure caused by widespread multiorgan metastases 2-mo later.

## DISCUSSION

ES most commonly arises from bone but can develop in extraskeletal sites. In contrast, half or more of primary adult cases are EOES[4]. ES exhibits the highest incidence in older adolescents, with patients aged over 40 years experiencing extraskeletal tumors, metastatic spread at the time of diagnosis, and shorter survival than younger patients. It shows aggressive clinical behavior with a high rate of local recurrence and distant metastasis[8]. Approximately 15%-46% of patients will demonstrate metastatic disease at presentation, reducing 5-year survival from approximately 35%-71% to a dismal 0-34%[9].

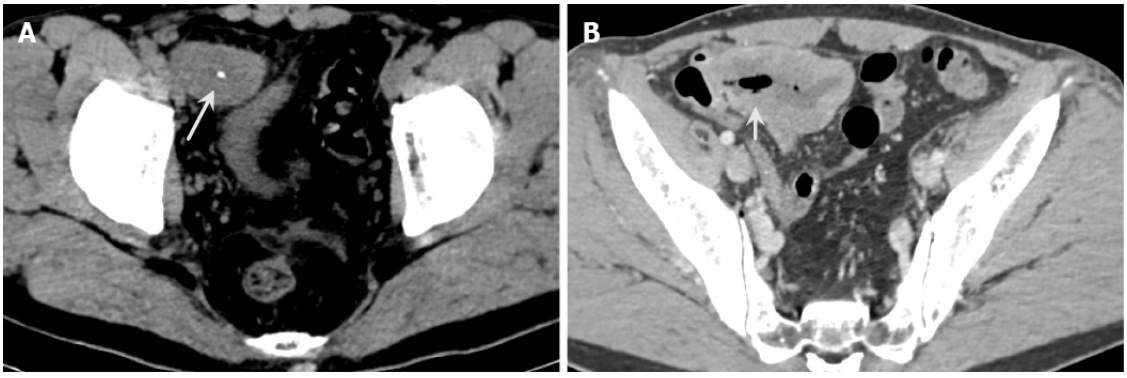


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**Figure 1 Abdominal computed tomography.** A: Axial computed tomography (CT) image shows a heterogenetic mass with calcification (white arrows); B: Contrast-enhanced CT shows mild heterogenetic enhancement and communication with the small intestinal lumen (short white arrows).

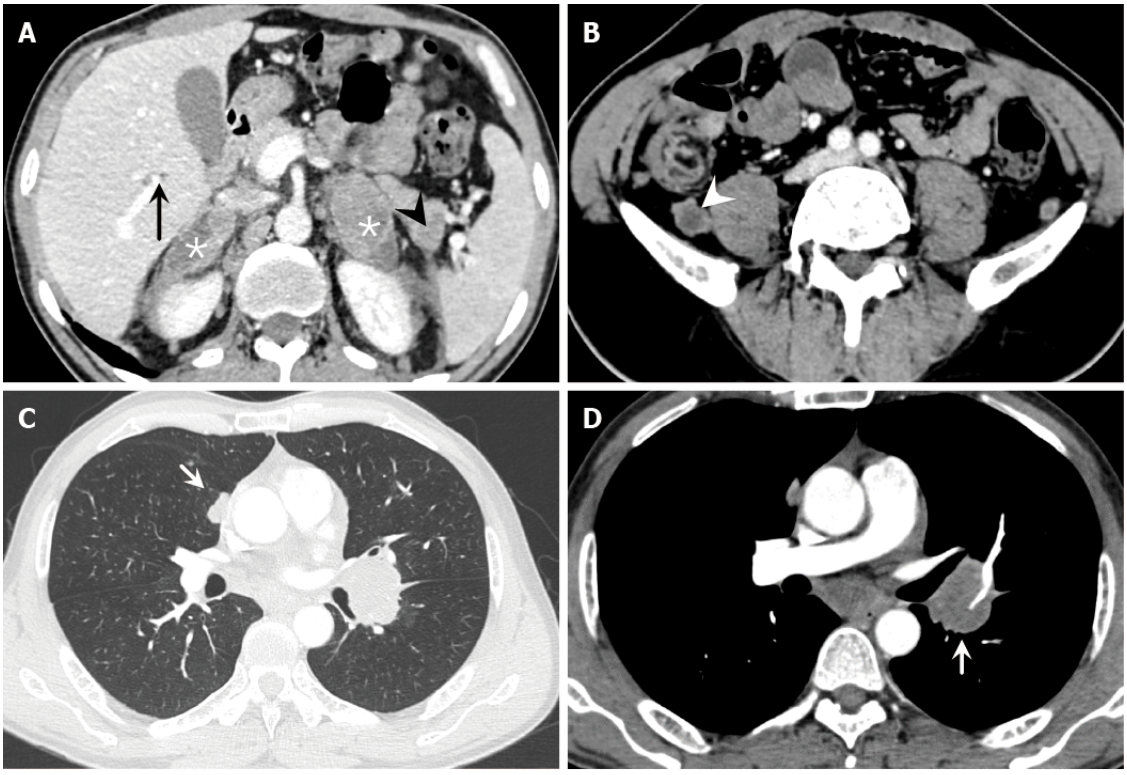


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**Figure 2 Abdominal and chest computed tomography.** A: Multiple metastatic lesions are observed on the bilateral adrenal gland (*), liver (black arrow) and pancreas (black arrowhead); B: Several enlarged lymph nodes (white arrowhead) are seen on the retroperitoneal area; C: A pulmonary metastatic nodule (short white arrow) is seen in lung windows; D: Several enlarged lymph nodes (short white arrow) are shown on the mediastinum area in contrast-enhanced computed tomography.

We have summarized all previous publications of small intestinal ES/PNET in Table 1[10-26]. The patient gender ratio (female/male) was 12/15. The ages ranged from 9 to 69 years, and 60% of patients with small intestinal ES were younger than 30 years. The most common sites in patients with metastatic disease are the liver and peritoneum. Adrenal metastases have rarely been described[7]. Seven patients had metastases to the liver and peritoneum solitarily. Only one patient had metastases to the adrenal gland and peritoneum at the time of diagnosis. Patients of more than 40 years of age or with metastatic spread at the time of diagnosis have shorter survival than younger patients. The form of distant metastasis included seeding, blood and lymphatic vessel metastasis. The mechanism of distant metastasis from the ES in the ileum to other organs could be explained for two reasons. First, hematogenous metastasis may occur because the tumor cells penetrate and spread from the vessels in the ileum. Second, there are abundant lymphatic networks in the submucosal layer of the ileum, and the lymphatics intermittently pierce the muscularis propria and drain into regional lymph nodes in the peritoneum. The tumor cells penetrate and spread from lymphatics to regional lymph nodes or even

**Table 1 Reported cases of Ewing Sarcoma of small bowel**

| Site | Age | Sex | Metastasis at diagnosis | Treatments | Follow-up | Ref. |
|---|---|---|---|---|---|---|
| Small intestine | 21 | F | - | Sx + Cx | 10 mo DFS | Adair *et al* [10], 2001 |
| Jejunum | 13 | M | - | Sx | 1 yr DFS | Sarangarajan *et al*[11], 2001 |
| Distalileum | 14 | M | - | Sx + Cx | 10 mo DFS | Graham *et al*[12], 2002 |
| Small intestine | 9 | F | - | Sx + Cx | Died 25 mo after diagnosis | Shek *et al*[13], 2001 |
| Terminal Ileum andJejunum | 63 | M | Adrenal glands + lymph nodes | Sx + Cx | ND | Kim *et al*[7], 2007 |
| Terminal Ileum | 44 | M | Intra-peritoneal | Sx + Cx | Died 13 mo after diagnosis | Sethi and Smith[14], 2007 |
| Ileum | 32 | M | - | Sx + Cx | 6 mo DFS | Rodarte-Shade *et al*[15], 2012 |
| Terminal Ileum | 15 | F | - | Sx + Cx | ND | Vignali *et al*[16], 2012 |
| Ileum | 18 | M | - | Sx + Cx | ND | Boehm *et al*[6], 2003 |
| Ileum | 18 | M | Liver | Sx | Died 8 mo after diagnosis | Milione *et al*[4], 2014 |
| Ileum | 20 | M | Liver | Sx + Cx | Died 28 mo after diagnosis | Milione *et al*[4], 2014 |
| Ileum | 42 | M | - | Sx + Cx | Died 11 16 mo after diagnosis | Milione *et al*[4], 2014 |
| Ileum | 45 | M | - | Sx + Cx | Died 13 mo after diagnosis | Milione *et al*[4], 2014 |
| Ileum | 15 | F | - | Sx + Cx + Rx | 28 mo DFS | Milione *et al*[4], 2014 |
| Ileum | 57 | M | - | Lost | Lost | Milione *et al*[4], 2014 |
| Ileum | 28 | F | Liver | Sx + Cx | 204 mo DFS | Milione *et al*[4], 2014 |
| ileum | 16 | F | - | Sx | 6 mo DFS | Li *et al*[17], 2017 |
| Ileum | 69 | M | Intra-peritoneal | Sx | Died 8 mo after diagnosis | Yang *et al*[18], 2021 |
| Terminal Ileum | 57 | F | - | Sx + Cx | 8 mo DFS | Bala *et al*[19], 2006 |
| Small intestine | 66 | M | - | Sx + Cx | 48 mo DFS | Batziou *et al*[20], 2006 |
| Ileum | 22 | M | Liver | Sx | NA | Peng *et al*[21], 2015 |
| Jejunum | 9 | F | Peritoneum | Sx + Cx | NA | Kim *et al*[7], 2017 |
| Jejunum | 67 | F | - | Sx | 3 mo DFS | Cantu *et al*[22], 2019 |
| Jejunum | 42 | M | - | Sx + Cx | 9 mo DFS | Yagnik *et al*[23], 2019 |
| Jejunum | 30 | F | - | Sx | 2 mo DFS | Kolosov *et al*[24], 2020 |
| Ileum | 17 | F | - | Sx | NA | Paricio *et al*[25], 2021 |
| Duodenum | 25 | F | - | Sx | Died 1 mo after diagnosis | Hassan *et al*[26], 2022 |

F: Female; M: Male; Sx: Surgery; Cx: Chemotherapy; Rx: Radiotherapy; DFS: Disease free survival; NA: Not available.

distal lymph nodes.

The most frequently presenting symptom is a rapidly growing mass with local pain. However, the accompanying symptoms depend largely on the sarcoma site[27]. Our patient complained of right lower quadrant pain accompanied by acid reflux, belching, and emesis. CT showed a large, sharply delineated mass of relatively lower or equal density to that of the adjacent muscle. After enhancement, the mass showed heterogenetic enhancement with intratumor necrosis and calcification. Calcification is seen in 25%-30% of previous cases[1]. This patient had metastases of the bilateral adrenal gland, liver, pancreas and lung and multiple regional lymph node metastases. These findings represent necrotic changes common in both EOES and its metastases, which reflect the disease's aggressive nature[2].

For differentiation of Ewing sarcoma from the other small round cell tumors, molecular detection of specific fusion genes is recommended, which accepted as the gold standard method for diagnosing Ewing sarcoma[17]. However, this patient did not do this due to a small tissue sample size. Immunohistochemistry has emerged as a compelling alternative. NKX2.2, CD99 and FLI-1 are good immunohistochemical markers for ES. NKX2.2 was shown to be a valuable immunohistochemical marker for ES in the differential diagnosis of small round cell tumors, which has been identified as an important target of EWS-FLI-1[28,29]. A few number of non-Ewing tumors can be positive for NKX2.2, such as synovial sarcomas, mesenchymal chondrosarcomas, and malignant melanomas. Nuclear spindling and TLE1 immunoreactivity favor synovial sarcomas[30]. NKX2.2-positive synovial sarcoma exhibited weak focal

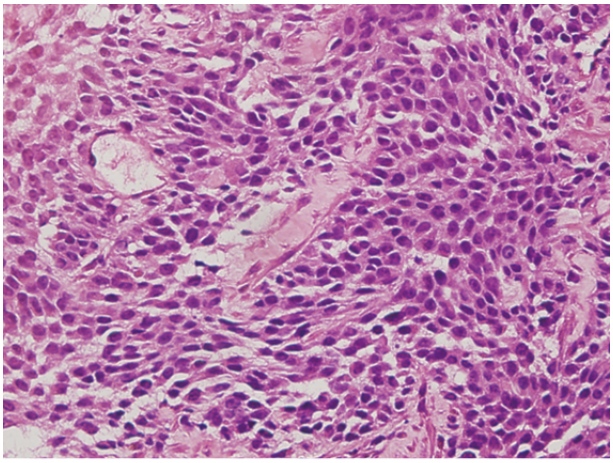

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**Figure 3 Pathologic findings.** Histopathology of the small intestinal tumor is composed of heteromorphic cells, and distributed in the shape of sheet or nest with round or oval cells and visible nucleoli. (Original magnification 400 ×; hematoxylin-eosin stains).

staining compared to diffuse labeling of ES. Mesenchymal chondrosarcomas could be excluded based on histology and immunohistochemical data[31]. Malignant melanomas could be excluded because the tumor did not express specific melanoma markers (*e.g.*, HMB45 and Melan A)[32]. According to the exclusive diagnosis, the present case was ultimately diagnosed as synchronous ES.

The imaging characteristics of the small intestinal ES are nonspecific as well. The major differential diagnosis for small intestinal ES includes GIST, lymphoma, adenocarcinoma, neuroendocrine neoplasm and metastatic lesions. GISTs are the most common mesenchymal tumors in the gastrointestinal tract and typically present as submucosal tumors of the gastrointestinal wall, occasionally accompanied by mucosal ulcers and tumor rupture[33]. GISTs occurring in the small intestinal characteristically have hemorrhage, necrosis, or cyst formation that appears as focal areas of low attenuation on computed tomographic images, and may present with cavity and fistula formation[34]. Moreover, GISTs rarely exhibit regional lymph node metastasis, unlike the mass presenting with multiple regional lymph node metastases in our patient. Intestinal lymphoma classically presents with a thickened wall and paradoxical dilatation but no obstruction, potentially with lymphadenopathy, splenomegaly[35]. And it often shows mild enhancement and the presence of vessel floating signs. In addition, lymphoma rarely presents with multiorgan metastases[36]. Intestinal adenocarcinoma typically shows irregular or annular thickening of the intestinal wall resulting in luminal narrowing, which may result in intestinal obstruction. Small intestinal neuroendocrine neoplasms may have mural transgression with the invasion of the serosa and mesentery and may conglomerate into spiculated masses with frequent calcification and surrounding lymphadenopathy[37,38]. Tumor metastasis to the small intestine is extremely rare, and few reports indicate in the literature[39].

Neoadjuvant chemotherapy was initially used to eliminate micrometastases and reduce the size of the primary tumor[40]. ES is quite radiosensitive, and some researchers have emphasized the important role of preoperative radiotherapy for successful local treatment in spinal ES[41]. However, improvements in surgical technique and the risks associated with radiation (secondary malignancies) have reduced the reliance on radiation[42]. Surgery alone does not appear to be effective for metastatic ES due to technical difficulties related to surgery and a low survival rate. This case will contribute to understanding the prognosis and determination of optimal management because small bowel ES is extremely rare and difficult to cure.

## CONCLUSION

In conclusion, EOES originating in the ileum with widespread multiorgan metastases is rare and easily misdiagnosed. When a small intestinal mass accompanied by calcification and wide multiorgan metastases is seen on CT, a suspicion of EOES should not be overlooked. Together with previous reports, this case has expanded knowledge about the spectrum of tumors in the small intestine.

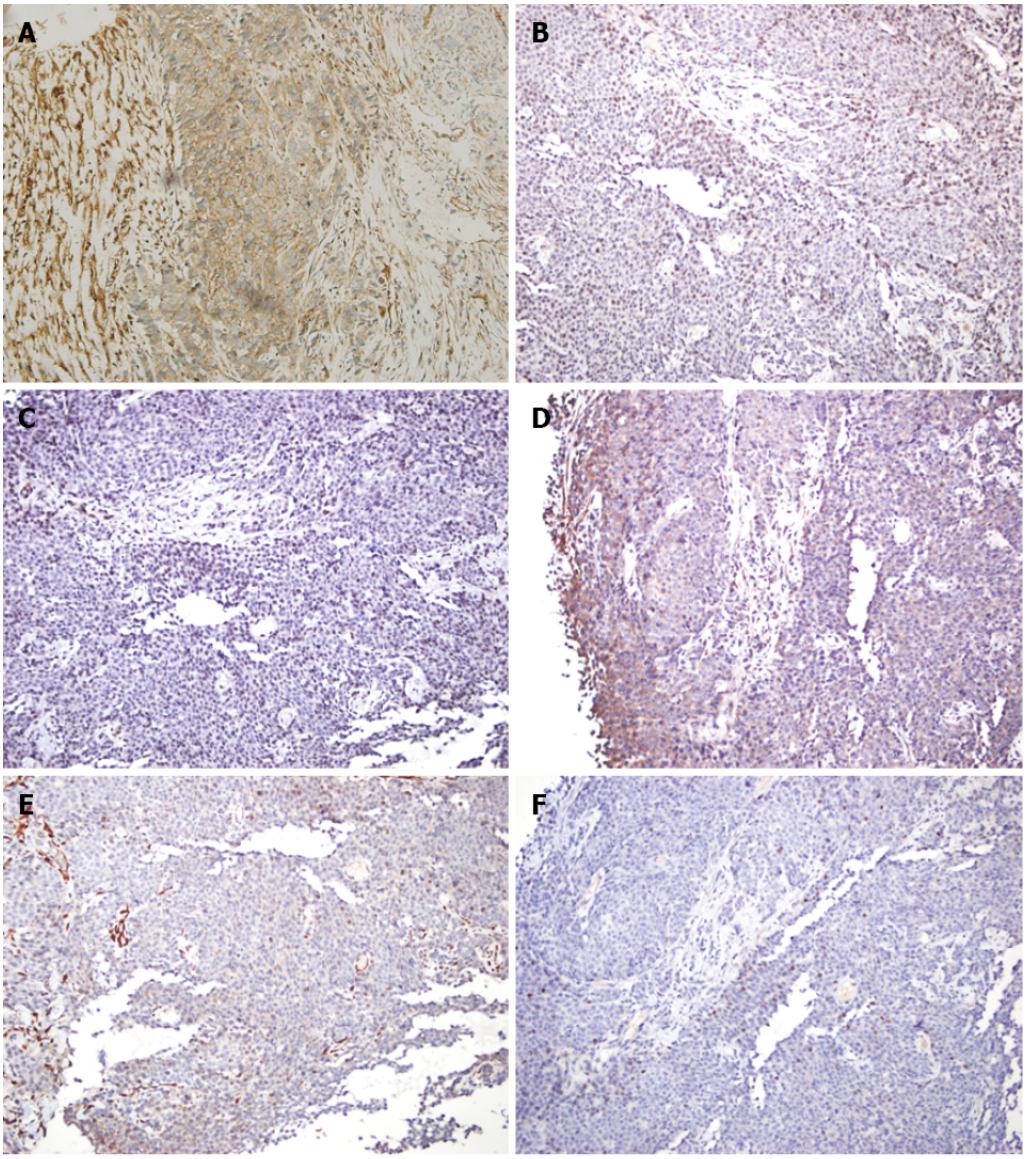


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**Figure 4 Immunohistochemistry findings.** A: Strong positive staining for CD99 (original magnification 200 ×); B: Positive staining for NKX2.2 original magnification 200 ×); C: Positive staining for FLI-1 (original magnification 200 ×); D: Positive staining for Syn (original magnification 200 ×); E: Negative immunoreactivity for CK (original magnification 200 ×); F: Negative immunoreactivity for CgA (original magnification 200 ×).

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## FOOTNOTES

**Author contributions:** Li H and Guo AW were responsible for the coordination of the project and contributed to the study design; Li H, Guo AW, Yuan Y and Liu YS collected, analyzed the data and edited the manuscript. Yuan Y and Li SX followed up the patient; Yuan Y and Li H supervised the study and reviewed this manuscript; All authors have read and approved the final manuscript.

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**Conflict-of-interest statement:** All authors report no relevant conflict of interest for this article.

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LETTER TO THE EDITOR

# Exosomes: Promising biomarkers and targets for cancer

Zhen Fang, Yi-Xuan Ding, Fei Li

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**Peer-review model:** Single blind

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Grade B (Very good): B, B
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## Abstract

The review article entitled "Exosomes as potential diagnosis and treatment for liver cancer " recently published in *World Journal of Gastrointestinal Oncology* 2022; 14: 334-347 concluded that exosomes can be used as effective biomarkers or therapeutic biotargets in liver cancer. Exosomes are a hot spot in the field of tumor diagnosis and treatment research. We had also previously published a review on exosomes and tumors. In this letter to the editor, we summarize the clinical application prospects and current challenges of exosomes.

**Key Words:** Exosomes; Cancer; Biomarkers; Diagnosis; Therapy



**Core Tip:** Exosomes have been shown to be major transmitters of cell-to-cell communication. Several advantageous features make exosomes effective therapeutic targets for cancer and ideal vehicles for drug delivery. This letter highlights the opportunities and challenges for clinical study and application of exosomes.

**Citation:** Fang Z, Ding YX, Li F. Exosomes: Promising biomarkers and targets for cancer. *World J Gastrointest Oncol* 2022; 14(8): 1594-1596
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## TO THE EDITOR

We read with great interest the systematic review "Exosomes as potential diagnosis and treatment for liver cancer" recently published in *World J Gastrointest Oncol* 2022; 14: 334-347[1]. The authors conducted a literature search to identify potential diagnostic and therapeutic markers of exosomes in liver cancer. Forty potential liver cancer

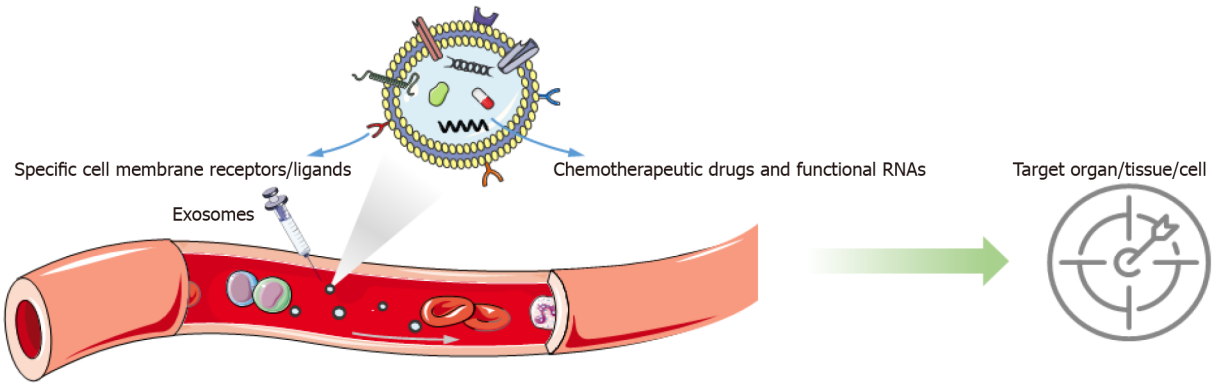


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**Figure 1 Schematic diagram of exosome drug delivery system.**

biomarkers, 13 potential biotherapeutics and 10 potential hepatocellular carcinoma therapeutic targets were identified, providing future directions for basic research and targeted therapy of liver cancer.

Exosomes, first discovered in 1983, are small lipid bilayer vesicles with a diameter of 40-160 nm, which are found in body fluids such as blood, urine, saliva, and cerebrospinal fluid[2]. Exosomes contain many biomolecules, including membrane-bound proteins, soluble proteins, lipids, DNA, microRNAs and non-coding RNAs[3]. In recent years, studies have found that exosomes are involved in intercellular communication in many physiological processes in the body, and play a crucial role in mediating tumorigenesis, development and metastasis[4-6]. Tumor-derived exosomes convey tumorigenic information and contribute to the tumor microenvironment for tumor proliferation and metastasis, and are a promising biomarker for cancer therapy[2]. The unique characteristics of tumor cell-derived exosomes make them potential biomarkers for early cancer diagnosis, tracking cancer patient's response to therapy, and detecting mechanisms of resistance to therapy, and making important contributions to precise and personalized cancer therapy.

A variety of cancer cell-specific proteins, lipids, DNA, RNA and metabolites can be isolated from cancer cell-derived exosomes, which can be used as cancer biomarkers[3]. Studies have reported that exosome-associated glypican-1 (GPC1) is a diagnostic biomarker for early pancreatic cancer[7]. Circulating exosome-derived lncRNA-GC1 can be used as a biomarker to detect early gastric cancer and monitor disease progression[8]. Tumor cell-specific molecules in exosomes can be used for early diagnosis and detection of cancer recurrence.

Exosomes have natural delivery capabilities as carriers for cancer therapeutics and functional RNAs [9]. Compared with traditional nanomaterial carriers, exosomes have the advantages of high bioavailability, non-cytotoxicity and non-immunogenicity. Transmembrane and membrane-anchored proteins within exosomes enhance endocytosis, thereby facilitating transfer of chemotherapeutics. Studies have found that neutrophil-derived exosomes deliver chemotherapeutics across the blood-brain barrier and effectively inhibit tumor growth[10]. Exosomes have the characteristics of small size, strong penetration and high biological stability. Using exosomes to deliver drugs or adding inhibitory immune checkpoints on the surface of exosomes to further enhance the anti-cancer effect is a new direction for exosomes in cancer treatment. In the future, exosome-based drug delivery systems are expected to be widely used in cancer therapy (Figure 1).

With the deepening of exosome research, a more comprehensive understanding of exosomes has been achieved, but there are still factors that restrict exosome research and clinical application. For example, the large-scale extraction, isolation and purification of exosomes are limited. At present, the exosome extraction method is mainly ultracentrifugation, but with low yield and high cost, thus it is difficult to achieve industrial production and large-scale clinical application.

In conclusion, much progress has been made in the field of exosome research, but the obstacles hindering the widespread clinical application of exosomes should also be highly concerned. The great prospect of exosomes for cancer diagnosis and treatment is undeniable.

## FOOTNOTES

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LETTER TO THE EDITOR

# Colitis and colorectal tumors should be further explored and differentiated

Dong-Hui Xu, Bo Zhou, Zhi-Peng Li, Lian-Ping He, Xin-Juan Wang

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## Abstract

The original article by Yuichi *et al* explored whether the Japan Narrow-Band Imaging Expert Team classification and the pit pattern classification are suitable for diagnosing neoplastic lesions in patients with ulcerative colitis. In this letter, we offer some other perspectives. Risk factors for colorectal tumors include type 2 diabetes. Among genetic factors, the deletion or mutation of some genes, such as the p53 gene, can lead to colorectal tumors. There are significant gender differences in the occurrence and development of colorectal tumors. Some non-genetic factors, such as smoking, are also associated with the development of colorectal tumors. These all suggest that colorectal tumors are not only caused by ulcerative colitis, and we suggest further exploration and differentiation between colitis and colorectal tumors.

**Key Words:** Colorectal cancer; Nicotine; p53; Tobacco; Ulcerative colitis



**Core Tip:** Among genetic factors, the deletion or mutation of some tumor suppressor genes can lead to colorectal tumors. Non-genetic factors are also associated with the development of colorectal tumors. The underlying disease can be a risk factor for colorectal tumors. There are significant gender differences in the occurrence and development of colorectal tumors. These all suggest that colorectal tumors are not only caused by ulcerative colitis, and we suggest further exploration and differentiation between colitis and colorectal tumors.

**Citation:** Xu DH, Zhou B, Li ZP, He LP, Wang XJ. Colitis and colorectal tumors should be further explored and differentiated. *World J Gastrointest Oncol* 2022; 14(8): 1597-1599
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## TO THE EDITOR

We read with great interest the study by Kida Y *et al*[1] which was published in the world journal of gastroenterology. The study focused on whether the Japan Narrow-Band Imaging Expert Team (JNET) classification and pit pattern classification are applicable for diagnosing neoplastic lesions in patients with ulcerative colitis (UC). This study found that The JNET and pit pattern classifications did not show high accuracy in diagnosing the pathology and invasion depth of neoplastic lesions in UC patients. Endoscopic diagnosis of neoplastic lesions in UC patients is still difficult and treatment strategies need to be carefully determined. Although the authors' findings provide new methods and ideas for existing diagnosis and treatment problems, our team agrees that there are still some issues that need further discussion in this paper.

In the case of genetic factors, environmental factors, living habits, and other adverse factors, everyone is theoretically at risk of developing colorectal tumors. The study by Simon[2] showed that genetic disorders such as Lynch syndrome, a personal history of inflammatory bowel disease, and type 2 diabetes are all predisposing factors for developing colorectal tumors. In genetic factors, deletion or mutation of some genes, such as the p53 gene, can also lead to colorectal tumors[3,4]. There are significant gender differences in the development of colorectal tumors, and the colorectum is a common tumor-producing organ in both men and women[5]. The study by Kim *et al*[6] showed that women over 65 had higher colorectal cancer mortality compared with men of the same age group. Colorectal cancer detection time and mortality are related to the site of colorectal cancer. Compared with right-sided colon cancer, left-sided colon cancer was detected later and more differentiated. In clinical work, it was found that the proportion of right-sided colorectal cancer in women is much higher than in men. All of the above evidence suggests that the mortality rate of female patients with colorectal cancer may be higher than that of male patients.

Some non-genetic factors, such as smoking, are also associated with the development of colorectal tumors. Among the etiologies of non-hereditary colorectal tumors, smoking has local and systemic effects on the colorectal mucosa through the production of carcinogens[7]. The nicotine in tobacco is potentially addictive and increases the patient's dependence on tobacco, thereby increasing the risk of colorectal cancer. In addition, the mutation rate of tumor suppressor genes in smokers was significantly higher than in non-smokers. Among the many mutant genes, the p53 gene mutation is the most important. These phenomena are related to the occurrence and development of colorectal tumors. The study by Siegel *et al*[8] shows that women under 49 are about 3% more likely to die than men.

In summary, colorectal tumors are not only caused by ulcerative colitis. Research by Curtin K shows that smoking (> 20 pack-years *vs* non-smokers) was associated with TP53 mutations (OR = 1.4, 95%CI 1.02-2.0), BRAF mutations (OR = 4.2, 95%CI 1.3-14.2), and MSI mutations (OR = 1.4, 95%CI 1.02-2.0) in rectal tumors and was associated with an increased risk of rectal cancer. Long-term exposure to > 10 h/wk of environmental tobacco smoke was associated with an increased risk of KRAS2 mutations (OR = 1.5, 95%CI 1.04-2.2)[9]. Colorectal cancer is also related to genetic factors, living habits, eating habits, *etc.* It may not be clear that patients with chronic ulcerative colitis developed colorectal tumors due to chronic inflammation in this study. To further explore whether chronic ulcerative colitis is a risk factor for colorectal tumors, genetic factors, dietary habits, lifestyle habits and other factors need to be further discussed.

Type 2 diabetes has been shown to be a risk factor for colorectal tumors. Among genetic factors, deletion or mutation of some genes, such as the p53 gene, can lead to colorectal tumors. There are significant gender differences in the occurrence and development of colorectal tumors. Some non-genetic factors, such as smoking, are also associated with the development of colorectal tumors. These all suggest that colorectal tumors are not only caused by ulcerative colitis. Therefore, we suggest further exploration and differentiation between colitis and colorectal tumors.

## FOOTNOTES

**Author contributions:** Wang XJ and He LP conceived of the presented idea and provided critical feedback to the final manuscript, revised the manuscript and approved the main conceptual ideas and outline; Xu DH, Zhou B, and Li ZP wrote the manuscript; all authors provided final edits and approved the manuscript.

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LETTER TO THE EDITOR

# Acute or chronic inflammation role in gastrointestinal oncology

Hong-Jin Chen, Gui-You Liang, Xiong Chen, Zhou Du

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Grade A (Excellent): 0
Grade B (Very good): 0
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## Abstract

The following letter to the editor highlights the review titled "Inflammatory bowel disease-related colorectal cancer: Past, present and future perspectives" in *World J Gastrointest Oncol* 2022 March 15; 14(3): 547-567. It is necessary to explore the role of inflammation in promoting tumorigenesis and development of gastrointestinal cancers.

**Key Words:** Inflammatory; Gastrointestinal cancers; Development; Letter to the Editor; Colorectal cancer



**Core Tip:** Gastrointestinal cancers are systematic tumors with the largest number of patients in the world. Most patients are prone to migration, invasion or other malignant phenotypes. The treatment strategies mainly include surgical resection, radiotherapy and chemotherapy in clinic. However, the survival rate of cases still cannot be significantly improved. Recently, the relationship between inflammation and gastrointestinal tumors has been gradually clarified, and chronic inflammation plays an important role in the occurrence and deterioration of tumors. The main purpose of this letter is to illustrate the key role of inflammation in tumor progression and potential therapeutic directions.

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## TO THE EDITOR

We read with interest the review by Majumder *et al*[1], which is titled "Inflammatory bowel disease-related colorectal cancer: Past, present and future perspectives." The tumor pathogenesis is complex and not yet clear. Recently, inflammation induced and promoted tumor occurrence and deterioration, and the presence of high levels of inflammatory factors in many tumor patients has gradually become clear. The gastrointestinal system is one of the most prone to inflammation. Patients with chronic inflammation are more likely to develop liver cancer, pancreatic cancer, stomach cancer and colon cancer than those without inflammation,. Studies have demonstrated that hepatitis B virus patients were more likely to get cancer of the liver, and prognosis and survival time is far less than the patients without hepatitis B virus[2]. Patients with pancreatitis had a 4.8-times significantly higher risk of developing cancer than those without pancreatitis[3]. *Helicobacter pylori* is one of the important risk factors for gastric cancer patients, and *Helicobacter pylori* will induce the occurrence of chronic gastritis[4]. In addition, patients with colitis have an increased mortality of colon cancer by 15%[1]. Therefore, if the potential biomarkers can be identified by early intervention of the synthesis, secretion and release of inflammatory factors, it may have great clinical significance for gastrointestinal tumors and improve the overall understanding of gastrointestinal tumors.

The interleukin (IL) family is the most common biomarker of inflammation. IL-1β, IL-6 and IL-10 are involved in the development and progression of gastrointestinal tumors. On the other hand, external stimuli, such as excessive oxidative stress, promote the secretion and release of the IL family, while the IL family itself has a certain feedback activation effect, thus exacerbating the inflammatory response[5]. In colitis-cancer, IL-6 and other factors promote epidermal cell damage, and prolonged inflammatory damage will lead to abnormal proliferation of epidermal cells, which if not controlled will eventually lead to gene epigenetic modification mutation and ultimately induce tumorigenesis[6,7].

Tumor necrosis factor (TNF), another classic inflammatory factor, can promote the activation of neutrophils or macrophages to aggravate tissue damage by regulating monocyte chemotactic protein-1 and other mRNAs[8]. Moreover, TNF accelerates the inflammatory process and thus leads to the occurrence of tumors[9]. In addition, the role of a c-x-c motif chemokine ligand (CCL) family in gastrointestinal tumors is gradually becoming clear. CCLs infiltrated tissues by recruiting macrophages and releasing IL family members or TNF, further leading to local inflammatory infiltration of tissues, gene mutation and ultimately tumorigenesis[10].

Interestingly, some papers showed that chronic inflammatory responses promoted tumorigenesis and development, while acute inflammation is currently considered to inhibit tumor progression (Figure 1)[11]. The new clinical research paper indicated that colon cancer patients with higher IL-6 and TNF (chronic inflammatory factors) developed a cancer recurrence. However, acute inflammatory factors, IL-10 and interferon γ, were lower in expression compared with those who did not recur[7]. IL-12 is an acute inflammatory factor that could inhibit tumor progression in gastrointestinal tumors, and its high expression leads to a longer survival time[12]. Additionally, the interferon family is a potential therapeutic biomarker, which could inhibit the occurrence and progression of gastrointestinal tumors by regulating cellular immunity, controlling cell cycle or promoting cell apoptosis[13,14]. Moreover, the interferon family has been approved by the Food and Drug Administration for the treatment of tumors[15].

In conclusion, inflammation is involved in the entire gastrointestinal tumor process. The worse inflammation is mainly chronic inflammation, which can be induced by many reasons, such as unhealthy high-fat diet, excessive use of antibiotics, imbalance of intestinal flora and so on[16]. Majumder *et al*[1] systematically summarized the role of inflammatory factors in colon cancer. However, they failed to study and consider the role of acute inflammation in colon cancer. Therefore, inflammatory factors should be considered as important triggers to optimize current diagnosis and treatment strategies for early tumor diagnosis.

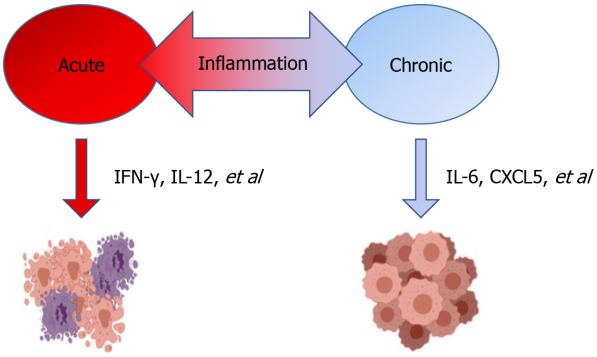


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**Figure 1 Relationship of inflammation and cancer.**

## FOOTNOTES

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