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REVIEW

Hepatic Hemangioendothelioma: An update

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Abstract

Primary epithelioid hemangioendotheliomas of the liver (EHL) are rare tumors with a low incidence. The molecular background of EHL is still under investigation, with WWTR1-CAMPTA1 mutation may function as a tumor marker. Commonly, this tumor is misdiagnosed with angiosarcoma, cholangiocarcinomas, metastatic carcinoma, and hepatocellular carcinoma (sclerosing variant). Characteristic features on imaging modalities such as ultrasound, computed tomography, magnetic resonance imaging and positron emission tomography/computed tomography guide in diagnosis and staging. The "halo sign" and the "lollipop sign" on computed tomography and magnetic resonance imaging are described in the literature. Currently, there are no standardized guidelines for treating EHL with treatment options are broad including: chemotherapy, ablation, surgery and liver transplantation with inconsistent results.

Key words: Epithelioid hemangioendotheliomas; Halo sign; Lollipop sign; Angiosarcoma; Cholangiocarcinomas; Hepatocellular carcinoma

Core tip: Primary epithelioid hemangioendotheliomas of the liver are rare tumors with an incidence rate of less than 0.1 per 100000 population. The molecular background of epithelioid hemangioendotheliomas is still under investigation. The "halo sign" and the "lollipop sign" on computed tomography and magnetic resonance imaging are described in the literature. The differential diagnosis includes angiosarcoma, cholangiocarcinomas, metastatic carcinoma, and hepatocellular carcinoma (sclerosing variant). Currently, there are no standardized guidelines for treating EHL with treatment options are broad and

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INTRODUCTION

Epithelioid hemangioendotheliomas (EH) are vascular tumors that may affect the liver, lungs, mediastinum, and multiple other sites. However, the most commonly involved organ is the liver[1]. Primary epithelioid hemangioendotheliomas of the liver (EHL) are rare tumors with an incidence rate of less than 0.1 per 100000 population. Due to the low incidence rate, not a lot is understood regarding the pathogenesis of this tumor. However, it has been shown that EHL have a predilection for females, with a female-to-male ratio of 3:2, and affects the right lobe of the liver more than the left lobe. These tumors may be asymptomatic (24.8%), or symptomatic, with right upper quadrant pain being the most common presenting symptom (48.6%)[2]. The lungs, regional lymph nodes, peritoneum, bone, spleen, and diaphragm are the most common sites of extrahepatic involvement[2,3].

GENETICS

The molecular background of EH is still under investigation. To date, many genes involved in cell cycle control, signaling pathways, epigenetic modification, and DNA repair have been implicated in the pathogenesis of EH. A study by Errani *et al*[4] sought to determine the genetic alterations of EH irrespective of the site of the tumor. They found that a WWTR1-CAMTA1 was a recurrent mutation in EH. WWTR1 is involved in transcriptional co-activation and is usually inhibited by the Hippo pathway, which causes the protein to translocate from the nucleus to the cytoplasm[5]. Mutations that cause WWTR1 to be retained in the nucleus by transcriptions factors such as TEAD 1-4, are thought to cause the oncogenesis[6]. On the other hand, CAMTA1 is a transcription activator that belongs to the calmodulin-binding protein family[7]. The downstream targets are yet to be determined. CAMTA1 seems to behave as a tumor suppressor gene, as its deletion also results in oncogenesis[8,9]. However, the conserved regions in the fusion protein function in transcription and binding calmodulin, which might help promote tumorigenesis[7,10]. Additionally, the WWTR1-CAMPTA1 fusion protein seems to be specific to EH, whereby Errani *et al*[4] demonstrated that it is not detected in tumors that mimic EH, such as epithelioid hemangioma and epithelioid angiosarcoma. As such, the authors recommended that WWTR1-CAMPTA1 may function as a tumor marker allowing physicians to identify EH when the diagnosis is unclear.

PATHOLOGY

Macroscopically, EHL seems to have two growth patterns, nodular and diffuse. The patterns represent different stages of the disease. Early disease presents as a nodular growth, and can be seen in 11.1% of patients. The nodular lesions are usually multiple (66.5%) and affect both lobes of the liver (82.2%)[11-13]. Later stages present as diffuse lesions due to nodular tumors growing in size and coalescing together. As the tumor infiltrates the surrounding structures, portal hypertension may develop[11,14].

The World Health Organization describe this tumor as malignant tumor with metastatic potential and variable clinical course (indolent to progressive)[15,16]. Microscopically, EH can have three cell types: intermediate, dendritic, and epithelioid cells. Epithelioid cells are present in all cases, are of endothelial origin, and may present with vacuoles causing it to resemble signet cell morphology (Figure 1). Dendritic cells are stellate in shape and have multiple processes. The intermediate cells share features of both cell types[17].

Due to its endothelial origin, these tumors usually express the FLI-1 protein, which

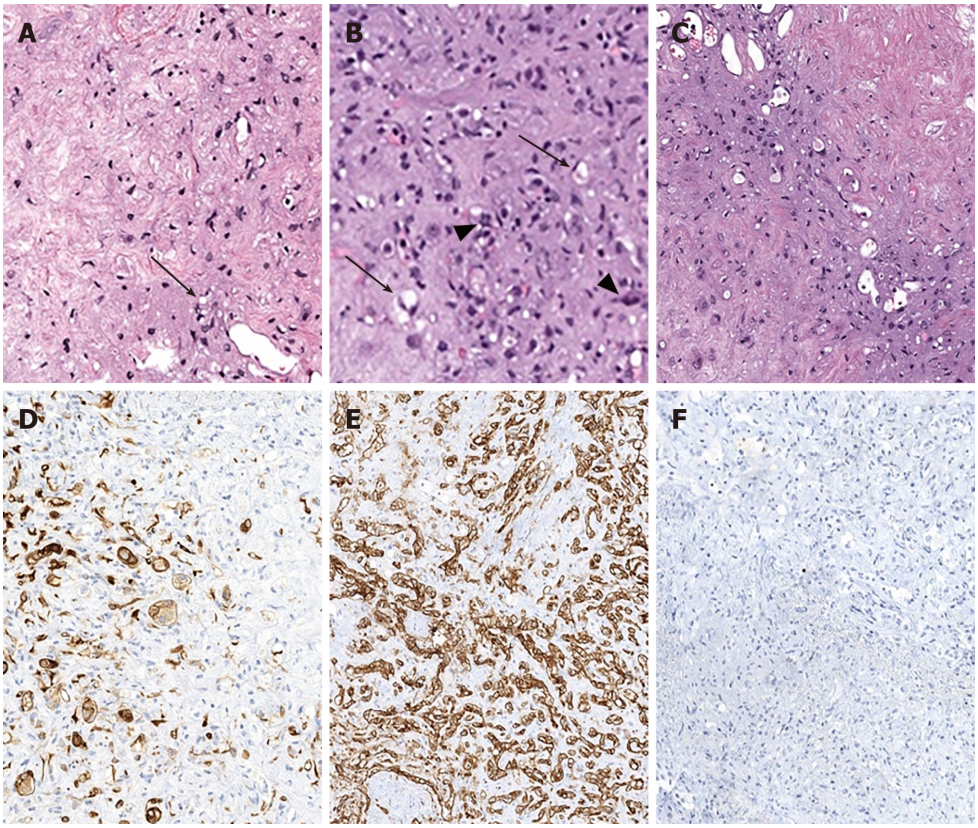

Figure 1 Epithelioid hemangioendothelioma microscopic features. There is variable cellularity, ranging from stromal-rich areas, mimicking cartilage to highly cellular regions. A: The tumor consists mostly of stellate cells with only rare epithelioid cells containing intracytoplasmic lumen/vacuoles (arrows); B: In more cellular areas, the intracellular lumens (arrows) are more prominent. In addition, the tumor infiltrates sinusoids forming tufted foci (arrowheads); C: Transitional areas between fibrous and moderately cellular areas. To confirm and differentiate epithelioid hemangioendothelioma from tumors with similar histologic features (most commonly cholangiocarcinoma, hepatocellular carcinoma and metastatic signet ring cell carcinoma), immunohistochemical stains are needed; D: Patchy staining for cytokeratin 7, a stain commonly expressed in adenocarcinomas of the upper gastrointestinal tract and pancreaticobiliary tree; E: Diffusely positive for the endothelial marker cluster of differentiation-31 (CD31); F: Negative for the hepatocellular marker, Hepatocyte Paraffin 1 (HepPar1). CD31: Cluster of differentiation-31; HepPar1: Hepatocyte Paraffin 1.

has been shown to be sensitive in identifying vascular tumors. In a single study, FLI-1 allowed for the identification of 100% of endometrioid hemangioendotheliomas with an 85% specificity. This marker showed to have a higher sensitivity than other endothelial markers such as CD31 and 34. Additionally, CD34 is not a specific marker for EH since it is expressed by most vascular tumors (90%). In the liver, the expression of podoplanin proved to be specific to EHL, allowing for accurate identification[18].

Pathologically, EH can be misdiagnosed in up to 80% of cases[2]. Most commonly, EH is misdiagnosed with angiosarcoma, cholangiocarcinomas (CC), metastatic carcinoma, and hepatocellular carcinoma (HCC) (sclerosing variant)[2,17,19,20]. Therefore, awareness of pathological differences is necessary to accurately diagnose these tumors.

EHL and angiosarcoma have similar presentations on immunohistochemistry profiling and hematoxylin-eosin staining, however differentiating features may still be found. On high power fields, angiosarcoma shows greater atypia, mitotic activity, and nuclear pleomorphism. Additionally, relative to angiosarcomas, EHL demonstrates greater sclerosis but less parenchymal destruction on low power fields[17]. Although both tumors may stain for CD34, factor VIII, and CD31, some markers such as D2-40 are more common in EHL[21].

Unlike EHL, intrahepatic cholangiocarcinomas (ICC), metastatic carcinomas, and HCCs do not usually express CD34, factor VIII, or CD 31, but are more likely to express cytokeratins. Finally, unlike EHL, HCC stains for CD10, arginase and HepPar-1 with polyclonal carcinoembryonic antigen and CD10 showing canalicular pattern. Some HCC's may contain vessels that express CD34 due to capillarization of sinusoids, however, this stain is not positive n the neoplastic cells proper and is only expressed along the affected sinusoids[21,22]. On the other hand, the neoplastic cell composing EHL expresses CD34 diffusely.

IMAGING

Imaging is an indispensable component in initial tumor staging, treatment response evaluation, and recurrence identification. Lung or multiorgan involvement, disease progression, the presence of ascites, age more than 55 years, and male gender were found to be associated with a worse prognosis[23]. Extrahepatic tumor extension beyond portal lymph nodes is a negative prognostic factor[1].

There are two different types of EHL with different stages. The early stages of the disease present as the nodular type, while advanced stages appear as diffuse disease with coalescence of different lesions that may invade hepatic vasculature. The discrete nodules of EHL range in size from 0.5 cm to 12 cm and may progress to complex and confluent masses[2,24-26]. They are located peripherally and extend up to the liver capsule. Fibrosis and compensatory hypertrophy of the unaffected liver segments causes flattening or retraction of the liver capsule[27]. Because of the tumors metastatic potential and risk of recurrence after surgical approaches, imaging is pivotal for identifying the prognostic markers that can guide treatment. Imaging findings should include the exact number, dimensions, location, vascular or ductal involvement, locoregional lymphadenopathy and extrahepatic extension, as they are important factors for surgical decision on resectability[1].

ULTRASOUND

The appearance of EHL on ultrasound is important to recognize, since ultrasound might be the first modality used to assess right upper quadrant pain, which is the most common presenting symptom of EHL[28]. On ultrasound (US), EHL usually appear as hypoechoic lesions that may demonstrate heterogeneous echotexture (Figure 2). The presence of capsular retraction, calcifications, and multifocal lesions may further support the diagnosis[29].

EHL shows intratumoral vascularity and better visualized on color Doppler. Multifocal disease may cause intrahepatic congestion which will decrease the portal vein flow and result in portal hypertension with splenomegaly and hepatomegaly[30].

Another beneficial form of US is contrast enhanced US (CEUS). In a single study, EHL mostly depicts rim enhancement in the arterial phase on CEUS, but some masses may display heterogeneous hyperenhancement. Additionally, in one study all the lesions expressed early wash-out of the contrast agent on late and portal phases, secondary to the absence of portal veins in the tumor[29]. Though this is not specific for EHL, the presence of early contrast wash-out on late and portal phases signifies that the lesion is not benign and requires further investigation, be it biopsy or other imaging modalities[28].

COMPUTED TOMOGRAPHY

On non-contrast enhanced computed tomography (CT) scans, most lesions appear hypodense relative to the surrounding liver parenchyma[31]. Occasionally, a hyperdense rim may be evident due to the hypercellularity of the periphery. Relative to contrast enhanced CT scans, non-contrast enhanced CT scans can better visualize the bulk of the tumor, since they can additionally detect capsular retraction and coarse/nodular intratumoral calcifications, which can be present in up to 25% of cases[32-34]. The capsular retraction is due to hypertrophy of normal tissue in response to fibrosis of diseased tissue[35-37]. However, though capsular retraction is suggestive of EHL, it is not specific to it and other pathologies such as metastatic lesions and cholangiocarcinomas may cause capsular retraction[35,38,39].

On contrasted enhanced studies, three patterns of enhancement have been described. In the arterial phase, some tumors demonstrate mild homogeneous enhancement that does not increase in the delayed or portal vein phases. Other tumors develop a ring like enhancement during the arterial phase, with central filling on the delayed and portal phases, which is termed a "halo sign". Lastly, some tumors demonstrate a heterogeneous pattern that progresses during the delayed and portal phases. The type of enhancement expressed seems to depend on the size of the tumor, as tumors more than 3 cm exhibited delayed heterogeneous enhancement, tumors 2 to 3 cm exhibited ring like enhancement, and tumors less than 2 cm exhibited homogenous enhancement (Figure 2)[40]. An imaging finding on contrast enhanced CT/MRI that is rather specific to EHL was first described by Alomari, which he termed the "lollipop sign" (Figure 3). EHL infiltrate sinusoids, venules, and veins, leading to narrowing or obstruction of these structures. Radiologically, this

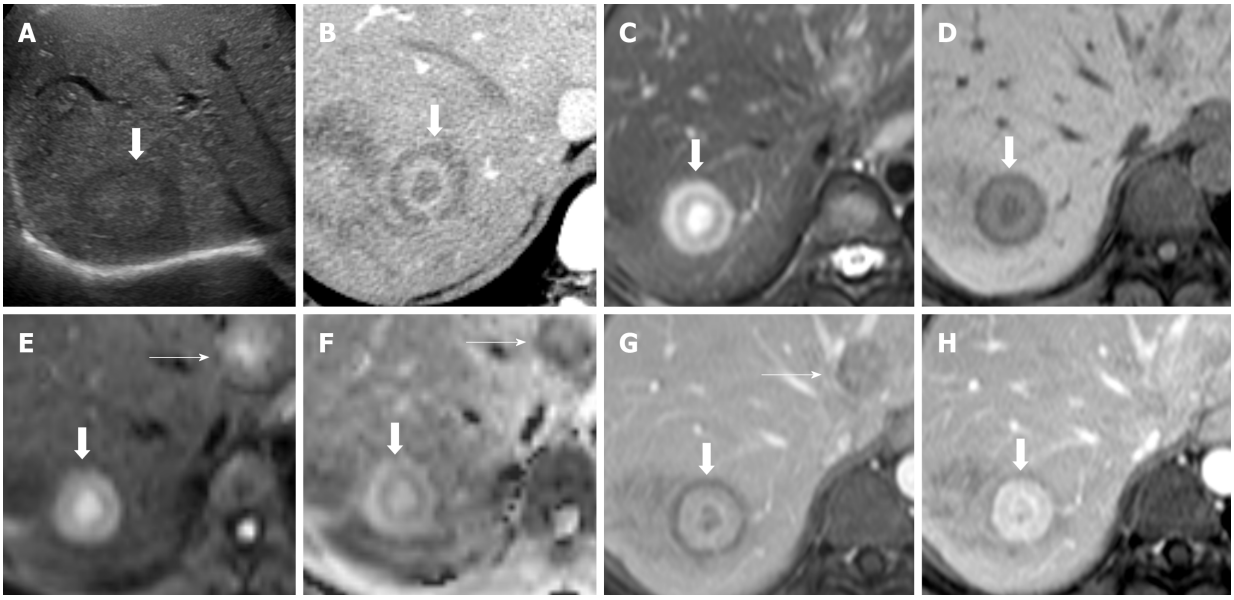

Figure 2 Hepatic hemangioepithelioma imaging feature. A: Transverse ultrasound of the right upper abdomen reveals a iso to mildly hypoechoic lesion (arrow) in the liver; B: Axial contrast enhanced CT image of the liver; C: Axial T2 weighted images (T2WI); D: Axial pre contrast T1 weighted images (T1WI); E: Axial diffusion weighted image (DWI); F: Apparent diffusion coefficient; G: Axial post contrast T1WI portovenous; and H: Axial post contrast T1WI venous magnetic resonance imaging (MRI) images. On T2WI, a target appearance consists of a core with high signal intensity (similar to fluid), a thin ring with low signal intensity, and a peripheral halo with slight hyperintense signal (thick arrows). On dynamic study, it consists of an hypodense/hypointense core, surrounded by a layer of enhancement and a thin peripheral hypodense/hypointense halo (thick arrows). Other hepatic hemangioepithelioma nodules are also noted (thin nodules). T2WI: T2 Weighted Images; T1WI: T1 weighted images; DWI: diffusion weighted image; MRI: Magnetic resonance imaging.

histological feature causes tapering and termination of the portal and hepatic vein and their branches as they approach the lesion. These occluded vessels are likened to the stick of the lollipop, while the hypodense well-defined tumor itself is likened to the candy, giving rise to the lollipop appearance. In its original description, only vessels that terminate within or at the edge of the rim meet the criteria of the lollipop sign. Additionally, lesions that enhance irregularly, are hyperdense, and/or have a central scar are excluded[41]. Relative to the other imaging characteristics, the lollipop sign is the least likely to be seen in other malignant or benign liver malignancies, making this imaging finding the most characteristic for EHL[40].

MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging (MRI) is preferred over CT for the diagnosis of EHL due to its ability to detect smaller subcapsular lesions[11,12,42,43]. On non-contrast enhanced images, EHL typically demonstrates a "halo sign", consisting of three layers of varying signal. These tumors usually demonstrate a hypointense core with a hyperintense rim on T1-Weighted Imaging (T1WI), while the tumor core is hyperintense on T2-Weighted Imaging (T2WI) with heterogeneous signal intensity with a hypointense rim[40]. This is secondary to a hypocellular center that may exhibit necrosis, prior hemorrhage, thrombosis, and/or calcification[44]. With contrast administration, EHL demonstrate findings similar to contrast enhanced CT, with three patterns of enhancement. In the arterial phase, tumors may demonstrate mild homogeneous enhancement, ring like enhancement, or a heterogeneous pattern depending on the size of the lesion (Figure 2 and 3).

Similar to T1WI and T2WI, most lesions (60%) exhibit a "target sign" on diffusion weighted imaging (DWI) with a hyperintense external rim and core. On higher b-values, the periphery's signal increases, signifying restricted diffusion. On the other hand, the core exhibits a lower signal, which once again highlights the hypocellular core and hypercellular periphery[28,34]. Likewise, on ADC map, tumors typically demonstrate high signal intensity centrally and low signal intensity peripherally. The high ADC values centrally help differentiate EHL from other tumors, as other tumors in the liver rarely exhibit high ADC values[34].

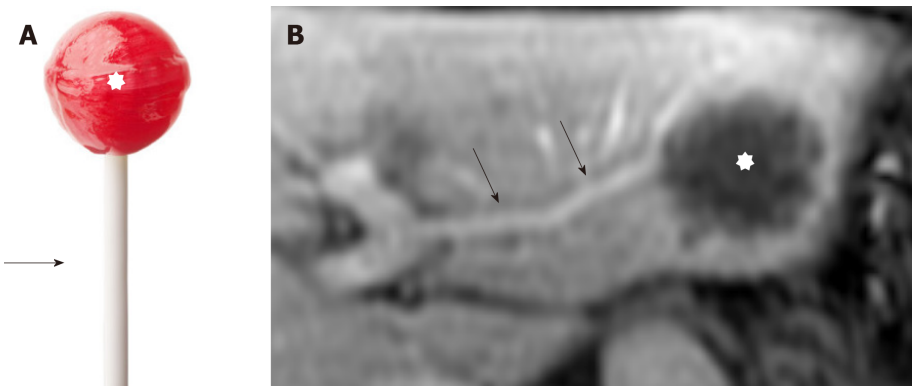

Figure 3 Hepatic hemangioepithelioma imaging feature. A: A lollipop; B: Axial post contrast T1 Weighted Images images. Hepatic hemangioepithelioma nodule (star) with portal veins entering and terminating in the periphery of the lesion (arrow). This configuration resembles a lollipop. The ''Lollipop sign'' is a combination of two structures: the well-defined tumor mass on enhanced images (the candy in the lollipop) and the adjacent occluded vein (the stick), because hepatic hemangioepithelioma has the tendency to spread within the portal and hepatic vein branches. The vein should terminate smoothly at the edge or just within the rim of the lesion; vessels that traverse the entire lesion or are displaced and collateral veins cannot be included in the sign.

POSITRON EMISSION TOMOGRAPHY/ COMPUTED TOMOGRAPHY

The healthy hepatocytes express high levels of glucose-6-phosphatase, which generally leads to rapid dissolution of the FDG avid signal relative to malignant tissue[45]. However, since EHL may exhibit variable levels of glucose-6-phosphatase, uptake and excretion of FDG is unpredictable, which limits evaluation of EHL in the early phase. Secondary to the unpredictability of FDG uptake, it has been recommended to review positron emission tomography/computed tomography (PET/CT) studies of EHL at two different time points to improve detection (Figures 4, 5, 6 and 7)[45-47].

Similar to MRI and CT, EHL tumors demonstrate different patterns of FDG uptake. The lesions in the same patient may demonstrate different behavior on PET/CT, as well as appear at different time interval following FDG administration. Most EHL lesions demonstrate increased uptake relative to the surrounding liver parenchyma, however, up to one third of EHLs may demonstrate similar FDG uptake to the surround tissue. Additionally, the hypercellular periphery may cause higher uptake near the edge of the tumor which appears as a hypermetabolic rim[40,48]. Hence, the performance of PET/CT in the evaluation of metastatic disease and recurrence requires further assessment.

MANAGEMENT

There are no standardized guidelines for treating EHL. The treatment options are broad with inconsistent results and include chemotherapy, ablation, surgery and liver transplantation[49-53]. In fact, one study reported that there was no significant difference in 5-year survival rates among the different treatments[54]. Consequently, few studies have been conducted on EHL and thus natural history of the disease is unpredictable ranging from localized and indolent to aggressive and metastatic.

Furthermore, observation alone can yield favorable outcomes including spontaneous regression or disease stabilization if the tumor is non-aggressive. For example, a retrospective study on pediatric cases reported spontaneous remission without treatment in 60% of patients with focal disease (n = 10) and 42% of patients with multifocal disease (n = 12)[55]. Consequently, another study also demonstrated that overall survival does not significantly differ between local and metastatic or unilateral and bilateral disease[54]. These findings reinforce the unpredictability of tumor behavior, thus complicating the treatment. However, the general consensus is to begin with observation to assess tumor behavior before intervention, if applicable at all.

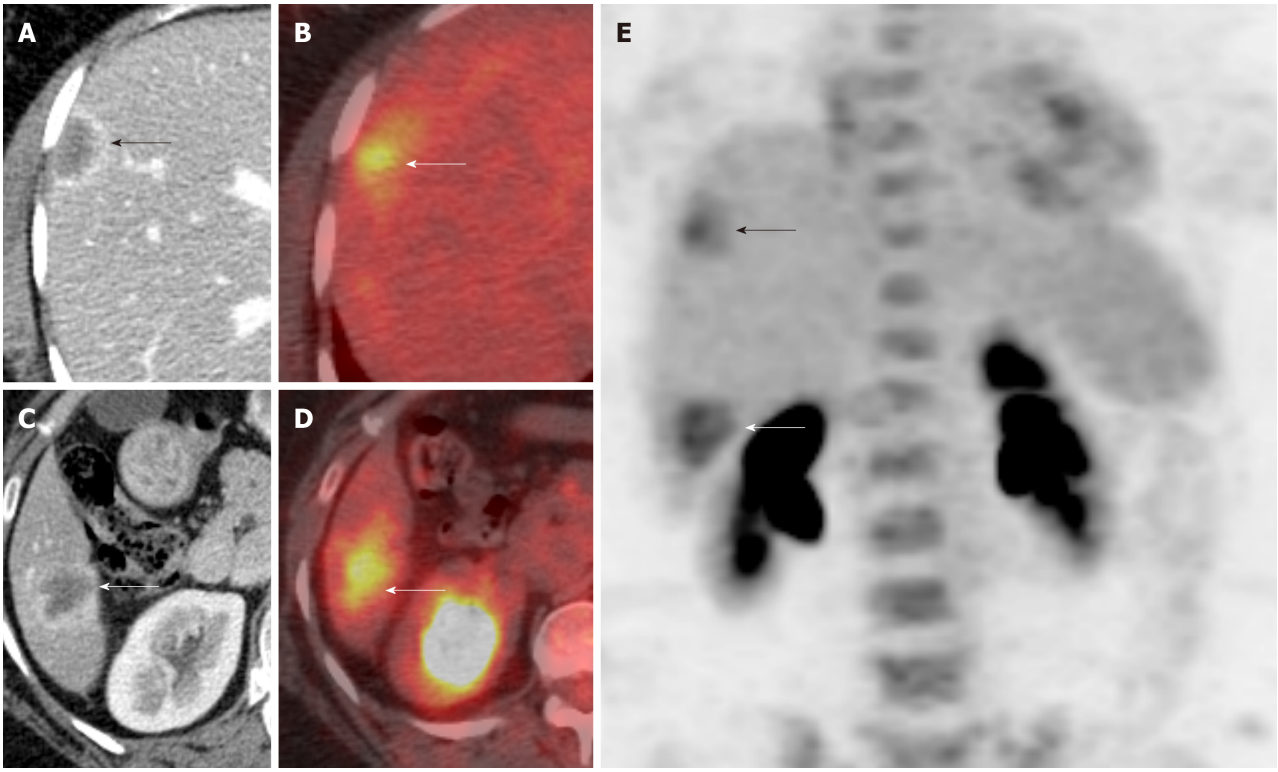

Figure 4 Imaging of hepatic hemangioepithelioma. A and B: Axial contrast enhanced (Computed Tomography) CT images show hepatic hemangioepithelioma nodules (arrows); C and D: Axial ^{18}F-labeled fluoro-2-deoxyglucose positron emission tomography/computed tomography; E: Coronal maximum intensity projection images shows fluoro-2-deoxyglucose avid hepatic hemangioepithelioma nodules (arrows).

MEDICATIONS

Prednisone and propranolol are described in literature for medical management of EHL with variable results (Table 1). In a group of nine children with focal, multifocal, and diffuse disease assigned to propranolol (n = 3) or a combination of propranolol/prednisolone (n = 6), 100% of them in each treatment achieved tumor regression. Interestingly, three out of six of all patients with diffuse disease and five out of twelve patients with multifocal disease achieved tumor regression after receiving propranolol or propranolol/prednisolone[55]. Due to EHL's association with hypothyroidism, L-thyroxine might be needed for patients with severely low T3 and T4 levels[55,56]. Emad *et al*[55] concludes that treatment for EHL should be escalated gradually according to disease's response to treatment beginning with close observation for focal disease, then medical therapy and followed by chemotherapy.

CHEMOTHERAPY

Chemotherapy such as interferon, vincristine, or cyclophosphamide have variable results and should be added in patients not responsive to medical therapy such as propranolol or prednisolone (Table 2). Furthermore, patients presenting with early and aggressive disease tend to respond poorly to the medical management[55]. A study reported that regardless of disease severity and initial diagnosed stage, chemotherapy consistently and significantly decreased overall survival (OS) compared to those without chemotherapy[54]. However, some studies have advocated that chemotherapy should be used to reduce tumor burden and slow disease progression, and hepatic transplantation adopted as the optimal management due to favorable prognosis[57-59]. Thalidomide has been suggested as a front-runner for tackling metastatic disease due to its anti-angiogenic properties[60]. In addition, sorafenib and intra-arterial 5-fluorouracil have demonstrated encouraging results for overall survival[14,61,62]. Due to its ability to block vascular endothelial growth factor (VEGF), a signaling protein highly expressed in EHL, Bevacizumab has been utilized for the management of EHL[19,63,64].

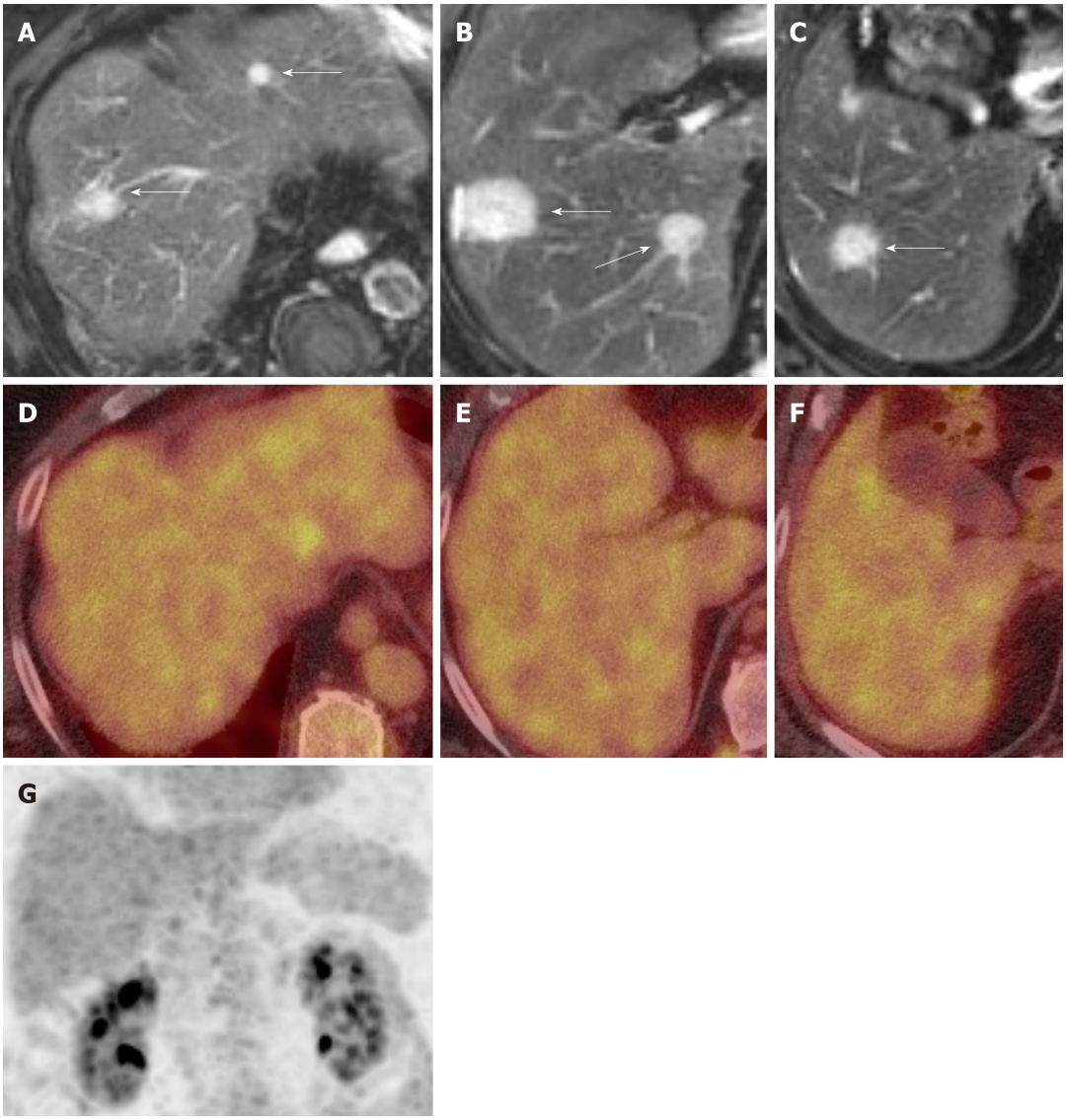

Figure 5 Imaging of hepatic hemangioepithelioma. A, B and C: Axial T2 Weighted Image Magnetic Resonance Imaging images show hepatic hemangioepithelioma nodules (arrows); D, E and F: Axial ^{18}F-labeled fluoro-2-deoxyglucose positron emission tomography/computed tomography and G: Coronal maximum intensity projection images show no fluoro-2-deoxyglucose update by the hepatic hemangioepithelioma nodules.

INTERVENTIONAL

Alternative treatment modalities include radiotherapy and radiofrequency (RFA)/microwave ablation (Figure 8). A case report described significant remission of EHL lesions two months after performing selective internal radiotherapy (SIRT), with a single dose 1.8 GBq 90-yttrium (48.6 mCi)[65]. On the other hand, RFA has proved to be a safe and efficient intervention for EHL lesions up to three centimeters large with up to 1% and 7% mortality and complication rates, respectively. RFA could eventually be proposed as an alternative to hepatic resection[66,67].

SURGERY

The hepatic resection is reserved for single, intrahepatic and resectable lesions (Table 3), while liver transplant is performed for patients with multiple bilobar hepatic lesions (Table 4). The surgical intervention for EHL includes hepatic resection, hepatic transplantation, and hepatic artery ligation (HAL)[68]. Due to the lack of established guidelines for EHL management, there has been a debate on the most effective surgical intervention. Rodriguez *et al*[69] argued that liver transplants should be adopted at higher rates due to EHL's ability to metastasize and difficulty to resect, and to avoid liver failure in complicated intrahepatic disease. Some studies provided conflicting recommendations for extrahepatic disease. For example, two studies

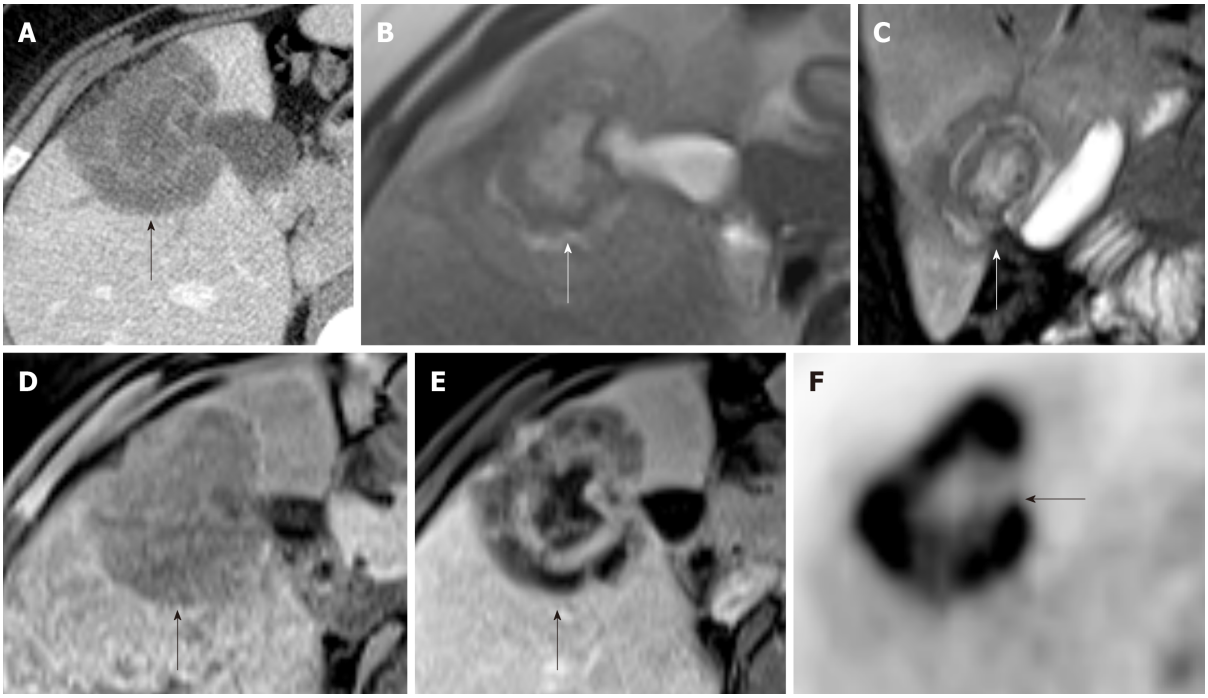

Figure 6 Imaging of hepatic hemangioepithelioma. A: Axial contrast enhanced CT image; B: Axial T2 weighted images; C: coronal T2 weighted images; D: Axial pre contrast T1 Weighted Image; E: Axial post contrast T1 Weighted Image MRI Images; and F: Axial ^{18}F-labeled fluoro-2-deoxyglucose positron emission tomography/computed tomography attenuation corrected image show a hepatic hemangioepithelioma nodule (arrow) with fluoro-2-deoxyglucose update.

suggested extrahepatic spread as an acceptable criteria for liver transplants, since its resection did not necessarily correlate with survival[2], while a study considered it as a surgical contraindication[57,70]. Therefore, clear criteria for surgical procedures are yet to be established.

In a retrospective single center study from 2003-2014, three out of six patients who underwent hepatic resection had disease relapse and all of them survived (Figure 9). On the other hand, one out of two patients who underwent liver transplants died from complications of metastasis[70]. Consequently, there was an overall decrease in disease free survival rates for both surgical interventions. For the hepatic resection group, the disease-free survival rate decreased from 83.3% to 44.4% for one and three years respectively, while only one out of two patients had recurrence after two months (with the other surviving without recurrence) in the liver transplant group. Another case report showed rapid recurrence after only 1-month post liver transplant[71]. Conversely, larger studies have demonstrated that hepatic resection has better OS rates than liver transplants. Mehrabi *et al*[2] reported that the 5-year survival rate of patients that underwent hepatic resection and liver transplant was 75% and 54.5%, respectively, while Grotz *et al*[57] reported 86% and 73% 5-year survival rates, respectively. Consequently, disease free survival is higher in hepatic resection compared to liver transplants 62% and 46%, respectively. However, due higher number of hepatic resections compared to liver transplants, large patient cohort studies are required to achieve statistically significant results.

Interestingly, a retrospective study of 149 patients from the European Liver and Transplant Registry proposed an EHL -LT scoring system to predict the risk of post-transplant recurrence[72]. This study recommended liver transplants rather than observation as the main intervention due to better prognosis and concluded that extrahepatic disease was not found to be a significant risk factor. In fact, this study suggested that lymph node metastasis should not necessarily delay liver transplant. Consequently, macrovascular invasion, waiting time of 120 d or less for transplant, and hilar lymph node invasion were all found to be risk factors for post-transplant recurrence in multivariate regression analysis. This can potentially impact management of EHL, since macrovascular invasion can be detected before transplant, but imaging modalities still need further refinement to increase sensitivity and specificity. This scoring system has the potential to guide health care providers on whether or not to pursue liver transplant and to determine the frequency of post-transplant follow up. On the other hand, in order to provide a chance for administering neoadjuvant therapy, a mandatory waiting time from diagnosis to liver transplant is recommended to provide a chance for administering neoadjuvant

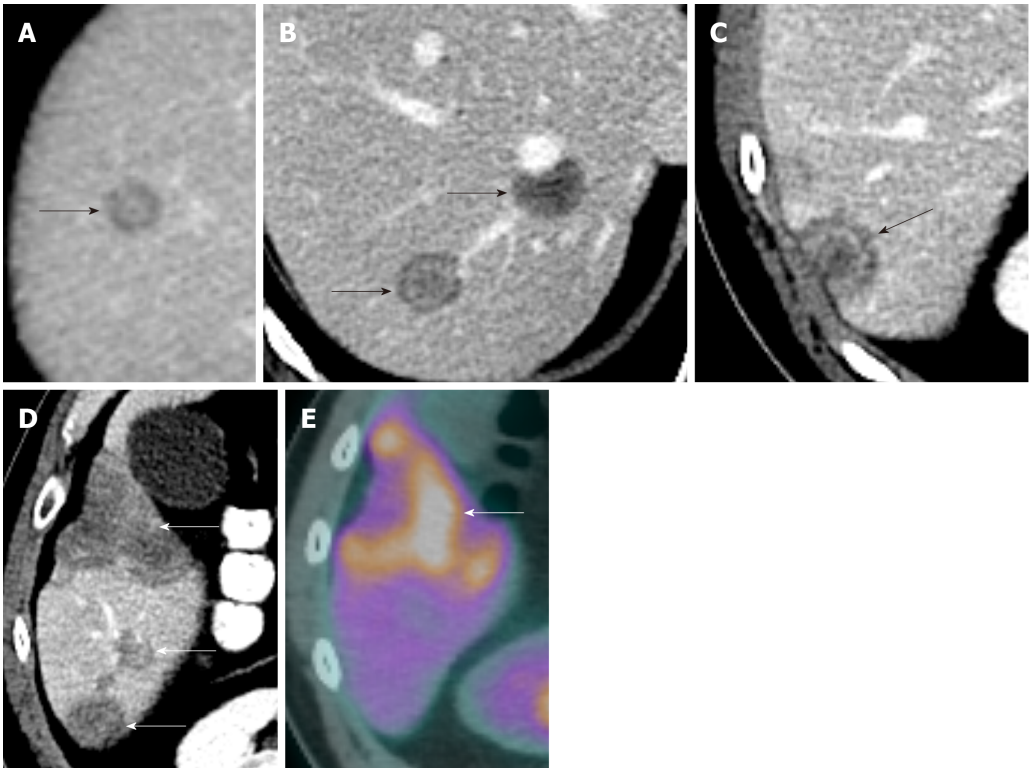

Figure 7 Imaging of hepatic hemangioepithelioma. A, B, C and D: Axial contrast enhanced CT images show hepatic hemangioepithelioma nodules (arrows); E: Axial ^{18}F-labeled fluoro-2-deoxyglucose positron emission tomography/computed tomography image shows a heterogeneous uptake by hepatic hemangioepithelioma nodule (arrow), while rest of the nodules showed no fluoro-2-deoxyglucose update.

therapy. In addition, increasing waiting time could help prevent avoid inappropriate liver transplants for cases misdiagnosed as EHL such as hepatic hemangiosarcoma which is associated with poorer prognosis post-transplant[72,73]. Consequently, a 93.9% 5-year survival rate was observed for patients with prognostic score of two or less based on the analysis of the European Liver Transplant Registry- European Liver and Intestinal Transplant Association (ELTR-ELITA) registry, while patients with a prognostic score of six or higher had a significantly lower ($P < 0.001$) 5-year survival rate of 38.5%[72]. Furthermore, adjuvant therapy could be assigned according to the new prognostic scoring system. Patients with a low score should have the priority for liver transplants, due to lower risk of post-transplant recurrence, while those with a high score should be given low doses of immunosuppression or antineoplastic immunosuppression combined with other neoadjuvant therapy[61,63,64,74,75].

The left hepatic artery ligation (HAL) has been used in treatment of disseminated disease complicated by severe congestive heart failure (CHF). Bachmann *et al*[68] described a case of neonatal CHF refractory to digitalis that resolved with uncomplicated left HAL under Doppler ultrasound. According to literature, HAL generates favorable outcomes for EHL related CHF refractory to medical treatment. However, it is discouraged for EHL related CHF associated with other severe symptoms due to higher rates of complication including death. For example, one patient with thrombocytopenia and another with portal hypertension both died from diffuse intravascular coagulation[76] and biliary complications[77], respectively. Therefore, more research is required to define optimum criteria for surgical candidates and prevent ineffective and unnecessary surgery.

CONCLUSION

EHL has a very low incidence rate, and the pathogenesis is not completely known. The imaging characteristic "halo sign" and "lollipop sign" on CT and MRI can aid in diagnosis. The differential diagnosis includes angiosarcoma, cholangiocarcinomas (CC), metastatic carcinoma, and HCC (sclerosing variant). The histological and IHC findings confirms the diagnosis. Currently, there are no standardized guidelines for the management. The treatment options are broad and include chemotherapy, ablation, surgery and liver transplantation, with inconsistent results.

Table 1 Summaries of medical management studies for hepatic hemangioepithelioma

Study	Year	Country	Patients	Medical management	Dose	Outcome	Duration of follow up
Salech *et al*[78]	2010	Chile	1	Thalidomide	300 mg daily	Partial response	109 mo
Raphael *et al*[79]	2010	United Kingdom	1	Thalidomide	400 mg daily	Stable disease	84 mo
Kassam and Mandel[80]	2008	Canada	1	Thalidomide	400 mg twice daily	Progressive disease	Not available
Bolke *et al*[81]	2006	Germany	1	Thalidomide	Unknown	Progressive disease/death	Not available
Mascarenhas *et al*[49]	2005	United States	1	Thalidomide	Unknown	Partial response	Not available
Soape *et al*[60]	2015	United States	1	Thalidomide	200 mg nightly	Progressive disease	12 mo

Table 2 Summaries of chemotherapeutics management studies for hepatic hemangioepithelioma

Study	Year	Country	Patients	Chemotherapy agent	Dose	Outcome	Duration of follow up
Emad *et al*[55]	2019	Egypt	9/28	Propranolol, prednisolone, vincristine, cyclophos-phamide	First line therapy: 0.6–1.2 mg/kg/d propranolol and/or 0.5-2 mg/kg/d prednisolone	Regression on propranolol, propranolol/prednisolone, propranolol/prednisolone/vincristine, propranolol/prednisolone/cyclophosphamide, propranolol/prednisolone/vincristine/cyclophosphamide, prednisolone/interferon (1/2)[1]	Minimum of 12 mo
					Salvage therapy: 1 million units/m^2/wk interferon, 1.5 mg/m^2/wk vincristine	Progression on prednisolone/interferon (1/2)[1], prednisolone/vincristine/cyclophosphamide, Prednisolone/embolization/cyclophosphamide	
Kim *et al*[82]	2010	Japan	1	Carboplatin, paclitaxel, and bevacizumab	15 mg/kg, every 21 d (bevacizumab)	Progression	Not available
Mizota *et al*[83]	2011	Japan	1	Carboplatin, paclitaxel, and bevacizumab	15 mg/kg, every 21 d (bevacizumab)	Progression	3 mo
Calabro *et al*[74]	2007	Italy	1	Interferon α-2a	Not available	Stable disease	Not available
Kayler *et al*[84]	2002	United States	1	Interferon α-2a	3 million units daily	Partial response	4 mo
Marsh R *et al*[85]	2005	United States	1	Interferon α	3 million units, 5 d/wk for 1 yr	Complete response	84 mo
Galvão *et al*[50]	2005	Brazil	1	Interferon alpha 2b	3 million units daily 9 weeks before and 1 week after liver resection	Complete response	36 mo
Agulnik *et al*[64]	2013	United States	1	Bevacizumab	15 mg/kg, every 21 d	Partial response	Not available
Lau *et al*[63]	2015	United States	1	Capecitabine and bevacizumab	Not available	Partial response	6 mo
Lakkis *et al*[86]	2013	France	2	Cyclophos-phamide	50 mg daily continuous	Complete response (1/2) and Partial response (1/2)	6 and 24 mo

Sangro *et al*[87]	2012	Spain	1	Sorafenib	200 mg every 36 hours	Partial response	6 mo
Kobayashi *et al*[62]	2016	Japan	1	Sorafenib	400-800 mg twice daily	Partial response	60 mo

[1]On prednisolone/interferon treatment, regression was reported in 1 patient and progression in the other patient.

Table 3 Summary of surgical management studies for hepatic hemangioepithelioma

Study	Year	Country	Patients	Study Design	Surgical management	Outcome	Duration of follow up
Bachman *et al*[68]	2003	Switzerland	1	Case report	Selective hepatic artery ligation	Stable, asymptomatic, heart failure signs disappeared	48 mo
Bostancı *et al*[65]	2014	Turkey	1	Case report	Selective internal radiotherapy	Partial response	12 mo
Grotz *et al*[57]	2010	United States	11/30	Retrospective	Hepatic resection	A 1-, 3- and 5-year overall survival of 100%, 86% and 86% and a disease free survival of 78%, 62% and 62%, respectively	60 mo
Wang *et al*[88]	2012	China	17/33	Retrospective	Hepatic resection	No significant difference in overall survival between the 17 patients who underwent liver resection alone 3-year survival rate 74.1%	1 patient underwent liver transplant and died 12 mo post-transplant

Table 4 Summary of liver transplant studies for hepatic hemangioepithelioma

Study	Year	Country	Liver transplant patients	Study Design	Reason for liver transplant	Outcome
Emamaullee *et al*[75]	2010	Canada	5/6 (1 patient did chemotherapy and surgical resection)	Retrospective	EHL (5/5), Recurrence (1/5)	1 patient had recurrence twice after two transplants but 2nd transplant resulted in stable disease. 1 patient had recurrence in less than 6 mo post-transplant and passed away less than 1 year post-transplant. 4 patients have stable disease post-transplant
Nudo *et al*[89]	2008	Canada	11/11	Retrospective	EHL	3/11 patients died (2 had recurrence while 1 died due to hepatic artery thrombosis). 4/11 patients had recurrence. 2/5 did surgical resection (both failed and 1/2 patients died at 61 mo post-resection while other patient did a second transplant and patient is still alive). 1/11 patients did radiotherapy. 1/11 patients assigned pegylated interferon and died 11 mo later
Rodriguez *et al*[69]	2007	United States	110/110	Retrospective	EHL	1/110 had operative death and 2/110 patients died within 30 d post-transplant. 1-year, 3-year, and 5-year overall survivals were 80%, 68%, and 64%, respectively. 31/110 were 5-year survivors. 38/110 patients died during follow-up. 12/38 patients died of recurrent EHL with distant involvement. 12/110 required re-transplantation including four patients who did a third transplant. For re-transplantation patients: 1-year, 3-year, and 5-year allograft survivals were 70%, 60%, and 55%, respectively
Mosoia *et al*[90]	2008	France	6/9	Retrospective	EHL	2/6 had recurrence and died (1 patient had recurrence and died at 56 mo while other patient had liver recurrence and died at 6 mo)

Lerut *et al*[58]	2007	France	59/59	Retrospective	EHL	Early (< 3 mo) and late (> 3 mo) post-LT mortality was 1.7% (1 patient) and 22% (14 patients). 14 (23.7%) patients with recurrence after a median time of 49 mo (range, 6-98). 9 (15.3%) patients died of recurrence and 5 survived with recurrent disease. Disease-free survival rates at 1, 5, and 10 yr post-liver transplant are 90%, 82%, and 64%
Mehrabi *et al*[2]	2006	Germany	128/286	Review	EHL	The most common management has been liver transplantation (44.8% of patients), followed by no treatment (24.8%), chemotherapy or radiotherapy (21%), and liver resection (9.4%). The 1-year and 5-year patient survival rates were 96% and 54.5%, respectively, after liver transplant; 39.3% and 4.5%, respectively, after no treatment, 73.3% and 30%, respectively, after chemotherapy or radiotherapy; and 100% and 75%, respectively, after liver resection
Jung *et al*[70]	2016	Korea	2/8	Retrospective	EHL	One patient died from tumor recurrence at 9 mo and the other is alive after 5 years without recurrence
Cardinal *et al*[91]	2009	United States	17/25	Retrospective	EHL	Mean survival of 172 (124-220) mo in the liver transplant group
Abdoh *et al*[71]	2017	Finland	1	Retrospective	EHL	Recurrence after 1 month and died 1 month later
Grotz *et al*[57]	2010	United States	11/30	Retrospective	EHL	1-, 3- and 5-year overall survival of 91%, 73% and 73% and a disease free survival of 64%, 46% and 46% respectively

EHL: Hepatic hemangioepithelioma.

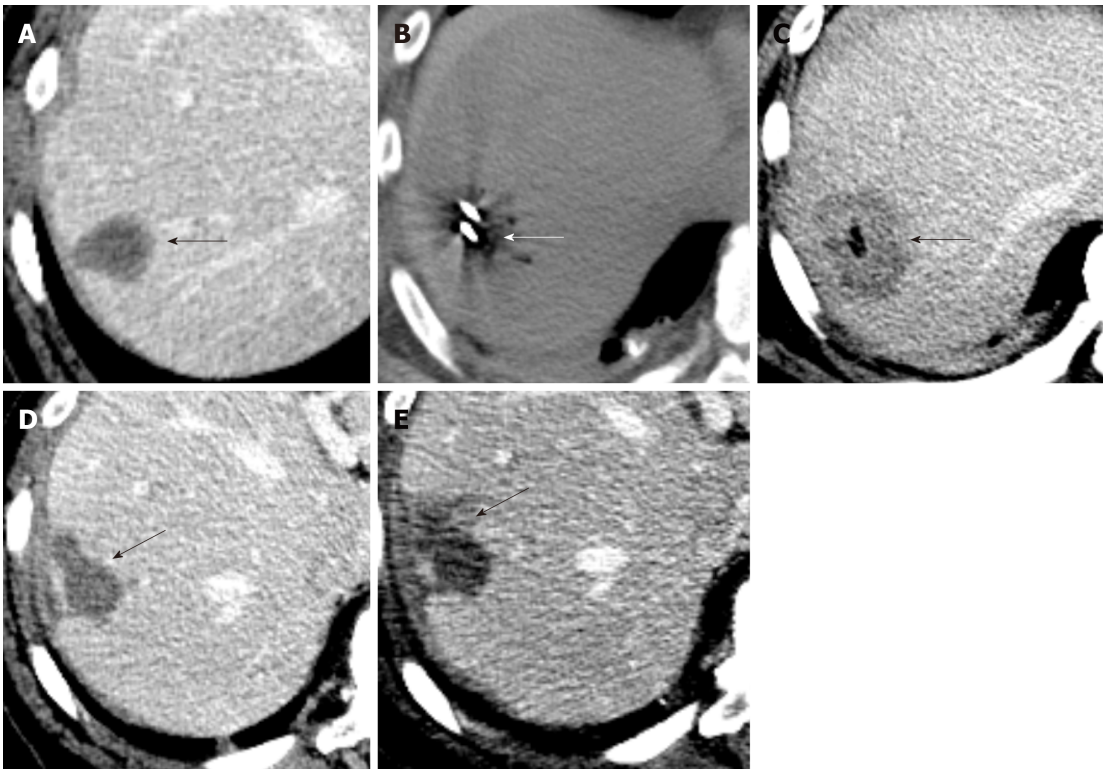

Figure 8 Ablation of hepatic hemangioepithelioma. A: Axial contrast enhanced CT image shows a 3 cm hepatic hemangioepithelioma nodule (arrow) in segment 7 of the liver; B: Axial non-contrast CT image shows microwave ablation of liver of the segment 7 nodule (arrow); C: Immediate post ablation axial contrast enhanced CT image shows an ablation cavity (arrow); D: Follow up axial contrast CT image after 3 mo and; E: Axial contrast CT image 6 mo show an evolving post ablation cavity and tract (arrow).

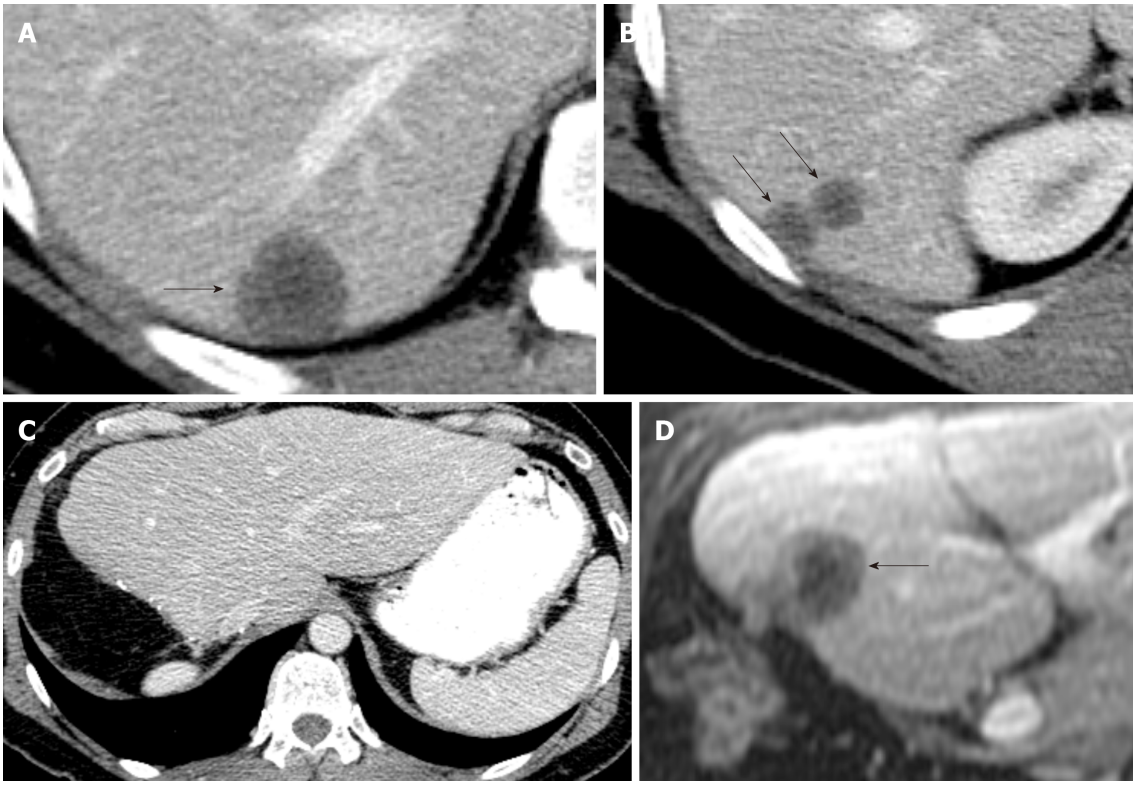

Figure 9 Recurrent hepatic hemangioepithelioma. A and B: Axial contrast enhanced CT images show hepatic hemangioepithelioma nodules (arrows); C: Axial contrast enhanced CT image shows post-surgical changes related to right hepatectomy; D: 3 mo follow up axial post contrast T1 Weighted Image Magnetic Resonance Imaging image shows a recurrent hepatic hemangioepithelioma (arrow).

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ORIGINAL ARTICLE

Basic Study

Changes in extracellular matrix in different stages of colorectal cancer and their effects on proliferation of cancer cells

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Author contributions: Wang ZJ designed the experiments; Li ZL, Wei GH, Yang Y, and Wang XW performed the research; Li ZL wrote the paper.

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Abstract

BACKGROUND

The extracellular matrix is the main component of the tumor microenvironment. Extracellular matrix remodels with the oncogenesis and development of tumors. Previous studies usually focused on the changes of proteins in normal colorectal tissues and colorectal cancers. Little is known about the changes in the extracellular matrix in different stages of colorectal cancer and the effects of these changes on the development of this cancer.

AIM

To test the changes of type I collagen, type IV collagen, matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and tissue inhibitor of metalloproteinase-3 (TIMP-3) in different stages of colorectal cancer and the effects of these changes on the proliferation of cancer cells.

METHODS

The extracellular matrix from various stages of colorectal cancer and normal colon tissue was obtained by using acellular technology. We used proteomics to detect the differential expression of proteins between normal colon tissues and colorectal cancer tissues, and then we used Western blot to observe their expression in each stage of colorectal cancer and in normal colon tissue. By co-culturing the extracellular matrix and HT29 colon cancer cells *in vivo* and *in vitro*, we tested the cancer cell proliferation rate *in vitro* by methyl thiazolyl tetrazolium (MTT) assay and *in vivo* by measuring the tumor volume.

RESULTS

The expression of type I collagen and MMP-2 increased with increased tumor stage. The expression of MMP-9 was higher in colorectal cancer tissues and was

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highest in stage III cancer. The expression of type IV collagen and TIMP-3 decreased with increased tumor stage. The proliferation rate of cancer cells in the extracellular matrix of colorectal cancer was higher than that in the extracellular matrix of the normal colon.

CONCLUSION
These data suggest that the extracellular matrix structure and composition become disorganized during the development of tumors, which is more conducive for the growth of cancer cells.

Key words: Colorectal cancer; Extracellular matrix; MMP; Proliferation; Collagen; TIMP

Core tip: The extracellular matrix remodels during the occurrence and development of tumor. In order to study the changes of extracellular matrix, we obtained the extracellular matrix of colorectal cancer by acellular technology. We found that type I collagen, MMP-2, and MMP-9 increased in the colorectal cancer tissue, while type IV collagen and TIMP-3 decreased in the colorectal cancer tissue. Furthermore, we co-cultured the extracellular matrix and HT 29 cancer cells *in vivo* and *in vitro*, and found that the cancer extracellular matrix was more conducive for the growth of cancer cells than the normal tissue extracellular matrix.

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INTRODUCTION

With the development of the "soil-seed" theory, the effect of the tumor microenvironment on tumor cells has received more attention[1]. The tumor microenvironment is a complex system consisting of the extracellular matrix (ECM), many types of cells, and bioactive factors, which control the complex interactions between tumor cells and stromal cells and between cells and the ECM[2]. During tumorigenesis, the ECM plays a role of "double-edged sword" in the process of tumor proliferation and invasion[3]. On the one hand, the ECM controls the proliferation, differentiation, and metastasis of tumor cells, and acts as a natural barrier. On the other hand, the remodeled ECM provides a loose "soil" for tumor cells to promote the occurrence and development of tumors[4,5]. This process of remodeling occurs at the same time with tumor formation, as shown by changes in the molecular composition, amount, and structure of the ECM[6].

The research on cell function is either in two-dimensional (2D) environment or in three-dimensional (3D) environment. Studies have shown that there are differences in cell proliferation, gene expression, and cell migration in 2D *vs* 3D cultures[6-8]. The 3D *in vitro* experiments were better than 2D cultures in imitating tumor cell microenvironment *in vivo*. The *in vitro* 3D culture refers to tumor cells cultured in collagen, Matrigel, or fibrin[9]. However, none of them can capture the complexity of the native matrix. The tumor ECM obtained by decellularization technology can contain almost all the proteins and their ratios in the natural tissue, which can more realistically simulate the tumor environment in which tumor cells live.

Previous studies have demonstrated that the composition of the ECM changes during cancer formation[10,11]. However, the relationship between the stages of colorectal cancer (CRC) and the changes in the ECM and the proliferation of cancer cells is unclear. Therefore, we obtained the tumor ECM by decellularization and aimed to observe the changes in the ECM during different stages of CRC and the effects of these changes on the proliferation of cancer cells.

MATERIALS AND METHODS

Patients and tissue samples

Tissues were removed from 60 patients with CRC during surgery at Beijing Chaoyang Hospital, Capital Medical University. All procedures in this study were approved by the Medical Ethics Committee of Beijing Chaoyang Hospital, and all patients provided written informed consent. Tumor stage was classified according to the 8th edition of the American Joint Committee on Cancer TNM staging system for CRC. The patients included 34 males and 26 females and none had received preoperative chemoradiotherapy. Of these patients, 10 were classified with stage I disease, 22 with stage II, 19 with stage III, and 9 with stage IV. Ten cases with a normal colon were selected as a control group. All samples were put into the digestive solution composed of 0.25% trypsin and 0.02% EDTA and were continuously oscillated at 37 ℃ for 24 h at 130 rounds per minute. After that, the tissues were put into 0.5% Triton X-100 buffer and continuously oscillated at 150 rounds per minute for 24 h. Then, the ECM was obtained. After freeze-drying, the ECM samples were sterilized by Co-60 radiation and stored at -20 ℃.

Western blot analysis

Western blot assays were performed to detect the protein level. The protein concentrations were tested with a BCA Protein Assay Kit (Pierce, United States). Equal amounts of protein (20 μg) were loaded. Type I collagen, type IV collagen,MMP-2, MMP-9, and TIMP-3 antibodies were purchased from Beijing Biosynthesis Biotechnology Co, LTD. All primary antibodies were used at a 1:1000 dilution. The enhanced chemiluminescence reaction was used to detect the protein bands.

Co-culture of cells and the extracellular matrix

The sterilized ECM was cut into 3 mm × 3 mm × 3 mm pieces under aseptic conditions and placed in a 96-well culture plate. One hundred microliter of colon cancer HT29 cells at a density of 1×10^{6} cells/mL (1×10^{5} cells) was slowly added vertically into the ECM, and then cultured in an incubator containing 5% CO_2.

Methyl thiazolyl tetrazolium assay

The culture medium in the 96-well plate was removed and treated with methyl thiazolyl tetrazolium (MTT) solution for 4 h. After removing the supernatant, 150 μL of DMSO was added to dissolve the tetrazolium salt and measure the optical density using a Multiskan Spectrum Microplate Reader (Thermo Labsystems, Milan, Italy) at 570 nm. The experiment was repeated three times.

Animal experiments

Six-week-old male nude BALB/c mice were randomly divided into five groups with 10 mice in each group. The density of colon cancer HT29 cells was adjusted to 1 × 10^{6} cells/mL. The ECM from each group was cut into 3 mm × 3 mm × 3 mm pieces under aseptic conditions and placed in a 96-well plate. In each well, 50 μL of the above cell suspension (5×10^{4} cells) was slowly added and allowed to stand for 1 h. Abdominal anesthesia was performed with 10% chloral hydrate (0.01 g/mL), and the right forearm underarm skin in each mouse was cut under aseptic conditions, and the ECM and cancer cell complex were embedded subcutaneously. The animals were killed on the 30th day, and the long diameter (a) and short diameter (b) of the tumor were measured with a Vernier caliper. Approximate tumor volume was obtained using the following equation: V = a × b × b/2.

All animals were housed under a 12/12 h light/dark cycle at 22 ℃ and 40%-60% relative humidity conditions. They were given free access to water and food. All animal experimental protocols were done according to the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Science Council, China.

Statistical analysis

Statistical analyses were carried out using the Statistical Package for the Social Sciences version 22.0 and figures were made by GraphPad Prism 6.0. All data are expressed as the mean ± SD. Statistical analyses were performed by means of *t*-tests when two groups were compared or one-way ANOVA when more than two groups. Statistical significance was set at $P < 0.05$.

RESULTS

Preparation of extracellular matrix

An overview of ECM preparation is provided in Figure 1. The normal tissue ECM and CRC ECM were obtained by decellularization. From outward appearance, there was no obvious difference in normal tissue ECM and CRC ECM. All of them presented milk white, sticky surface and soft texture.

High expression of type I collagen, MMP-2, and MMP-9 and low expression of type IV collagen and TIMP-3 in extracellular matrix of colorectal cancer

We used proteomics to analyze the differential expression of proteins in normal colorectal tissue and colorectal cancer tissue, and some of them were selected for analysis in each stage of colorectal cancer (Table 1). The expression of type I collagen was highest in stage III and stage IV and lowest in normal tissue and stage I. Spearman correlation analysis showed that the expression of type I collagen was positively correlated with the stage of CRC (Figure 2). The expression of MMP-2 was higher in the colorectal cancer tissues and it increased with the increased tumor stage. The expression of MMP-9 was higher in the colorectal cancer tissue, but it was highest in the stage III CRC (Figure 3). However, the expression of type IV collagen and TIMP-3 gradually decreased with increased CRC stage. Spearman correlation analysis showed that type IV collagen was negatively correlated with the stage of CRC (Figures 2 and 3).

Extracellular matrix of colorectal cancer is conducive to the proliferation of tumor cells in vitro and in vivo

To study the growth of cancer cells in each group, we co-cultured cancer cells and ECM *in vivo* and *in vitro*. The proliferation of cancer cells was determined *in vitro* by the MTT assay. We found that cancer cells co-cultured with CRC ECM grew significantly better than cancer cells with normal tissue ECM (Figure 4). *In vivo* tumor volume in each group was larger and was greatest in stage IV CRC ECM. Compared to the normal tissue ECM, the CRC ECM was more conducive to the proliferation of cancer cells (Figure 5).

DISCUSSION

The occurrence of malignant tumors is a complex process of interactions between cancer cells and the tumor microenvironment[12,13]. Paget described the relationship between cancer cells and the tumor microenvironment as the seed and soil, indicating that the tumor microenvironment is very important for tumorigenesis and tumor progression[14]. The tumor microenvironment is a unique environment that emerges during the course of tumor progression[12,15]. The tumor microenvironment is composed of ECM, cells, and interstitial fluids, and the ECM is the major component[16]. During cancer progression, the structure and composition of the ECM become disorganized, and this change can promote cellular transformation and metastasis[4,16,17].

Collagen is an important component of the ECM and is considered a structural barrier against tumor invasion[18,19]. Paradoxically, increased expression of collagen is associated with an elevated incidence of proliferation and invasion[20,21]. Abnormal expression of collagens and pathological collagen crosslinking ultimately resulted in increased tissue stiffness and altered tissue homeostasis[6]. The stiff ECM affects many aspects of the cell, such as motility, proliferation, and chemotherapeutic drug efficiency[22,23]. In our study, we found increased expression of type I collagen and decreased expression of type IV collagen in the ECM of CRC. The imbalance of ECM composition could result in an increase in ECM stiffness, which provides enough traction for cell proliferation and migration[24].

MMP-2 and MMP-9 are members of the MMP family, and they play an important role in the degradation and remodeling of the ECM[25]. TIMP-3 exists only in the ECM and could inactivate the MMPs by binding to MMPs[26]. Thus, reaching a balance between TIMPs and MMPs is conducive to the stability of the ECM. The remodeled ECM affects the motility and proliferation of cancer cells[22]. In our study, we found that the expression of MMP-2 and MMP-9 was higher in CRC tissue than in the normal tissue. The expression of MMP-2 increased with increased tumor stage. The expression of MMP9 was highest in stage III and we speculated that this is associated with the deactivation of MMP9.

In conclusion, our study showed that the expression of type I collagen, MMP-2, and MMP-9 increases in CRC while the expression of type IV collagen and TIMP-3

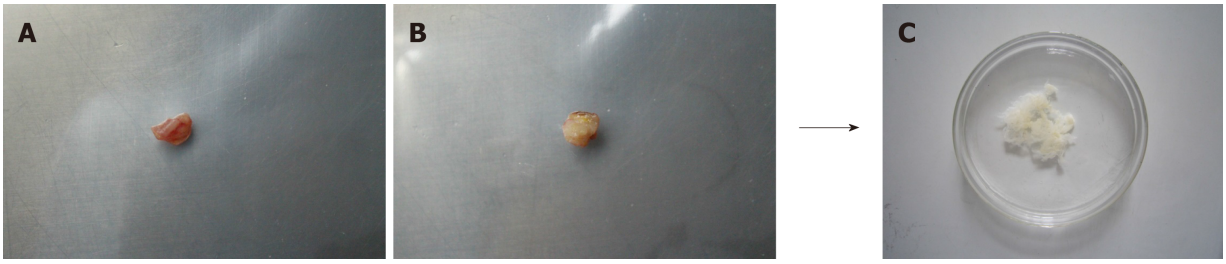

Figure 1 Overview of extracellular matrix preparation. A: Normal human colon tissue; B: Human colorectal cancer tissue; C: Normal human colon tissue or human colorectal cancer tissue were decellularized and shown in a glass culture dish.

decreases in this malignancy. The changes in the composition of the ECM are conducive to cell proliferation. Thus, these findings will provide a new platform for the future design of anticancer drugs based on the biophysical properties of the tumor microenvironment.

Table 1 Analysis of differential expression of proteins in normal colorectal tissues and colorectal cancer tissues

Protein	Description	T *vs* N fold-change	Regulated type	*P* value
A0A024R6R4	MMP2	1.2683	Up-regulated	0.042
P14780	MMP9	1.3930	Up-regulated	0.031
P35625	TIMP3	0.5057	Down-regulated	0.026
P02462	Collagen IV	0.4551	Down-regulated	0.049
P02452	Collagen I	1.9724	Up-regulated	0.018

N: Normal colorectal tissues; T: Colorectal cancer tissues. Statistically significant ($P < 0.05$).

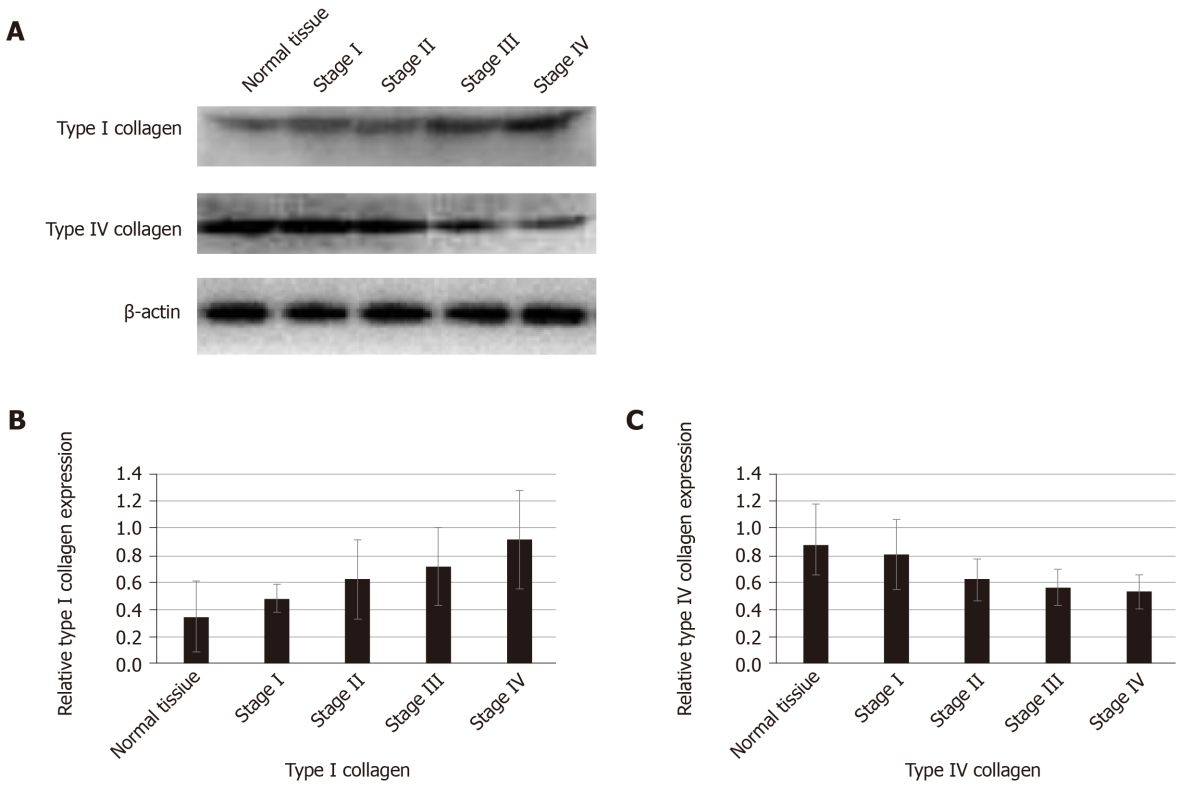

Figure 2 Expression of type I collagen and type IV collagen in normal tissue and colorectal cancer. A: Western blot showing the expression of type I collagen and type IV collagen; B: The expression of type I collagen in the extracellular matrix (ECM) of stages III and IV colorectal cancer was highest. In the ECM of stages I and II colorectal cancer, the expression was relatively low. The expression of type I collagen was positively associated with the stage of colorectal cancer ($r = 0.706$, $P < 0.01$); C: The expression of type IV collagen was negatively correlated with the stage of colorectal cancer ($r = -0.796$, $P < 0.01$).

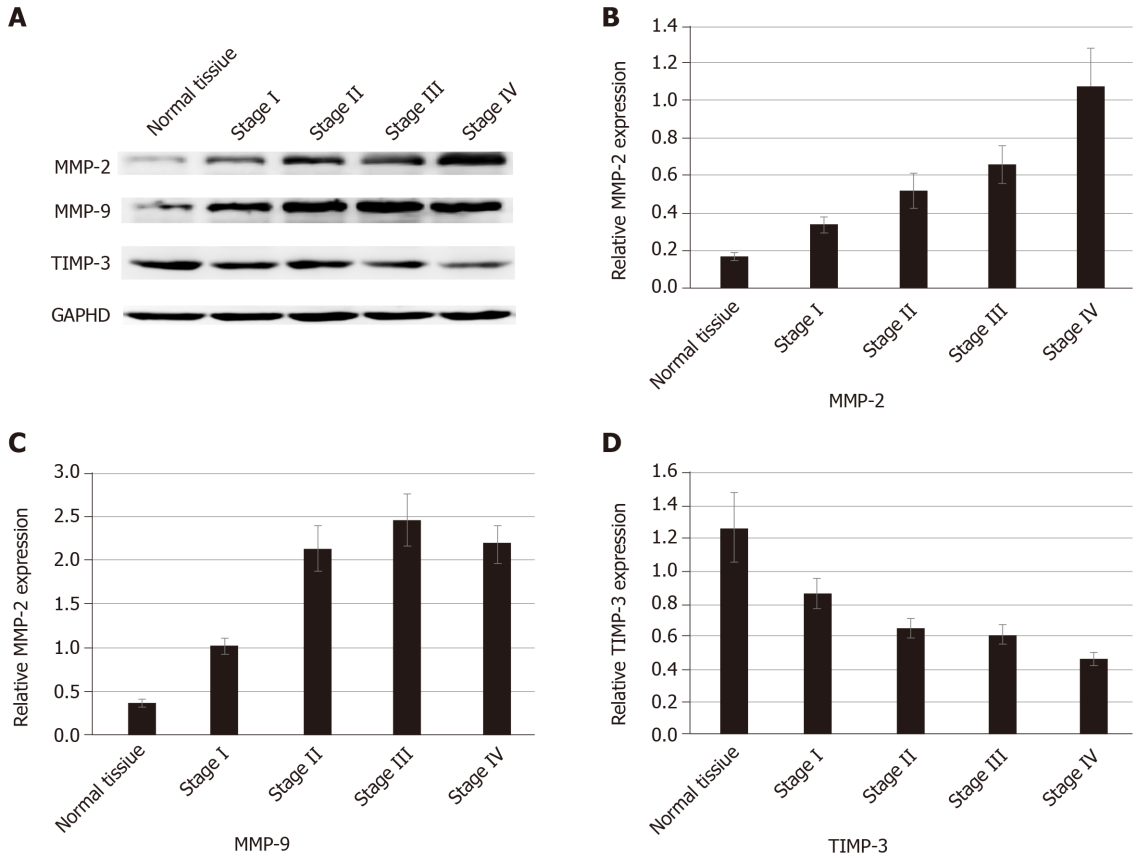

Figure 3 Expression of MMP-2, MMP-9, and TIMP-3 in normal tissue and colorectal cancer. A: Western blot showing up-regulated expression of MMP-2 and MMP-9 and down-regulated expression of TIMP-3 in colorectal tissues; B: The expression of MMP-2 increased with increased tumor stage; C: The expression of MMP-9 in the colorectal cancer tissues was higher than that in the normal tissue and it was highest in the stage III colorectal cancer; D: The expression of TIMP-3 decreased with increased tumor stage.

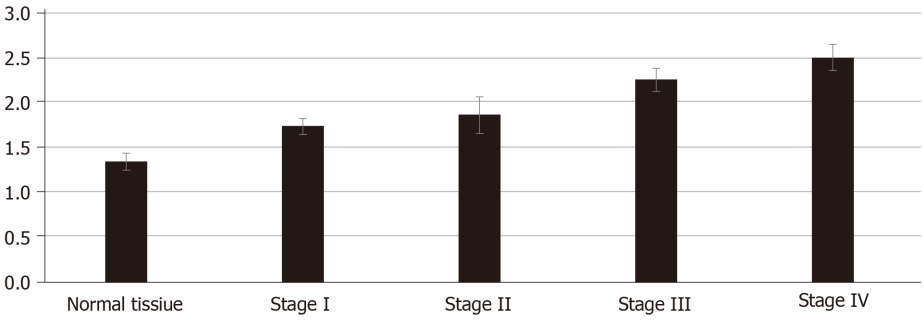

Figure 4 Cancer cells co-cultured with colorectal cancer extracellular matrix and normal tissue extracellular matrix *in vitro*. Compared to the optical density (OD) value of the normal tissue extracellular matrix (ECM), the OD value of colorectal cancer (CRC) ECM was higher. When comparing every two OD values of the CRC ECM, the difference between stage I ECM and stage II ECM was not statistically significant (P = 0.138).

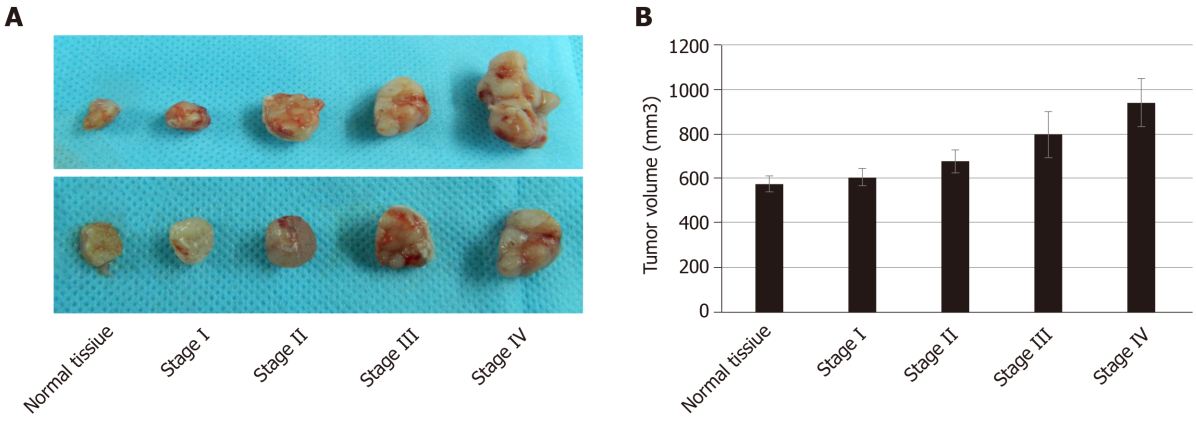

Figure 5 Cancer cells co-cultured with colorectal cancer extracellular matrix and normal tissue extracellular matrix *in vivo*. A: The volume of tumor in colorectal cancer extracellular matrix (ECM) was bigger than that in the normal tissue ECM; B: When comparing every two tumor volumes, the differences between stage I ECM and normal tissue ECM and between stage I ECM and stage II ECM were not statistically significant (P = 0.526 and 0.152, respectively).

ARTICLE HIGHLIGHTS

Research background

The extracellular matrix is not only the substantial support for tumor cells but also promotes the occurrence and development of tumors.

Research motivation

The extracellular matrix changes in the structure and composition during the process of oncogenesis and development of tumors. However, little is known about the changes of the extracellular matrix in different stages of colorectal cancers and the effect of these changes on the development of colorectal cancer. The answer to this may provide a new platform for the future design of anticancer drugs.

Research objectives

In this study, the authors aimed to study the changes of the extracellular matrix in different stages of colorectal cancer and the relationship between the changes of the extracellular matrix with the proliferation of cancer cells.

Research methods

The extracellular matrix was obtained by acellular technology from 60 colorectal cancer patients. Type I collagen, type IV collagen, MMP-2, MMP-9, and TIMP-3 were analyzed by Western blot. Besides, the extracellular matrix and the cancer cells were co-cultured *in vivo* and *in vitro* to study the effect of the extracellular matrix on the cancer cell proliferation.

Research results

The expression of type I collagen, MMP-2, and MMP-9 increased with increased tumor stage. The expression of type IV collagen and TIMP-3 decreased with increased tumor stage. The changed extracellular matrix promotes the cancer cell proliferation.

Research conclusions

This study showed that the extracellular matrix plays an important role in the development of tumor and this provides a certain theoretical basis for anti-tumor therapy.

Research perspectives

The tumor microenvironment is a complex system. The extracellular matrix obtained by decellularization provides an ideal tumor model to study the occurrence and development of tumor.

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ORIGINAL ARTICLE

Basic Study

Increased KIF21B expression is a potential prognostic biomarker in hepatocellular carcinoma

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Abstract

BACKGROUND

The kinesin superfamily protein member KIF21B plays an important role in regulating mitotic progression; however, the function and mechanisms of KIF21B in cancer, particularly in hepatocellular carcinoma (HCC), are unknown.

AIM

To explore the role of KIF21B in hepatocellular carcinoma and its effect on prognosis after hepatectomy.

METHODS

First, data on the differential expression of KIF21B in patients with HCC from The Cancer Genome Atlas database was analyzed. Subsequently, the expression levels of KIF21B in HCC cell lines and hepatocytes were detected by reverse transcription-polymerase chain reaction, and its biological effect on BEL-7404 cells was evaluated by *KIF21B* knockdown. Immunohistochemical analysis was used to validate the differential expression of KIF21B in HCC tissues and adjacent normal tissues from 186 patients with HCC after hepatectomy. The Kaplan-Meier method was used to assess prognosis significance.

RESULTS

KIF21B expression levels were significantly higher in HCC tissues than in corresponding adjacent normal tissues. The expression levels of KIF21B in four HCC cell lines were higher than that in normal liver cells. Functional experiments

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showed that *KIF21B* knockdown remarkably suppressed cell proliferation and induced apoptosis. Moreover, immunohistochemistry results are consistent with The Cancer Genome Atlas analysis, with KIF21B expression levels being increased in HCC tissues compared to adjacent normal tissues. Univariate and multivariate analyses revealed KIF21B as an independent risk factor for overall survival and disease-free survival in patients with HCC after hepatectomy.

CONCLUSION
Taken together, our results provide evidence that KIF21B plays an important role in HCC progression and may be a potential diagnostic and prognostic marker for HCC.

Key words: Hepatocellular carcinoma; KIF21B; Proliferation; Apoptosis; Prognosis

Core tip: The kinesin superfamily protein member KIF21B plays an important role in regulating mitotic progression; however, the function and mechanisms of KIF21B in cancer, particularly in hepatocellular carcinoma, are unknown. We explored the role of KIF21B in hepatocellular carcinoma and elucidated its clinical significance. Our findings suggest that KIF21B may be a potential biomarker for hepatocellular carcinoma.

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INTRODUCTION

According to the global cancer statistics in 2018, hepatocellular carcinoma (HCC) ranked sixth and third in incidence and mortality, respectively[1]. In China, its incidence and mortality ranked fourth and third, respectively[2]. Patients with HCC who are diagnosed at an early stage have a relatively favorable prognosis, with a 5-year survival rate of 75%[3]. However, most HCC patients are diagnosed at advanced stages, and recurrence and metastasis remain major challenges in HCC treatment, leading to an extremely poor prognosis[4]. Thus, there is an urgent need to identify novel diagnostic and prognostic biomarkers for HCC.

Microtubule kinesin motor proteins participate in a series of cellular processes, such as mitosis, motility, and organelle transportation, and are involved in human carcinogenesis[5]. Kinesins are molecular motor proteins that move along microtubule tracks and perform various functions in intracellular transport and cell division[6]. Previous studies have reported that kinesins play critical roles in tumorigenesis and the progression of malignancies[7,8]. KIF21B, a member of the kinesin superfamily of proteins, plays an important role in regulating mitotic progression. Studies have confirmed that KIF21B was found in several types of cells, including neurons and immune cells[9-11]. Genetic alterations in the KIF21B protein have been linked with several neurodegenerative diseases[12]. KIF21B performs in excitatory synaptic transmission and silencing the expression of KIF21B inhibits its function and affects its biological behavior[13]. However, no previous studies have focused on the function and mechanisms of KIF21B in cancer, particularly in HCC. The role of KIF21B in HCC and its effect on prognosis have not yet been investigated and remain unknown.

In this study, we explored the role of KIF21B in HCC and elucidated its clinical significance. We first assessed the differential expression of KIF21B using the Cancer Genome Atlas (TCGA) database. Then, we measured its biological effect on BEL-7404 cells after transfection with *KIF21B*-specific small interfering RNA (siRNA). Kaplan-Meier survival analysis demonstrated that KIF21B was an independent risk factor for overall survival and disease-free survival in patients with HCC. KIF21B may serve as a novel prognostic marker and as a therapeutic target in the treatment of HCC.

MATERIALS AND METHODS

Clinical tissue specimens

A total of 186 HCC tissue specimens and matched adjacent normal tissues collected at Gansu Provincial Hospital between 2013 and 2018 were included in the study. In accordance with the protocol used to obtain the tissue samples, informed consent was obtained from all donors and recipients. The study was approved by the Ethics Committee of Gansu Provincial Hospital. The characteristics of patients with HCC are summarized in Table 1. Two independent pathologists diagnosed HCC based on the World Health Organization criteria. None of the patients had received chemotherapy, radiation therapy, or transarterial chemoembolization prior to surgery. HCC tissues and adjacent normal tissues were collected, fixed with 10% formaldehyde solution, and embedded in paraffin.

The Cancer Genome Atlas dataset and analysis of differential expression of KIF21B

The KIF21B expression profile and relative clinicopathologic features of patients with HCC were selected from the TCGA database (https://portal.gdc.cancer.gov/legacy-archive/search/f). The database included 50 paired cancer and adjacent normal tissues, which were used to examine the differential expression of KIF21B between HCC and adjacent normal tissues. The data were standardized using the Trimmed Mean of M-values method, and biological coefficient of variation was performed for the quality control. Differentially expressed genes were accessed using the TCGA analyze-DEA function considering a log2 fold change > 1 or < −1. The fold change was the ratio of gene expression in the cancer samples to that in the adjacent normal samples. Differences with $P < 0.05$ were considered statistically significant.

Cell culture and transfection

HCC cell lines Hep-G2, BEL7402, BEL-7404, and SMMC-7721 and the normal liver cell line Chang liver were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). BEL7402, BEL-7404, and Chang liver cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco, Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc.). SMMC-7721 and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum. The cell lines were cultured in a 37 °C incubator with 5% CO_2. To further probe the role of KIF21B in HCC cells, KIF21B expression in BEL-7404 cells was silenced using lentivirus-mediated siRNA. In brief, cells were transfected for 24 h with lentiviral constructs expressing short hairpin RNA (shRNA) specific for KIF21B (shKIF21B; Shanghai Genechem Co., Ltd.) or control shRNA (shCtrl; Shanghai Genechem Co., Ltd.). Green fluorescence was used to estimate the efficiency of transfection. Stable knockdown cells were selected using puromycin.

Real-time quantitative reverse transcription-polymerase chain reaction analysis

TRIzol reagent (Shanghai Pufei Biotech Co., Ltd, Shanghai, China) was used to extract total RNA from BEL-7404 cells, which was used as a template for synthesis of cDNA using M-MLV Reverse Transcriptase (Promega, Beijing, China). For real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR), cDNA was mixed with SYBR Master Mixture (TAKARA, Kyoto, Japan) and amplified using a real-time PCR thermocycler (Agilent Technologies, Beijing, China). The PCR primers used have the following sequences: *KIF21B* forward, 5-GGATGCCACAGATGAGTT-3 and reverse, 5-TGTCCCGTAACCAAGTTC-3; *GAPDH* forward, 5-TGACTTCAACAGCGACACCCA-3 and reverse, 5-CACCCTGTTGCTGTAGCCAAA-3. PCR amplification was quantitated using the $2^{-\Delta\Delta Ct}$ method. Each sample was amplified in triplicate, and *GAPDH* was used as an internal control. The *KIF21B* and *GAPDH* primers were designed by Shanghai Genechem Co., Ltd.

Western blot analysis

Concentrations of proteins extracted from cells were measured using a Bicinchoninic acid Protein Assay Kit (Beyotime Biotechnology, China) based on the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the total proteins, and the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, United States). The membranes were incubated with the following primary antibodies overnight at 4 °C followed by incubation with the corresponding secondary antibodies: Mouse-human KIF21B antibody (1:400; Sigma-Aldrich) and rabbit-human anti-GAPDH (1:2000; Santa Cruz Biotechnology, CA, United States); goat anti-mouse IgG (1:2000; Santa Cruz Biotechnology CA, United States) and goat anti-rabbit IgG (1:2000; Santa Cruz Biotechnology). GAPDH was used as an internal control.

Table 1 Correlation between KIF21B expression and clinicopathological characteristics

Characteristic	*n*	KIF21B expression		*P* value
		Low	High	
Age (yr)				0.877
> 55	115	42	73	
≤ 55	71	27	44	
Gender				0.286
Male	102	34	68	
Female	84	35	49	
Tumor size (cm)				0.229
> 5	89	29	60	
≤ 5	97	40	57	
Liver cirrhosis				0.543
Yes	103	36	67	
No	83	33	50	
TNM stage				0.046[a]
I-II	105	32	73	
III-IV	81	37	44	
HBsAg				0.003[b]
Yes	110	31	79	
No	76	38	38	
AFP (ng/mL)				0.061
> 400	112	40	72	
≤ 400	74	29	45	
Vascular invasion				0.012[a]
Yes	117	35	82	
No	69	34	35	

$P < 0.05$ was considered significant,
[a]$P < 0.05$,
[b]$P < 0.01$; Statistical analyses were performed using the χ^2 test.

Cell growth assay

BEL-7404 cells were transfected with shKIF21B or shCtrl. Three days later, the cells were seeded into 96-well plates at a density of 2000 cells/well. The cells were incubated at 37 °C with 5% CO_2 for 5 d. The cells were counted daily using a Celigo Imaging Cytometer (Nexcelom Bioscience, Lawrence, MA, United States). Each experiment was performed in triplicate.

MTT assay

Lentivirus-infected BEL-7404 cells were seeded into 96-well plates at a density of 2000 cells/well, and cell viability was assessed using MTT (Genview, Beijing, China). MTT (5 mg/mL) was added to each well (20 μL) and incubated for 4 h at 37 °C. Dimethyl sulfoxide (Shanghai Shiyi Chemical Technology Co., LTD, Shanghai, China) was added to each well (100 μL). The MTT colorimetric assay was performed to detect cell proliferation after 1, 2, 3, 4, and 5 d of incubation. Absorbance by the resulting formazan crystals (solubilized with DMSO) was read at 490 nm using an enzyme-linked immunosorbent assay plate reader.

Fluorescence-activated cell sorting assay

To quantify the effects of shKIF21B on cell apoptosis, the transfected cells were fixed with ice-cold 75% ethyl alcohol at 4 °C overnight. The cells were stained using an Annexin V-APC/7-AAD Kit (eBioscience, Shanghai, China) according to the manufacturer's instructions. The cells were incubated with Annexin V–APC for 15–20 min at room temperature in the dark.

Colony formation assay

Colony formation assays were used to detect the growth of cells. BEL-7404 cells were transfected with shKIF21B or shCtrl. Three days later, 1000 cells/well were seeded

into six-well plates and cultured for 14 d at 37 °C. Cell status was evaluated, and the medium replaced every 3 d. At the end of the culture period, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30–60 min. The fixed cells were washed with PBS, stained with Crystal Violet Staining Solution (Shanghai Yuanye Bio-Technology Co., Ltd. Shanghai, China) for 10–20 min, and washed several times with double-distilled water. The number of colonies formed was counted using a microscope and photographed.

Immunohistochemical assay

Sections of each specimen (5 μm thick) were placed on glass slides, deparaffinized, dehydrated, and boiled in 10 mmol/L citrate buffer for antigen retrieval. After inhibition of endogenous peroxidase activity by incubation for 10 min with methanol containing 0.3% H_2O_2, the sections were blocked with 2% bovine serum albumin for 30 min and incubated overnight at 4 °C with a primary polyclonal rabbit-human KIF21B antibody (1:200; Bioss, Beijing, China). After three washes with PBS, the slides were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG for 30 min, reacted with diaminobenzidine, and counterstained with hematoxylin.

Evaluation of immunohistochemical staining for KIF21B

Expression of KIF21B was independently evaluated by two pathologists who were blinded to the clinical and pathological stage of the patients. Staining results were assessed by taking into consideration both the percentage of positive cells and the intensity of staining. The positive percentage of KIF21B in the cells was evaluated as follows; 0 (< 10%); 1 (10%-25%); 2 (25%-50%); 3 (50%-70%); and 4 (> 70%). Staining intensity was considered as: 0 (negative); 1 (weak); 2 (moderate); and 3 (strong). According to the overall score of the percentage of positive cells × the intensity of staining, which ranged from 0-12, we graded the staining as follows: 0 (negative); 1-4 (weak); 5-9 (moderate); 10-12 (strong). An overall score > 5 was considered as high expression.

Statistical analysis

All the data shown are the results of at least three independent experiments and are expressed as the mean ± SD. The differences between groups were compared using Student's *t*-test. Differences between groups were analyzed using one-way analysis of variance (ANOVA) or χ^2 test. Cumulative survival was compared using the Kaplan–Meier method with the log-rank test. A multivariate Cox proportional-hazards regression model was used to identify independent prognostic factors. Statistical significance was set at $P < 0.05$.

RESULTS

Differentially expressed KIF21B in HCC

Based on the screening of the 50 pairs of HCC/adjacent normal tissues in TCGA database, KIF21B expression in HCC tissue was upregulated in 31 specimens, unchanged in 14, and downregulated in 5 compared to expression in the corresponding normal tissues (Figure 1A-B). We utilized RT-qPCR to detect the expression of KIF21B in the HCC cell lines (BEL-7404, BEL-7402, HepG2, and SMMC7721) and the normal liver cell line (Chang liver). The results showed that *KIF21B* mRNA expression was higher in HCC cell lines than in normal liver cells (Figure 1C). The BEL-7404 cell line was selected for use in subsequent investigations due to the KIF21B expression being at the highest level in this cell line among the five HCC cell lines evaluated.

Efficiency of shRNA-mediated KIF21B knockdown in BEL-7404 cells

To investigate the role of KIF21B, we knocked down KIF21B expression in BEL-7404 cells using shRNA-mediated interference. As shown in Figure 2A, at 72 h after transfection, the proportion of infected cells in both the shCtrl and shKIF21B groups had reached 80%. Based on the RT-qPCR results (Figure 2B) and Western blot analysis (Figure 2C), the expression of *KIF21B* mRNA and KIF21B protein in the shCtrl group was significantly higher than that of the shKIF21B group.

Silencing of KIF21B expression suppresses HCC cell growth, proliferation, and colony formation and induces apoptosis

BEL-7404 cells transfected with shKIF21B or shCtrl were quantitated for 5 d using Celigo Imaging Cytometer analysis to evaluate cell growth. Silencing KIF21B significantly decreased the total number of cells and the rate of cell growth (Figure 3A-B).

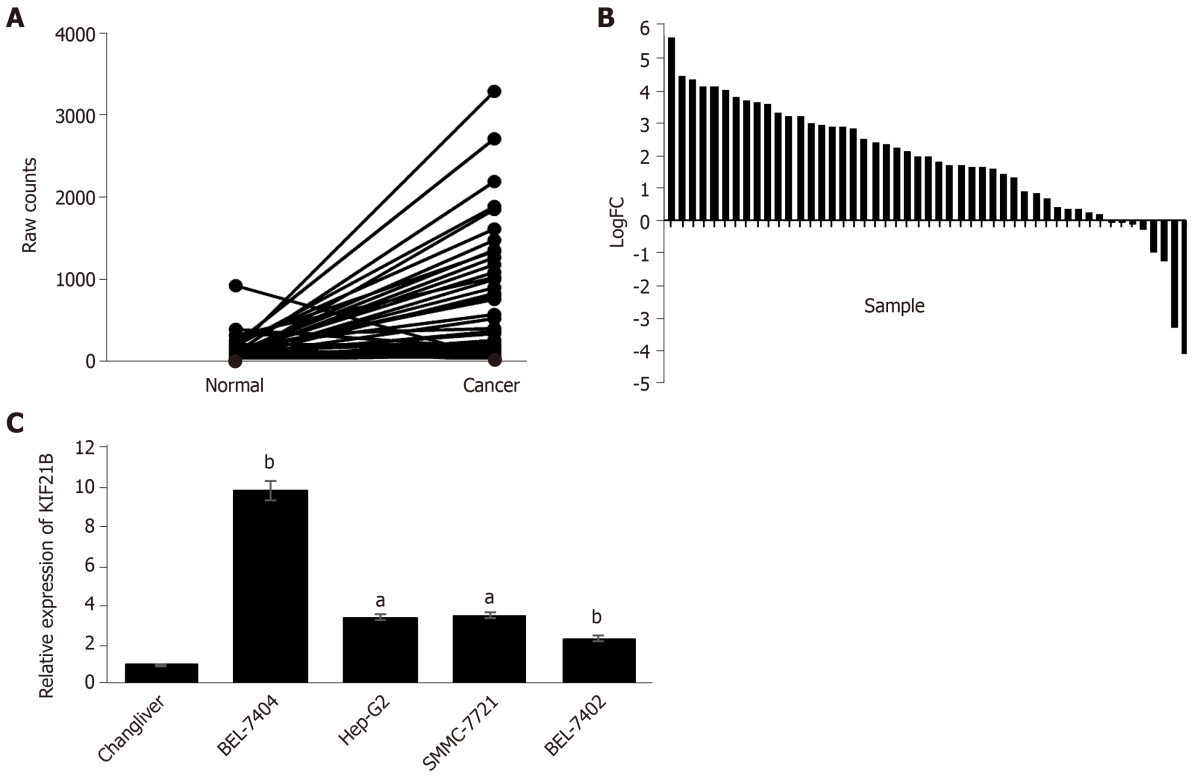

Figure 1 Differential expression of KIF21B in The Cancer Genome Atlas database and hepatocellular carcinoma cell lines. A and B: Differential expression levels of KIF21B in 50 pairs of matched hepatocellular carcinoma and adjacent normal tissues from the The Cancer Genome Atlas database ($P < 0.01$); C: KIF21B expression was examined in BEL-7404, BEL-7402, Hep-G2, SMMC-7721, and Chang liver cells. All the data were normalized to mRNA expression levels of human *GAPDH* using the $2^{-\Delta\Delta CT}$ method. All the experiments were conducted in triplicate. Data were analyzed by ANOVA or *t*-test. The data are reported as the mean ± SD. $P < 0.05$ was considered significant, $^{a}P < 0.05$, $^{b}P < 0.01$.

MTT analysis was used to evaluate the effect of KIF21B on cell proliferation. As shown in Figure 4, the shKIF21B group had lower cell viability compared to the shCtrl group with the difference being statistically significant at 4 d and 5 d post-transfection. Thus, silencing the expression of KIF21B inhibited cell proliferation.

Fluorescence-activated cell sorting analysis was used to determine the effect of KIF21B knockdown in inducing apoptosis. The results showed that the level of apoptosis was significantly increased in shKIF21B-transfected cells compared with that of the shCtrl group. This result suggested that knockdown of KIF21B induced cell apoptosis (Figure 5).

Colony formation assay was used to confirm the effect of KIF21B knockdown on the self-renewal capacity of HCC cells. As shown in Figure 6, the results after transfection of BEL-7404 cells with shKIF21B or shCtrl demonstrated that knockdown of KIF21B significantly reduced the level of apoptosis in the shKIF21B group compared to the shCtrl group.

Relationship between KIF21B expression and clinicopathological characteristics

Immunohistochemistry was used to analyze the relationship between KIF21B expression and clinicopathological characteristics for the 186 patients with HCC. The results revealed that KIF21B was significantly higher in tumor tissues compared to adjacent normal tissue, and it exhibited cytoplasmic expression (Figure 7A-D). Moreover, the results validated the TGCA data regarding KIF21B expression in HCC tissues and adjacent normal tissues. As shown in Table 1, high expression of KIF21B correlated with vascular invasion ($P < 0.05$), TNM stage ($P < 0.05$), and HBsAg ($P < 0.01$). However, KIF21B expression did not correlate with gender, age, tumor size, or alpha fetoprotein (AFP) level ($P > 0.05$).

Relationship between KIF21B expression and survival

Kaplan-Meier analysis of the 186 patients with HCC was performed to evaluate the relationship between KIF21B expression and prognosis. The Kaplan-Meier survival curves suggested that HCC patients with higher KIF21B expression had significantly lower OS ($P = 0.0002$) and DFS ($P = 0.0005$) after hepatectomy (Figure 7E and F). Univariate analysis showed that liver cirrhosis ($P < 0.05$) and KIF21B expression ($P < 0.01$) significantly correlated with OS while age ($P < 0.05$) and KIF21B expression ($P <$

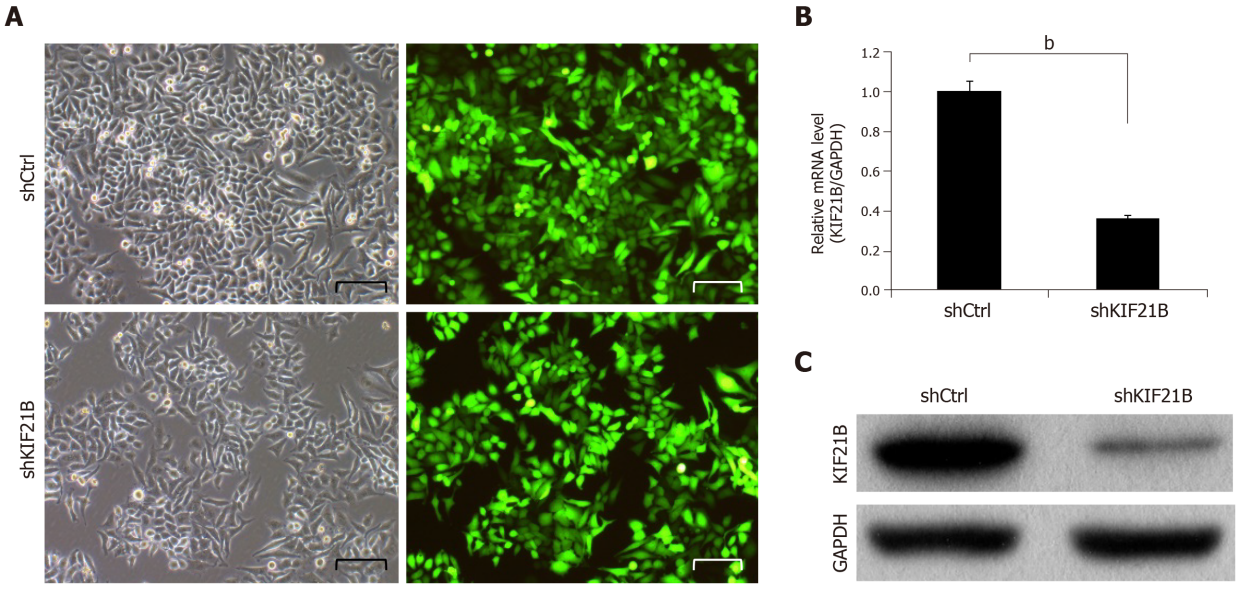

Figure 2 Knockdown of KIF21B using lentivirus-mediated small interfering RNA in hepatocellular carcinoma BEL-7404 cells. A: The proportion of infected cells in both the shCtrl and shKIF21B groups reached 80%; B: Expression level of *KIF21B* mRNA in BEL-7404 cells was significantly decreased in the shKIF21B-treated group compared to the shCtrl-treated group; C: Expression level of KIF21B protein significantly increased in the shCtrl-treated group compared to the shKIF21B-treated group ($P < 0.01$). All the experiments were conducted in triplicate. Data were analyzed by ANOVA or *t*-test. The data are reported as the mean ± SD. $P < 0.05$ was considered significant, $^{b}P < 0.01$.

0.01) significantly correlated with DFS (Tables 2 and 3, respectively). Multivariate analysis indicated that the level of KIF21B expression ($P < 0.01$) was an independent risk factor for OS (Table 2). KIF21B expression level ($P < 0.01$) and age were independent risk factors for DFS (Table 3). Overall, the univariate and multivariate analyses confirmed the potential of KIF21B as an independent risk factor for poor prognosis in HCC.

DISCUSSION

HCC is one of the most common liver malignancies worldwide, leading to extremely high incidence and mortality rates[4]. Due to continued tumor growth and metastasis, current treatment options are limited. Therefore, it is of great significance to determine the underlying mechanism of HCC to identify novel targets for the diagnosis and treatment of patients with HCC.

Kinesins are a superfamily of motor proteins that participate in mitosis, intracellular transportation, and cytoskeletal reorganization[14]. Changes in kinesins play pivotal roles in cell proliferation, invasion, and metastasis of cancer[15,16]. The KIFs member protein KIF21B is a microtubule motor protein that is dependent on ATP[15,17,18]. Recent studies suggest that KIF21B is not only a classic kinesin protein but also a regulator of microtubule dynamics[19-21]. *In vitro* reconstitution studies show that KIF21B increases the microtubule growth rate and catastrophe frequency. Moreover, the purified protein surprisingly associates primarily with depolymerizing microtubule and microtubule ends[22]. A recent study published in 2017 confirmed that KIF21B is a potential microtubule-pausing factor[21]. Microtubules and kinesins play roles in intercellular signal transduction, transport, malignant tumorigenesis, and tumor progression, invasion, and metastasis[11,23]. Therefore, in the current study we performed specific experiments to describe the role of KIF21B in HCC.

Specifically, we evaluated the differential expression of KIF21B across human cancer types by using the TCGA database. Importantly, KIF21B expression levels were found to be significantly increased in HCC tissues compared with adjacent normal tissues. In addition, compared to normal liver cells, the expression of KIF21B was significantly higher in HCC cell lines. Consistent with these observations, immunohistochemistry revealed that KIF21B expression was increased in most HCC tissues. We also measured the growth, proliferation, apoptosis, and colony formation ability of the HCC cell line BEL-7404 after transfection with *KIF21B* siRNA. The results showed that KIF21B qualified as an oncogene in hepatocellular cells by resisting the induction of apoptosis and by promoting cell proliferation and clone formation. Moreover, survival analysis demonstrated that HCC patients with high

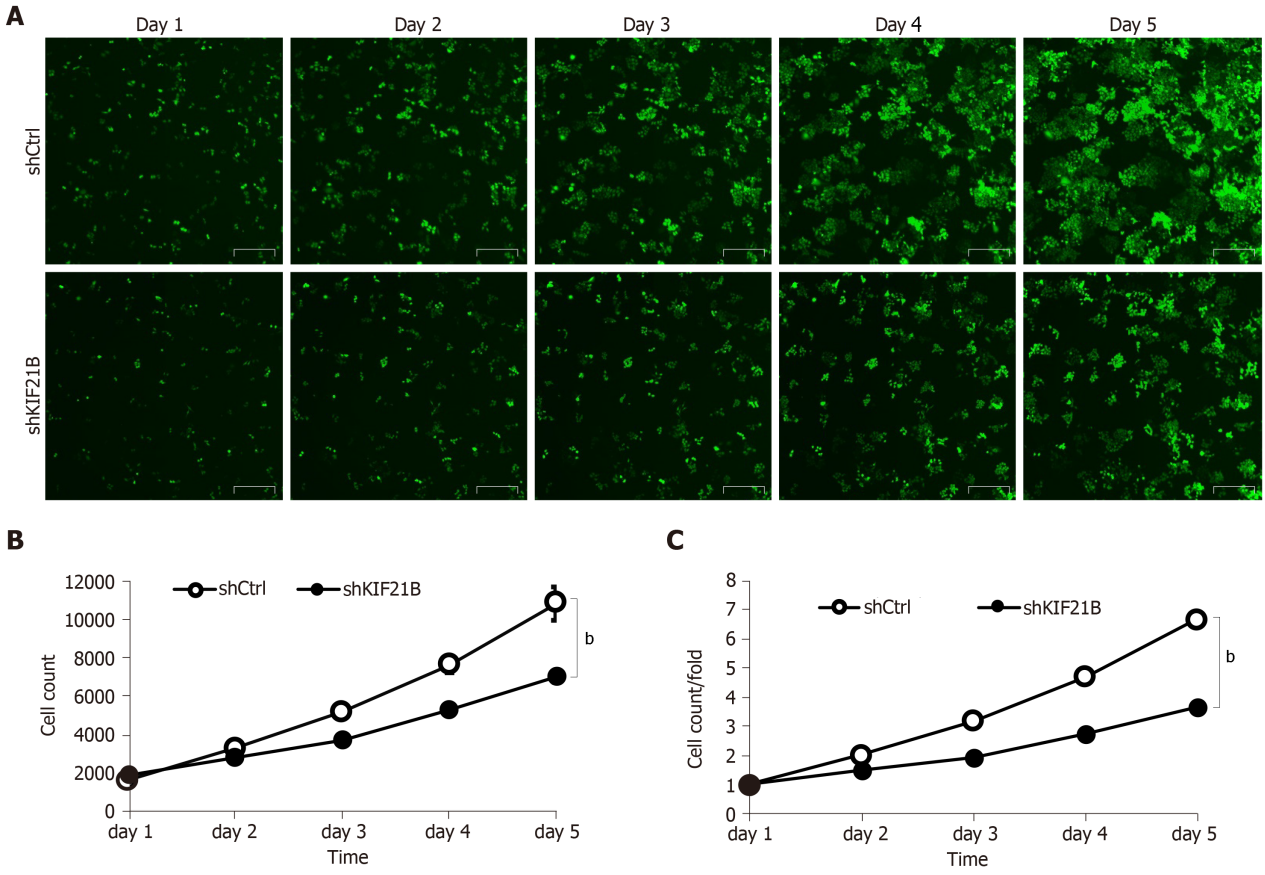

Figure 3 Growth of BEL-7404 cells detected using a Celigo Imaging Cytometer after transfection with short hairpin RNA shCtrl or shKIF21B. A and B: Fluorescence images of cells for each day post-transfection. Data were analyzed by ANOVA or *t*-test. The data are reported as the mean ± SD. $P < 0.05$ was considered significant, $^{b}P < 0.01$.

expression levels of KIF21B exhibited poor OS and DFS.

While the precise mechanisms of KIF21B remain unclear, to our knowledge, this study was the first to explore the biological behavior of KIF21B in HCC. We believe that the special cell lines used may allow KIF21B to decrease cell growth, proliferation, and self-renewal and to induce apoptosis. Furthermore, KIF21B may prove useful as an ideal target for gene therapy for HCC. More studies are needed to determine the effect that KIF21B may have on other tumor cell lines.

In conclusion, KIF21B plays a pivotal role in HCC carcinogenesis. The current cell culture studies confirmed that suppression of KIF21B expression by siRNA inhibited cell proliferation and induced apoptosis in BEL-7404 cells. Our results provide evidence that KIF21B may be considered as a novel prognostic biomarker and a new therapeutic molecular target for HCC.

Table 2 Univariate and multivariate analyses of overall survival in 186 patients with hepatocellular carcinoma

Variable	Overall survival					
	Univariate			Multivariate		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (> 55 yr *vs* ≤ 55 yr)	0.489	0.195-1.225	0.293			
Gender (Male *vs* Female)	0.726	0.291-1.809	0.491			
Tumor size (> 5 cm *vs* ≤ 5 cm)	1.536	0.549-4.297	0.414			
Liver cirrhosis (Yes *vs* No)	2.145	1.083-4.251	0.029[a]	1.188	0.712-1.81	0.510
TNM stage (I-II *vs* III-IV)	0.718	0.387-1.333	0.294			
HBsAg (Yes *vs* No)	0.844	0.476-1.495	0.560			
AFP (> 400 ng/mL *vs* ≤ 400 ng/mL)	1.576	0.842-2.949	0.155			
Vascular invasion (Yes *vs* No)	0.554	0.233-1.316	0.181			
KIF21B expression (low *vs* high)	0.212	0.093-0.482	< 0.001[b]	0.264	0.130-0.539	< 0.001[b]

$P < 0.05$ was considered significant,
[a]$P < 0.05$,
[b]$P < 0.01$. Statistical analyses were performed using Cox regression analysis.

Table 3 Univariate and multivariate analyses of disease-free survival in 186 patients with hepatocellular carcinoma

Variable	Disease-free survival					
	Univariate			Multivariate		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (> 55 yr *vs* ≤ 55 yr)	0.413	0.188-0.909	0.028[a]	0.464	0.284-0.759	0.002[b]
Gender (Male *vs* Female)	0.636	0.281-1.439	0.277			
Tumor size (> 5 cm *vs* ≤ 5 cm)	1.928	0.775-4.975	0.158			
Liver cirrhosis (Yes *vs* No)	1.285	0.724-2.280	0.392			
TNM stage (I-II*vs* III-IV)	0.709	0.423-1.188	0.192			
HBsAg (Yes *vs* No)	0.790	0.480-1.313	0.356			
AFP (> 400 ng/mL *vs* ≤ 400 ng/mL)	1.116	0.657-1.896	0.684			
Vascular invasion (Yes *vs* No)	0.905	0.410-1.997	0.805			
KIF21B expression (low *vs* high)	0.320	0.168-0.611	0.001[b]	0.373	0.216-0.643	< 0.001[b]

$P < 0.05$ was considered significant,
[a]$P < 0.05$,
[b]$P < 0.01$. Statistical analyses were performed using Cox regression analysis.

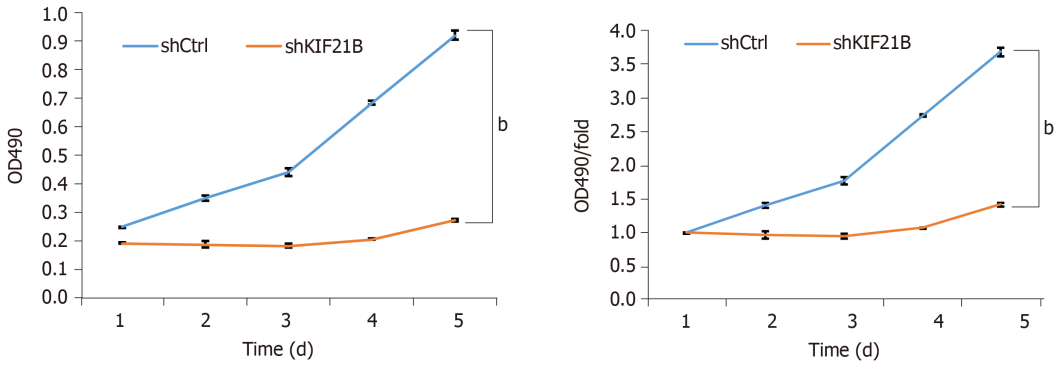

Figure 4 Growth of BEL-7404 cells detected by MTT assay after transfection with short hairpin RNA shCtrl or shKIF21B. Cell proliferation was significantly inhibited in the shKIF21B-transfected group cells. All the experiments were conducted in triplicate. Data were analyzed by ANOVA or *t*-test. The data are reported as the mean ± SD. $P < 0.05$ was considered significant, [b]$P < 0.01$.

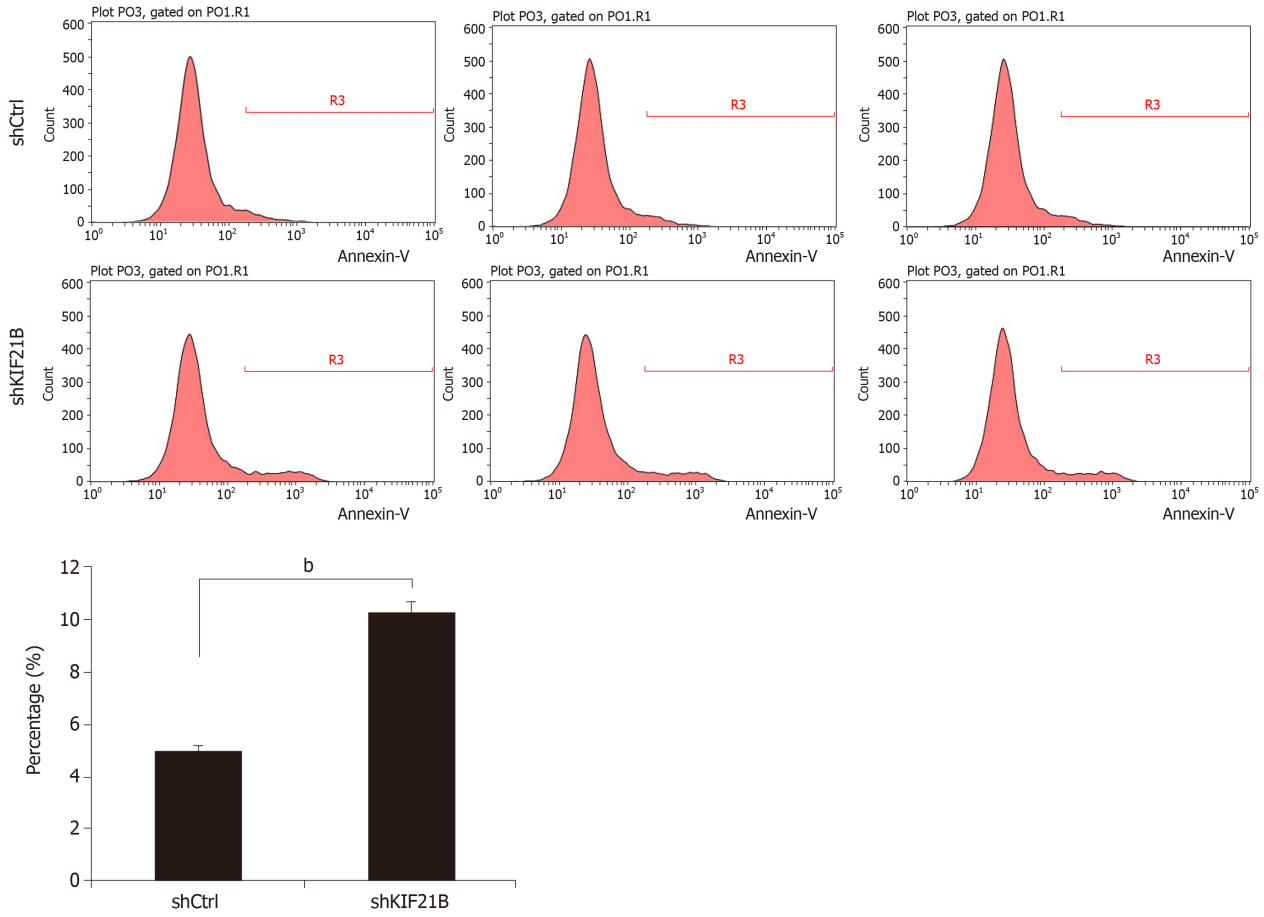

Figure 5 Apoptosis of BEL-7404 cells detected by fluorescence-activated cell sorting after transfection with short hairpin RNA shCtrl or shKIF21B. Cell apoptosis was significantly inhibited in the shCtrl-transfected group compared with the shKIF21B-transfected group. All the experiments were conducted in triplicate. Data were analyzed by ANOVA or *t*-test. The data are reported as the mean ± SD. $P < 0.05$ was considered significant, $^{b}P < 0.01$.

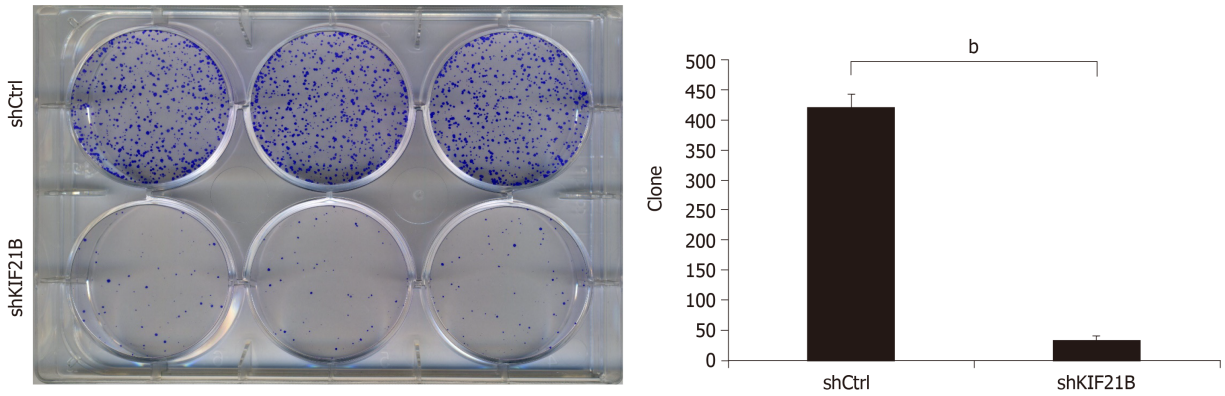

Figure 6 Self-renewal capacity of BEL-7404 cells detected using colony-forming assays after transfection with short hairpin RNA shCtrl or shKIF21B. Self-renewal capacity of cells was significantly inhibited in the shKIF21B-transfected group cells compared with the shCtrl-transfected group. All the experiments were conducted in triplicate. Data were analyzed by ANOVA or *t*-test. The data are reported as the mean ± SD. $P < 0.05$ was considered significant, $^{b}P < 0.01$.

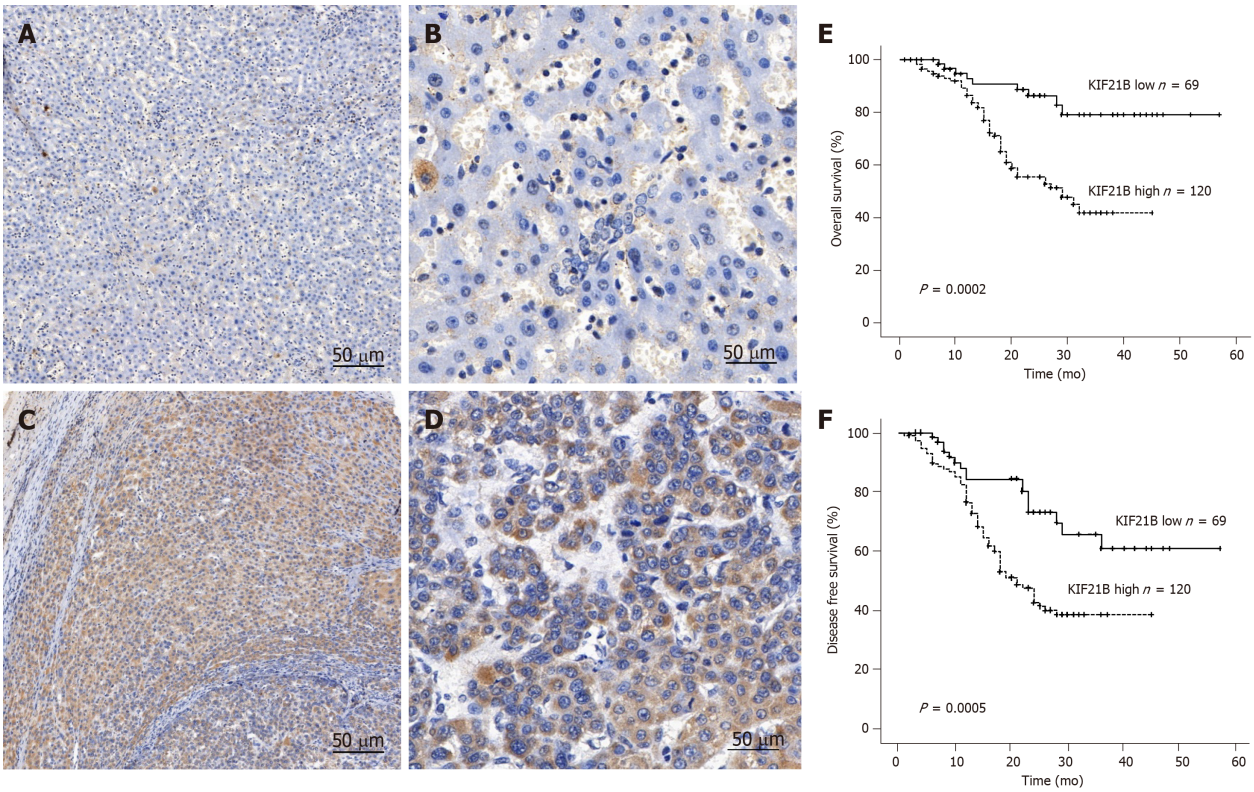

Figure 7 KIF21B expression in hepatocellular carcinoma tissues and normal adjacent tissues. A and B: Low expression of KIF21B in normal adjacent tissues (magnification: ×100 for A and ×400 for B); C and D: High expression of KIF21B in hepatocellular carcinoma tissues (magnification: ×100 for C and ×400 for D); E and F: Kaplan-Meier's survival curves using a log-rank test demonstrating the correlation between KIF21B expression and overall survival or disease-free survival. Dashed lines indicate high KIF21B expression, and solid lines indicate low KIF21B expression.

ARTICLE HIGHLIGHTS

Research background

As one of the most frequent cancers, the morbidity and mortality of hepatocellular carcinoma (HCC) is increasing year by year. The kinesin superfamily protein member KIF21B plays an important role in regulating mitotic progression; however, the function and mechanisms of KIF21B in cancer, particularly in HCC, are unknown.

Research motivation

To explore the role of KIF21B in hepatocellular carcinoma and its clinical significance.

Research objectives

The study aimed to investigate the function of KIF21B in HCC and its effect on prognosis after hepatectomy.

Research methods

First, we analyzed the differential expression of KIF21B in The Cancer Genome Atlas, and used immunohistochemical staining to validate it. Subsequently, after silencing KIF21B expression, the function of KIF21B in HCC lines was investigated by cell growth assay, MTT assay, fluorescence-activated cell sorting assay, and colony formation assay. The Kaplan-Meier method was used to assess its prognostic significance.

Research results

KIF21B expression levels were significantly higher in HCC tissues. Functional experiments showed that KIF21B knockdown remarkably suppressed cell proliferation and induced apoptosis. Statistical analyses revealed KIF21B as an independent risk factor in patients with HCC after hepatectomy.

Research conclusion

KIF21B plays an important role in HCC progression and may be a potential diagnostic and prognostic marker for HCC.

Research perspective

In the future, more studies are needed to determine the effect that KIF21B may have on other tumor cell lines and to investigate the underlying mechanism.

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ORIGINAL ARTICLE

Case Control Study

Association between *interleukin-21* gene rs907715 polymorphism and gastric precancerous lesions in a Chinese population

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Abstract

BACKGROUND

The single nucleotide polymorphisms of interleukin-21 (*IL-21*) gene were confirmed to be related to various diseases, but no studies have examined the possible role of *IL-21* single nucleotide polymorphisms (SNPs) (rs907715, rs2221903, and rs12508721) in gastric precancerous lesions.

AIM

To explore the associations between SNPs of *IL-21* gene (rs907715, rs2221903, and rs12508721) and gastric precancerous lesions in a Chinese population.

METHODS

Three SNPs of *IL-21* were genotyped using polymerase chain reaction–ligase detection reaction in 588 cases and 290 healthy controls from May 2013 to December 2016 in northwestern China. Gastric precancerous lesions were confirmed by endoscopic examination and categorized as non-atrophic gastritis, atrophic gastritis, and intestinal metaplasia. Descriptive statistic and logistic regression were used for data analyses.

RESULTS

IL-21 rs907715 genotype CC and C frequencies were higher in in patients with gastric precancerous lesions than in the controls (OR = 1.59, 95%CI: 1.06-2.38, P = 0.013; OR = 1.28, 95%CI: 1.01-2.22, P = 0.044, respectively) after adjusting for confounding factors. For SNP rs907715 in intestinal metaplasia patients,

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significant differences between cases and controls were observed in the frequencies of genotype CC and C (OR = 1.92, 95%CI: 1.24-2.98, $P = 0.004$; OR = 1.53, 95%CI: 1.04-2.24, $P = 0.028$, respectively); for non-atrophic gastritis and atrophic gastritis patients, the CC and C genotypes showed no significant association with risk in all models. No association between either rs2221903 or rs12508721 and gastric precancerous lesions was found in the present study. In the haplotype analysis, the TC haplotype (rs907715 and rs12508721) and TT haplotype (rs2221903 and rs907715) were more frequent in the case group than control group ($P < 0.05$).

CONCLUSION

Our findings indicate that SNP rs907715 of *IL-21* gene is associated with gastric precancerous lesions. The TC haplotype (rs907715 and rs12508721) and TT haplotype (rs2221903 and rs907715) increased the risk of gastric precancerous lesions. If confirmed, these findings will shed light on the etiology of precancerous lesions.

Key words: *Interleukin-21* gene; Single nucleotide polymorphisms; rs907715; Gastric precancerous lesions; Intestinal metaplasia

Core tip: This study investigated the associations between single nucleotide polymorphisms of interleukin-21 (*IL-21*) gene (rs907715, rs2221903 and rs12508721) and gastric precancerous lesions in a Chinese population. The results showed an association between *IL-21* rs907715 polymorphism and gastric precancerous lesions. *IL-21* rs907715 genotype CC and C frequencies were higher in patients with gastric precancerous lesions than in the controls. Single nucleotide polymorphism rs907715 increased in CC and C genotypes were associated with intestinal metaplasia patients when examined separately. These findings may help clarify the etiology of gastric cancer.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy worldwide, and ranks second in incidence and mortality among all malignancies in China[1,2]. GC is considered to be a multistep progression from non-atrophic gastritis (NAG), atrophic gastritis (AG), intestinal metaplasia (IM), dysplasia, to gastric adenocarcinoma[3]. The worldwide prevalences of AG and IM were 33% and 25% respectively[4]. As the specific recognizable stages of the precancerous cascade[5], gastric precancerous lesions can increase the risk of GC[6,7], so clarifying the etiology of gastric precancerous lesions is of great significance in preventing the development of GC[8]. Multiple factors contribute to the occurrence and development of gastric precancerous lesions, including environmental factors, such as *Helicobacter pylori* infection[9,10], high salt intake[11,12], alcohol consumption[12], and smoking status[13]. Some studies have explored genetic risk factors for precancerous lesions such as *interleukin (IL)-1*, *IL-8*, *IL-10*, and *IL-22*[14-18], but less attention has been given to *IL-21*.

IL-21 is an immune modulatory cytokine produced mainly by activated $CD4^+$T cells and natural killer (NK) cells, and has multiple effects on innate and adaptive immune responses[19]. The activity of *IL-21* is mediated via binding to a compound receptor consisting of *IL-21R* and γ chain[20,21], and the biological functions of *IL-21* include promoting T-cell proliferation, stimulating B-cell differentiation, and enhancing NK-cell activation[22,23]. *IL-21* plays important roles in inflammatory, antiviral, and antitumor responses[24]. Single nucleotide polymorphisms (SNPs) of the *IL-21* gene can change the expression level of mRNA, resulting in a change in protein expression or

autoantibody production[25]. SNPs of *IL-21* have been associated with various diseases of the immune system including systemic lupus erythematosus[26], Graves' disease[27], rheumatoid arthritis[28], and hepatitis B virus (HBV) infection[29]. Several SNPs of *IL-21* (rs907715, rs2221903 and rs12508721) have also been associated with the susceptibility to cancer[30-33]. For example, SNPs rs907715 and rs2221903 reduce the susceptibility to non-small cell lung cancer[30], and SNP rs12508721 is related to thyroid cancer[31], breast cancer[32] and HBV-related hepatocellular carcinoma[33]. Previous studies have found that *IL-21* may be associated with the risk of gastric precancerous lesions[34,35]. However, no studies have examined the possible role of *IL-21* SNPs (rs907715, rs2221903 and rs12508721) in gastric precancerous lesions. Therefore, the present study explored associations between SNPs of *IL-21* (rs907715, rs2221903 and rs12508721) and risk of gastric precancerous lesions in a northwestern Chinese population.

MATERIALS AND METHODS

Subjects

This study was conducted from May 2014 to December 2016 in hospitals from three cities (Yulin, North; Xi'an, Middle; Hanzhong, South) in Shaanxi Province, China (Figure 1). Men and women with gastrointestinal symptoms requiring upper endoscopy examination were screened for study eligibility. Individuals diagnosed with GC were excluded, while a total of 1674 subjects who had undergone upper gastrointestinal endoscopy, completed pathological and 24-hour urine testing were included. The medical records of all subjects were reviewed retrospectively.

Of the eligible and willing subjects, 588 with NAG, AG, or IM (cases) and 290 without any diagnosis of gastric diseases or *H. pylori* infection (controls) were enrolled. This study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Institutional Review Board of Xi'an Jiaotong University Health Science Center. Informed consent was obtained from all subjects.

Data measurements and collection

Demographic information was obtained from subjects' medical records including age, gender, smoking status, drinking status, height, and weight. For smoking status and drinking status, subjects were dichotomized as "yes" or "no". Body mass index was calculated as weight in kilograms divided by height in meters squared.

Daily salt intake was determined by 24-hour urine sodium excretion and was dichotomized as "high salt" and "non-high salt" according to the median of the controls (representing the general population). Subjects were asked to excrete and discard their first urine at 7 a.m. and to collect all urine over the following 24-hours, including the next day first urine at 7 a.m. Total volumes of the collection were measured. Urinary sodium levels were measured by the ion selective electrode method using by Olympus AU 680 autoanalyser.

In this study, NAG, AG, and IM were diagnosed by endoscopic findings and based on updated Sydney system criteria[36] and Atrophy Club criteria[37]. The serum *H. pylori* IgG antibody test was performed by an enzyme-linked immunosorbent assay on the same day of endoscopy. IgG values ≥ 10 U/mL were considered as "*H. pylori* infection" and < 10 U/mL as negative results[38].

Genotyping

Three SNPs (rs907715, rs2221903, and rs12508721) of IL-21 were genotyped in cases and controls. Genomic DNA was extracted from 5 mL peripheral blood samples using the Blood DNA Kit (Tiangen, Beijing, China), and stored at -80°C until subsequent assay. SNPs were genotyped using the polymerase chain reaction (PCR)–ligase detection reaction method using assay-on-demand probes and primers: C_8949748_10 for rs907715, C_16167441_10 for rs2221903, C_1597500_10 for rs12508721. The forward and reverse primers are shown in Table 1. All primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The reaction was performed in a total volume of 20 μL, containing genomic DNA (1 μL), buffer (2 μL), $MgCl_2$ (0.6 μL), dNTPs (2 μL), *Taq* polymerase (0.2 μL), 2 μL of each primer, and 12.2 μL ddH_2O. PCR conditions were as follows: denaturation at 95 °C for 2 min, 94 °C for 30 s; annealing at 56 °C for 90 s; extension at 40 cycles of 65 °C for 30 s, and a final extension at 65 °C for 10 min. Following amplification, PCR products were submitted for DNA sequencing.

Trizol was used for extraction of mRNA from six intestinal epithelium tissues according to the manufacturer's instructions. Quantification of mRNA of rs907715

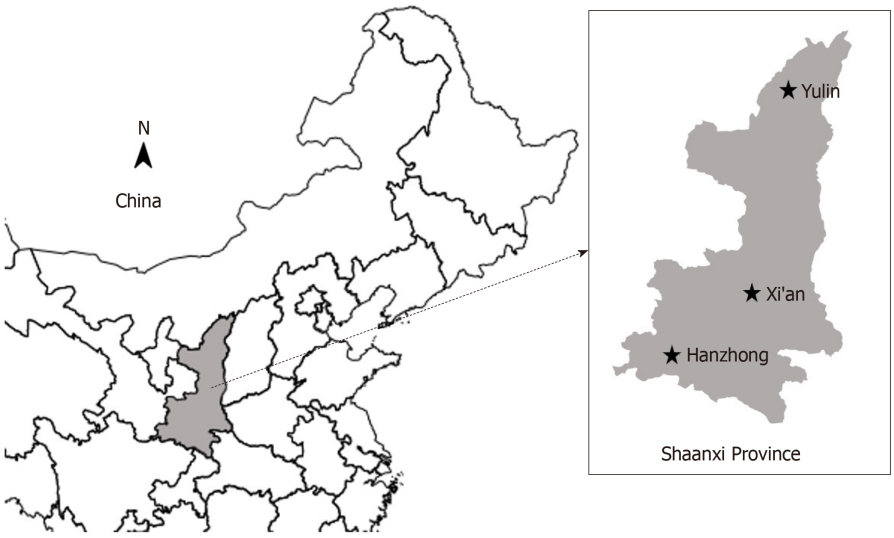

Figure 1 Location map of three selected cities in Shaanxi province, China.

was performed using BioEasy SYBR Green Real Time PCR Kit in a 20 μL reaction volume, containing SYBR Green Master Mix (10 μL), PCR Forward Primer (0.8 μL), cDNA (2 μL), ROX (0.4 μL) and nuclease-free water (6 μL). Extension was performed under the following conditions: Initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. All reactions were performed in duplicate. Using the $2^{-\Delta\Delta Ct}$ method[39] to calculate the relative mRNA expression levels.

Statistical analysis

Descriptive statistics were used to describe demographic characteristics of all subjects in our study. Genotype frequencies of three SNPs (rs907715, rs2221903, and rs12508721) were obtained by statistical description, and Hardy-Weinberg equilibrium was analyzed using the chi-squared goodness of fit test. Logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) of gastric precancerous lesions for genotype, controlling for demographic and lifestyle factors (age, gender, body mass index, drinking status, smoking status, daily salt intake and region). Distribution normality of *IL-21* mRNA expression was assessed using the Kolmogorov–Smirnov test, differences among three genotypes were measured using an independent sample Student's *t*-test. All analyses were performed with SPSS 22.0 software (IBM, Chicago, IL, United States). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Demographic and lifestyle associations

A total of 1674 subjects were included in the study, aged 26 to 88 with a mean age of 49.8 (SD = 11.4). The incidences of NAG, AG, and IM were 6.9%, 6.3%. and 21.9%, respectively (Table 2). The NAG incidence in the south region was higher than that in other two regions, while AG and IM incidences in middle region were higher than those in other two regions.

High salt intake was associated with an increased risk of NAG (OR = 2.58, 95%CI: 1.21-4.88, $P = 0.011$) (Table 3); *H. pylori* infection was correlated with decreased risk of AG (OR = 0.39, 95% 95%CI: 0.65-0.99, $P = 0.041$); and smoking was related to increased risk of NAG (OR = 2.15, 95%CI: 1.19-4.44, $P = 0.015$) and IM (OR = 1.97, 95%CI: 1.40-2.58, $P = 0.005$). Compared with the south region, subjects in the middle region had a lower risk of NAG (OR = 0.33, 95%CI: 0.28-0.51, $P = 0.009$) and a higher risk of IM (OR = 2.95, 95%CI: 1.45-4.33, $P = 0.007$); subjects in the north region had a lower risk of AG (OR = 0.33, 95%CI: 0.21-0.83, $P = 0.010$).

Association of IL-21 gene polymorphisms and gastric precancerous lesions

The genotype distributions of each group were consistent with Hardy-Weinberg equilibrium ($P > 0.05$) (Table 4). In univariate analyses, differences in the distribution frequency of rs907715 genotypes CC and C between cases and controls were statistically significant (OR = 1.77, 95%CI: 1.19-2.63, $P = 0.005$; OR = 1.43, 95%CI: 1.02-

Table 1 Probe primary information for genotyping *interleukin-21* gene polymorphisms

Primer	Type	Primer sequences, 5'→3'
rs907715	F	5'-ATAGATGAGGAAAGTGAGATC-3'
	R	5'- CTTTGCTTATTTGATATATTCC-3'
rs2221903	F	5'-GGACCACATATTGCCAG ACAC-3'
	R	5'-GACACTGACGCCCATATTGAT-3'
rs12508721	F	5'-ATGGGACTAAAGT CAAGGTG-3'
	R	5'-AGATGGCTTCTAGAGTCTGG-3'

2.01, P = 0.039, respectively). Results were similar after adjusting for confounding factors (OR = 1.59, 95%CI: 1.06-2.38, P = 0.013; OR = 1.28, 95%CI: 1.01-2.22, P = 0.044, respectively). Results were also similar for IM when examined separately (OR = 1.92, 95%CI: 1.24-2.98, P = 0.004; OR = 1.53, 95%CI: 1.04-2.24, P = 0.028, respectively). The distribution frequencies of genotype CC and C were not statistically different between cases and controls in all models.

Analyses of rs907715 mRNA expression in intestinal epithelium tissue from six subjects with IM showed significant differences between CC genotype and TT genotype (P < 0.001), CC genotype and CT genotype (P < 0.01) (Figure 2). Similarly, rs907715 mRNA expression levels in six NAG tissues and six atrophic gastritis tissues were conducted. For NAG tissues, the expression level of rs907715 CC genotype was significantly different from that among the rs907715 CT genotype (P < 0.05) and TT genotype (P < 0.01) (Figure 3); for atrophic gastritis tissues, the expression level showed significant difference between rs907715 CC genotype and TT genotype (P < 0.05) (Figure 4).

Haplotype Analyses

The results showed that the TC haplotype (rs907715 and rs12508721) was significantly associated with AG and IM (OR = 3.91, P = 0.003; OR = 2.02, P = 0.004, respectively), and it appeared to be a risk haplotype; the TT haplotype (rs2221903 and rs907715) was significantly associated with IM (OR = 1.44, P = 0.023) and it appeared to be a risk haplotype (Table 5).

DISCUSSION

Our results suggested that rs907715 genotypes CC and C confer increased susceptibility to IM and total gastric precancerous lesions, whereas no association was found for rs2221903 or rs12508721. Because we are not aware of any previous study that directly addressed these associations, our findings should be interpreted cautiously.

Regarding rs907715, Liu *et al*[30], for example, reported that genotype AA and A allele of rs907715 were associated with the decreased susceptibility to non-small cell lung cancer. Xiao *et al*[31] revealed that the G allele of rs907715 increased the susceptibility to Graves' disease. Moreover, a case-control study found that serum *IL-21* levels in HBV patients with rs907715 genotype AA were lower than those in patients with genotype AG/GG; this genotype was independently related to sustained virological response[40]. A meta-analysis showed that the genotype distribution of *IL-21* rs907715 was significantly different between systemic lupus erythematosus patients and healthy controls in all genetic models[26]. All of these findings suggest that rs907715 of *IL-21* may to some extent exert effects on antitumor, antiviral and/or inflammatory processes. However, other studies have shown no association between rs907715 and thyroid cancer[31] and breast cancer[32]. Hence, the associations we observed in our study should be addressed in future studies.

The *IL-21* gene is located on human chromosome 4q26-27 and plays an important role in anti-tumor immunopathology[41]. Previous studies have found that *IL-21* is overexpressed in *H. pylori*-infected gastric mucosa[42], and is correlated with the occurrence and development of gastritis[34,35]. Moreover, studies have found that the concentration of *IL-21* is increased in both tissue and serum of GC patients[43,44]. Thus, IL-21 may play a role in the development and progression of GC and gastritis-related diseases. Previous evidence has shown that SNP rs907715 is associated with increased *IL-21* transcription and expression[40,45]. The rs907715, locating in the third intron of *IL-21* gene, may be a surrogate marker for mutations with functional consequences[25]. SNPs including rs907715 may be in linkage disequilibrium with a variant correlated

Table 2 Demographic and lifestyle characteristics of participants in different regions

Characteristic	North		Middle		South		Total	
	n = 742	44.3 (%)	*n* = 488	29.2 (%)	*n* = 444	26.5 (%)	*n* = 1674	100 (%)
Gender								
Female	464	62.5	128	26.2	136	30.6	728	43.5
Male	278	37.5	360	73.8	308	69.4	946	56.5
BMI	742	20.0 ± 3.2	488	20.6 ± 2.8	444	20.3 ± 2.2	1674	20.6 ± 2.0
Age in yr	742	52.9 ± 15.1	488	46.3 ± 12.8	444	47.5 ± 15.0	1674	49.8 ± 11.4
< 40	110	36.2 ± 5.4	174	36.9 ± 5.2	76	36.7 ± 3.9	360	35.8 ± 2.9
40-49	220	45.1 ± 4.2	202	44.7 ± 4.5	288	44.8 ± 2.1	710	45.5 ± 2.0
50-59	150	54.6 ± 6.1	28	51.8 ± 1.9	8	54.4 ± 2.5	186	54.2 ± 2.0
≥ 60	262	66.1 ± 5.0	84	68.3 ± 11.1	72	77.0 ± 10.4	418	68.0 ± 8.4
Lifestyle								
High salt	230	31.0	112	23.0	56	12.6	398	23.8
Smoking	314	42.3	142	29.1	318	71.6	774	46.2
Drinking	286	38.5	116	23.8	184	41.4	586	35.0
Clinical diagnosis								
H. pylori infection	512	69.0	296	60.7	360	81.1	1168	69.8
NAG								
None	700	94.3	470	96.3	388	87.4	1558	93.1
Mild	23	3.1	10	2.1	35	7.9	68	4.1
Moderate	16	2.2	6	1.2	17	3.8	39	2.3
Severe	3	0.4	2	0.4	4	0.9	9	0.5
AG								
None	722	97.3	438	89.8	408	91.9	1568	93.7
Mild	13	1.8	33	6.8	23	5.1	69	4.1
Moderate	6	0.8	14	2.8	10	2.3	30	1.8
Severe	1	0.1	3	0.6	3	0.7	7	0.4
IM								
None	612	82.5	328	67.2	368	82.9	1308	78.1
Mild	87	11.7	104	21.3	48	10.9	239	14.3
Moderate	34	4.6	45	9.2	23	5.1	102	6.1
Severe	9	1.2	11	2.3	5	1.1	25	1.5

Data presented as (mean ± standard deviation). BMI: Body mass index; *H. pylori*: *Helicobacter pylori*; NAG: Non-atrophic gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia.

with mRNA translation, thereafter may lead to the change of protein expression[27]. Therefore, SNP rs907715 of *IL-21* gene may alter the mRNA expression levels and regulate the function of *IL-21*. This suggested that rs907715 may be related to the risk of gastric precancerous lesions by influencing the activities of *IL-21*.

The data regarding TC haplotype frequency (rs907715 and rs12508721) in AG and IM patients compared to controls showed that this haplotype may be a risk for gastric precancerous lesions. Similarly, the TT haplotype frequency (rs2221903 and rs907715) in IM patient compared to controls showed that this haplotype may be a risk for IM. This result suggested that the two haplotypes, according to the *IL-21* polymorphisms, might be the important genetic factors for susceptibility to gastric precancerous lesions.

The present study selected subjects in three cities from north to south in Shaanxi province, which enhanced the power of population representation and made our results more credible. However, this study also has several limitations. First, all cases and controls were selected from participants experiencing upper gastrointestinal symptoms, which may cause a potential selection bias and increase the positive results of the study. Second, controls were screened from subjects with non-*H. pylori* infection and non-precancerous lesions to represent the general population, this led to a mismatched number of cases and controls, which may weaken the testing effectiveness. Third, this study was a case-control study and unable to draw a causal relationship.

Table 3 Association between risk factors and gastric precancerous lesions

Risk factors		NAG		AG		IM	
		OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age		1.09 (0.99-1.03)	0.315	0.90 (0.88-1.01)	0.412	1.01 (0.99-1.02)	0.313
BMI		1.01 (0.93-1.11)	0.421	0.89 (0.78-1.32)	0.587	1.11 (0.98-1.24)	0.134
Gender	Female	-		-		-	
	Male	1.52 (0.79-2.14)	0.156	0.91 (0.42-1.83)	0.792	1.30 (0.88-1.91)	0.199
High salt	No	-		-		-	
	Yes	2.58 (1.21-4.88)	0.011	0.59 (0.24-1.77)	0.315	1.01 (0.55-1.41)	0.699
H. pylori infection	No	-		-		-	
	Yes	0.59 (0.37-1.08)	0.141	0.39 (0.65-0.99)	0.041	0.90 (0.77-1.31)	0.555
Smoking	No	-		-		-	
	Yes	2.15 (1.19-4.44)	0.015	1.11 (0.77-2.10)	0.515	1.97 (1.40-2.58)	0.005
Drinking	No	-		-		-	
	Yes	1.00 (0.99-1.39)	0.057	0.731 (0.49-1.19)	0.161	0.95 (0.89-1.33)	0.668
Region	South	-		-		-	
	Middle	0.33 (0.28-0.51)	0.009	0.71 (0.46-1.99)	0.669	2.95 (1.45-4.33)	0.007
	North	0.55 (0.40-1.01)	0.053	0.33 (0.21-0.83)	0.010	1.41 (0.89-2.01)	0.313

BMI: Body mass index; *H. pylori*: Helicobacter pylori; NAG: Non-atrophic gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia.

In conclusion, our study found that SNP rs907715 was associated with gastric precancerous lesions, and the TC haplotype (rs907715 and rs12508721) and TT haplotype (rs2221903 and rs907715) increased the risk of gastric precancerous lesions, which may help clarify the etiology of GC. Further studies are required to elucidate the roles of rs907715 in development of gastric precancerous lesions at a molecular level, which may provide new targets for therapeutic interventions.

Table 4 Comparison of the genotype distribution of the *IL-21* gene polymorphisms in gastric precancerous lesions

SNPs set	Geno-type	Control (*n* = 290, %)	NAG (*n* = 116, %)	AG (*n* = 106, %)	IM (*n* = 366, %)	OR_1	P_1 value	OR_2	P_2 value	OR_3	P_3 value	OR_4	P_4 value	OR_5	P_5 value
rs907715	TT	70 (24.1)	25 (21.6)	19 (17.9)	63 (17.2)	-		-		-		-		-	
	CT	143 (49.3)	53 (45.7)	50 (47.2)	170 (46.5)	1.04 (0.60-1.81)	0.896	1.29 (0.71-2.35)	0.408	1.32 (0.88-1.98)	0.179	1.25 (0.87-1.80)	0.230	1.13 (0.62-1.74)	0.439
	CC	77 (26.6)	38 (32.7)	37 (34.9)	133 (36.3)	1.38 (0.76-2.52)	0.290	1.77 (0.93-3.36)	0.079	1.92 (1.24-2.98)	0.004	1.77 (1.19-2.63)	0.005	1.59 (1.06-2.38)	0.013
	C	220 (75.9)	91 (78.4)	87 (82.1)	303 (82.8)	1.16 (0.69-1.94)	0.578	1.46 (0.83-2.56)	0.190	1.53 (1.04-2.24)	0.028	1.43 (1.02-2.01)	0.039	1.28 (1.01-2.22)	0.044
rs12508721	TT	39 (13.4)	14 (12.1)	18 (17.0)	54 (14.8)	-		-		-		-		-	
	CT	151 (52.1)	57 (49.1)	55 (51.9)	178 (48.6)	1.05 (0.53-2.08)	0.885	0.79 (0.42-1.50)	0.467	0.85 (0.54-1.36)	0.498	0.87 (0.57-1.33)	0.525	0.82 (0.55-1.28)	0.618
	CC	100 (34.5)	45 (38.8)	33 (31.1)	134 (36.6)	1.25 (0.62-2.54)	0.529	0.72 (0.36-1.42)	0.335	0.97 (0.60-1.57)	0.895	0.96 (0.62-1.50)	0.863	0.88 (0.59-1.33)	0.879
	C	251 (86.6)	102 (87.9)	88 (83.0)	312 (85.2)	1.13 (0.59-2.17)	0.709	0.76 (0.41-1.40)	0.375	0.90 (0.58-1.40)	0.634	0.91 (0.60-1.36)	0.639	0.83 (0.49-1.19)	0.801
rs2221903	TT	231 (79.7)	90 (77.6)	83 (78.3)	271 (74.0)	-		-		-		-		-	
	CT	52 (17.9)	24 (20.7)	21 (19.8)	80 (21.9)	1.19 (0.69-2.04)	0.539	1.12 (0.64-1.98)	0.685	1.31 (0.89-1.94)	0.173	1.25 (0.87-1.79)	0.223	1.08 (0.53-1.55)	0.459
	CC	7 (2.4)	2 (1.7)	2 (1.9)	15 (4.1)	0.73 (0.15-3.60)	0.701	0.80 (0.16-3.91)	0.777	1.83 (0.73-4.56)	0.190	1.41 (0.59-3.41)	0.441	1.19 (0.41-2.87)	0.503
	C	59 (20.3)	26 (22.4)	23 (21.7)	95 (26.0)	1.13 (0.67-1.91)	0.643	1.09 (0.63-1.87)	0.769	1.37 (0.95-1.99)	0.092	1.27 (0.90-1.79)	0.171	1.08 (0.81-1.48)	0.311

OR_1: NAG cases compared with controls; OR_2: AG cases compared with controls; OR_3: IM cases compared with controls; OR_4: Total gastric precancerous lesions cases compared with controls; OR_5: Adjusted by age, gender, BMI, drinking status, smoking status, daily salt intake and region. SNPs: Single nucleotide polymorphisms; NAG: Non-atrophic gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia.

Table 5 Haplotype analysis of polymorphisms in patients and controls

SNPs	Haplo-type	Control (*n* = 290, %)	NAG (*n* = 116, %)	AG (*n* = 106, %)	IM (*n* = 366, %)	χ^2_{-1}	P_1 value	OR_1	χ^2_{-2}	P_2 value	OR_2	χ^2_{-3}	P_3 value	OR_3
rs907715|rs12508721	CC	0.496	0.543	0.575	0.571	0.713	0.397	1.20 (0.78-1.86)	1.936	0.164	1.37 (0.88-2.15)	3.612	0.057	1.35 (1.00-1.84)
	CT	0.066	0.060	0.057	0.071	0.037	0.847	1.09 (0.45-2.67)	0.104	0.747	1.17 (0.45-3.01)	0.077	0.781	0.92 (0.50-1.69)
	TC	0.163	0.138	0.047	0.087	0.368	0.544	1.21 (0.66-2.23)	8.984	0.003	3.91 (1.51-10.11)	8.509	0.004	2.02 (1.25-3.26)
	TT	0.275	0.259	0.321	0.271	0.125	0.724	1.09 (0.67-1.78)	0.763	0.382	0.81 (0.50-1.31)	0.024	0.878	1.03 (0.73-1.45)
rs2221903|rs907715	CC	0.128	0129	0.123	0.158	0.002	0.963	0.99 (0.52-1.87)	0.017	0.896	1.05 (0.53-2.06)	1.264	0.264	0.78 (0.50-1.21)
	CT	0.006	0.026	0.019	0.014	2.450	0.118	0.26 (0.04-1.59)	1.113	0.292	0.36 (0.05-2.60)	0.701	0.404	0.50 (0.10-2.60)
	TC	0.434	0.474	0.509	0.484	0.527	0.468	0.85 (0.55-1.31)	1.759	0.185	0.74 (0.47-1.16)	1.571	0.210	0.82 (0.60-1.12)
	TT	0.432	0.371	0.349	0.344	1.244	0.265	1.29 (0.83-2.00)	2.158	0.142	1.41 (0.89-2.24)	5.157	0.023	1.44 (1.05-1.98)

Haplotype of rs907715(C/T), rs2221903 (T/C) and rs12508721(C/T). OR_1: NAG cases compared with controls; OR_2: AG cases compared with controls; OR_3: IM cases compared with controls. SNPs: Single nucleotide polymorphisms; NAG: Non-atrophic gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia.

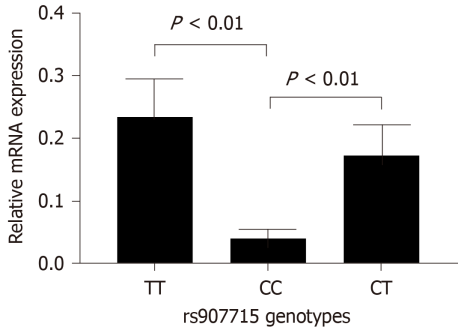

Figure 2 Interleukin-21 mRNA expression level in six intestinal epithelium tissues among three rs907715 genotypes.

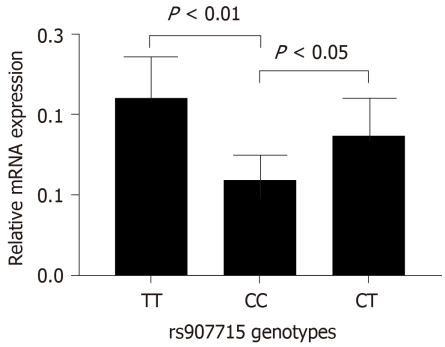

Figure 3 Interleukin-21 mRNA expression level in six non-atrophic gastritis tissues among three rs907715 genotypes.

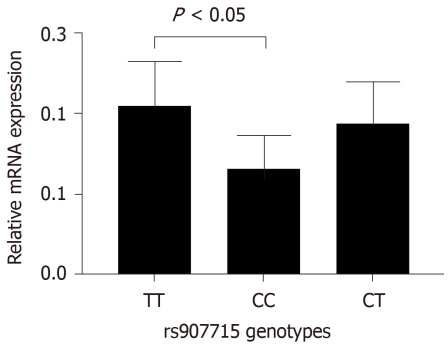

Figure 4 Interleukin-21 mRNA expression level in six atrophic gastritis tissues among three rs907715 genotypes.

ARTICLE HIGHLIGHTS

Research background

Previous studies have found that interleukin-21 (*IL-21*) may be associated with the risk of gastric precancerous lesions, and single nucleotide (SNPs) of the *IL-21* gene are associated with various diseases or cancer. Clarifying the possible role of *IL-21* SNPs (rs907715, rs2221903 and rs12508721) in gastric precancerous lesions is of great significance in preventing the development of gastric cancer.

Research motivation

However, no studies have examined the possible role of *IL-21* SNPs (rs907715, rs2221903 and rs12508721) in gastric precancerous lesions.

Research objectives

Therefore, the present study explored the associations between SNPs of *IL-21* (rs907715, rs2221903 and rs12508721) and risk of gastric precancerous lesions in a north western Chinese population, which may help clarify the etiology of gastric cancer and provide new targets for therapeutic interventions.

Research methods

Gastric precancerous lesions were confirmed by endoscopic examination and categorized as non-atrophic gastritis, atrophic gastritis, and intestinal metaplasia. Three SNPs of *IL-21* (rs907715, rs2221903 and rs12508721) were genotyped using polymerase chain reaction–ligase detection reaction in 588 cases and 290 healthy controls. Descriptive statistic and logistic regression were used for data analyses.

Research results

We found an association between *IL-21* rs907715 polymorphism and gastric precancerous lesions. *IL-21* rs907715 genotype CC and C frequencies in patients with gastric precancerous lesions were higher than in controls. SNP rs907715 increased in CC and C genotypes were associated with intestinal metaplasia patients when examined separately. However, the exact role of rs907715 in development of gastric precancerous lesions at a molecular level remains to be studied.

Research conclusions

In conclusion, our findings indicate that SNP rs907715 of *IL-21* gene is associated with gastric precancerous lesions.

Research perspectives

If confirmed by other studies, the results of our study suggest that *IL-21* rs907715 polymorphisms may shed light on the etiology of precancerous lesions.

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ORIGINAL ARTICLE

Retrospective Study

Circulating cytokines and outcome in metastatic colorectal cancer patients treated with regorafenib

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Author contributions: Ricci V and Merlano MC designed the research; Ricci V, Granetto C and Fea E enrolled the patients; Fabozzi T, Granetto C and Lo Nigro C revised the manuscript; Falletta A, Paccagnella M and Abbona A performed the research; Lo Nigro C collected the samples; Falletta A, Ricci V and Fabozzi T wrote the paper and Paccagnella M analyzed the data; Merlano MC revised the manuscript for important intellectual content.

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Abstract

BACKGROUND

Regorafenib is an oral small-molecule multikinase inhibitor approved in third or later line of treatment for patients with metastatic colorectal cancer (mCRC). Regorafenib has shown significant benefits in overall survival and progression free survival in two phase III trials compared to placebo in patients with mCRC who had progressed on previous therapy.

AIM

To identify an immune profile that might specifically correlate with the outcome in patients treated with regorafenib.

METHODS

Blood samples were collected from 17 patients before treatment with regorafenib and from 6 healthy volunteers. The proteins evaluated (TNF-α, TGF-β, VEGF, CCL-2, CCL-4, and CCL-5) were selected on the basis of their roles in angiogenesis and colorectal cancer pathogenesis.

RESULTS

We found that TNF-α basal level was significantly higher in mCRC patients compared to healthy individuals. Non Responder (NR) patients showing progression of disease (n = 12) had higher basal level of TGF-β, TNF-α, VEGF, CCL-2 and CCL-5 compared to Responder (R) patients (complete response CR, n

additional data are available.

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= 1; partial response PR, *n* = 1; Stable Disease SD, *n* = 3). On the contrary, plasma basal level of CCL-4 was higher in R compared to NR patients. High values of TGF-β and TNF-α negatively correlated with progression free survival.

CONCLUSION
These results suggest a cytokine signature potentially able to discriminate between R and NR patients to treatment with regorafenib.

Key words: Colorectal cancer; Multikinase inhibitor; Cytokines; Angiogenesis

Core tip: We analyzed levels of specific cytokines in plasma of metastatic colorectal cancer patients before treatment with regorafenib. Our aim was to identify biomarkers useful to select metastatic colorectal cancer responder patients and an immune profile potentially correlated with the outcome.

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INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death and the third most commonly diagnosed cancer in humans in the world[1].

For many years treatment of metastatic CRC (mCRC) consisted mainly of single agent 5-fluorouracil but the addition of irinotecan and oxaliplatin to 5-fluorouracil increased the median progression free survival (PFS) to 8 mo[2], which further improved with the later addition of the vascular endothelial growth factor (VEGF) and EGFR inhibitors[3].

Regorafenib is an oral multikinase inhibitor that inhibits the activity of vascular endothelial growth factor receptor 1, 2, 3 (VEGFR-1,-2,-3), tyrosine kinase receptor with immunoglobulin-like and EGF-like domains 2 (TIE2), platelet-derived growth factor receptors, fibroblast growth factor receptors and oncogenic receptor tyrosine kinases (KIT, RET, RAF-1, BRAF, $BRAF^{V600E}$)[4]. Regorafenib monotherapy significantly increased overall survival (OS) and PFS in the CORRECT and the CONCUR trials, which compared regorafenib treatment to placebo[5,6].

Cytokine levels could be potentially useful in monitoring disease progression and treatment outcome. Suenaga *et al*[7] demonstrated that baseline serum CCL-5 and VEGF-A levels may act as potential predictive markers for survival or treatment-specific toxicities in mCRC patients receiving regorafenib in salvage-line setting.

TGF-β is a multifunctional polypeptide promoting angiogenesis and expression of cell adhesion proteins, and inhibiting growth of epithelial and immune cells. High plasma levels of TGF-β were associated with progression of disease[8].

TNF-α might serve as a predictive marker for treatment efficacy and clinical outcome. Olsen *et al*[9] demonstrated that higher levels of TNF-α, as well as of other cytokines, are associated with a worse prognosis and mortality in CRC patients.

The expression of CCL-2, also known as monocyte chemotactic protein 1, in colorectal cancer cells is strictly related to advanced tumor stage, accumulation of tumor-associated macrophages, and negative prognosis[10,11].

Several reports demonstrated that the releasing of CCL-5 (also known as RANTES) promotes cancer cell invasiveness and decreases antitumor immunity by recruitment of C-C chemokine receptor type 5 $(CCR\text{-}5)^{+}$ T-regulatory cells[12]. The interaction between CCL-5 and its receptor also participates in VEGF up-regulation in the osteosarcoma microenvironment[13].

CCL-4 is a chemokine released by a variety of immune and epithelial cells that interacts with CCR-5 to attract macrophages, T cells and, most important, immature dendritic cells (DCs) whose presence is essential for the activation of immune response. Unlike the two above mentioned chemokines, high serum levels of CCL-4 in CRC patients are associated with improved disease free survival and this might be

related to increased recruitment of Th1 cells, which frequently express CCR-5[14].

In this "proof of concept" study we analyzed the level of all these soluble cytokines in the plasma of mCRC patients before treatment with regorafenib, with the aim to identify biomarkers potentially useful to select mCRC patients who could benefit from regorafenib therapy.

MATERIALS AND METHODS

Study design

This exploratory study was performed in a single centre. The aim of the study was to evaluate the role of specific biomarkers potentially involved in the clinical activity of regorafenib: TNF-α, TGF-β, VEGF, CCL-2, CCL-4 and CCL-5. The selected cytokines were measured at baseline as described below and the clinical outcome of each patient was correlated to the cytokines profile. Six healthy volunteers (2 men and 4 women) were also analyzed. Patients were treated following standard clinical practice and followed accordingly.

All enrolled patients signed an informed consent for the storage and analysis of their biological material approved by the local ethical committee (prot n° 24347; August 7, 2015).

Blood samples

We enrolled in the present study 17 mCRC patients treated at the Oncology Department, S. Croce and Carle Teaching Hospital in Cuneo from April 2016 to June 2018. All patients received 160 mg regorafenib once a day for 3 wk, followed by 1 wk treatment free.

Blood samples were collected into EDTA vacutainer tubes at baseline immediately before the first administration of regorafenib. Plasma samples were obtained through centrifugation step and stored in aliquots at −80°C in the Biobank of the Oncology Department until use.

Patient characteristics

The mCRC cohort consisted of 53% males and 47% females. Median age was 63 (52 to 77 years). In 53% of the cases, the tumour was located in the colon (3 right colon and 6 left colon) and in 47% in the rectum.

The mCRC group included 82% of patients at third-line treatment, 12% of patients at fourth-line treatment, and 6% of patients at fifth-line treatment line. RAS mutations were detected in 76% of the cases. Patients' main characteristics are reported in Table 1.

Analysis method

Plasma levels of six cytokines were evaluated with ELISA kits from R and D Systems (TNF-α, TGF-β, VEGF, CCL-4 and CCL-5) and Invitrogen (CCL-2). For TGF-β analysis, the samples were incubated with 1 N HCL for 10 min followed by with 1.2 N NaOH/0.5 mol/L HEPES prior to perform the assay, in order to activate the latent TGF-β to the immunoreactive form. The ELISA assay employs the quantitative sandwich enzyme immunoassay technique. Analysis was performed according to the manufacturer's protocol. In brief, 100 µL of sample was used and incubated for two hours in a 96 well plate, coated with antibody against each cytokine. After washing, 200 µL of horseradish peroxidise-conjugated antibody was added to each well and incubated for one/two hours. After further washing, a substrate solution was added. Optical density was determined by reading the absorbance with a plate reader (Multiscan Ascent, Thermo fisher®) at 450 nm. Patient samples, standards and controls were assayed in triplicate, and the average values were recorded. The protein concentrations were expressed in pg/mL. CCL-2 was evaluated with uncoated ELISA using Corning Costar 9018 plates. Plates were incubated overnight at 4°C with the capture antibody anti-human CCL-2 overnight 4 °C. The assay was then carried out as described above.

Statistical analysis

Differences in the medians were tested by the Mann–Whitney *U* test. In order to find the optimal cut-off point at baseline, which might help in predicting survival, the receiver operating characteristic curve (ROC) analysis was performed. The cut-off was defined as the point on the ROC curve with the largest average sensitivity and specificity. Subgroups divided using the cut-off value were compared for PFS and OS. PFS was defined as the interval between the date of starting regorafenib treatment and the date of confirming disease progression, last follow-up or death. OS was calculated from the date of starting regorafenib treatment and the date of death or last

Table 1 Patient characteristics (*n* = 17)

Patient characteristics	*n* (%)
Sex (male/female)	9/8 (53/47)
Median age (range)	63 (52-77)
Primary tumor site (colon/rectum)	9/8 (53/47)
Number of previous anticancer therapies (III/IV/V)	14/2/1 (82/12/6)
Mutational RAS status (mutated/wild type)	13/4 (76/24)

follow-up.

PFS and OS were estimated by the Kaplan-Meier method and they were compared using the log-rank test, with predictive or prognostic factors being identified by univariate analysis. It was not possible to perform the Cox analysis due to the small number of patients.

Correlation analysis was used to describe the relationship between PFS and basal TGF-β and TNF-α levels and was performed using the Spearman test.

The statistical analyses were carried out using SPSS software version 24.0 (IBM Corporation, Armonk, NY, United States) and GraphPad Software 5.0 (San Diego, CA, United States). $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Correlation between basal cytokines levels and response

Patients population was divided into two groups according to the best response to regorafenib treatment by instrumental evaluation every 3 mo. The 12 Non Responder (NR) patients showed disease progression. The 5 Responder (R) patients showed either complete response (CR, $n = 1$), partial response (PR, $n = 1$) or stable disease (SD, $n = 3$).

We measured cytokine levels in all the mCRC patients. We found that the plasma basal level of TNF-α was significantly higher in NR compared to R patients ($P = 0.011$).

Also TGF-β was significantly higher in NR compared to R patients ($P = 0.031$).

NR patients had a higher median level of VEGF with respect to R, but only the difference between NR and healthy controls was significant ($P = 0.044$).

The CCL-2 plasma level showed a trend similar to VEGF: Higher in NR *vs* R patients, but significantly higher only between NR and healthy controls ($P = 0.035$).

CCL-4 basal level, on the contrary, showed an opposite trend, in particular NR had lower median value than R.

The levels of CCL-5 did not show any difference among all the three groups (Figure 1).

Correlations between TNF-α, TGF-β levels and PFS

We further investigated the possible association between basal cytokine levels and PFS.

TNF-α ($r_s = -0.51$, $P = 0.033$) and TGF-β ($r_s = -0.52$, $P = 0.038$) negatively correlated with PFS in the patient cohort (Figure 2A and B).

Furthermore there was a positive correlation between TNF-α and TGF-β basal values of all the mCRC patients ($r_s = 0.53$, $P = 0.028$) (Figure 2C).

Instead there was no significant difference among the other cytokines at baseline and between them each of them and PFS.

ROC and Cox analysis, PFS and OS Kaplan-Meier curves

Median PFS was 2.97 mo [95% confidence interval (CI): 2.384–3.549 mo], median OS was 9.03 mo (95%CI: 6.704–11.356 mo). Median follow-up was 6.60 mo (95%CI: 5.285–7.915 mo).

Using the ROC analysis (Figure 3A) we identified a cut-off value of 7.41 pg/mL for TNF-α basal level (AUC: 0.908, 95%CI: 0.758-1.000, $P = 0.010$). Then, using this cut-off value we clustered all patients into two groups, observing a higher PFS (5.20 mo, 95%CI: 4.198-6.202 *vs* 2.60 mo, 95%CI: 2.284-2.850, $P = 0.005$) in patients with baseline TNF-α below the cut-off point (Figure 3B).

We also observed in the same patients a better OS (16.60 mo 95%CI: 16.171-17.029 *vs* 7.30 mo, 95%CI: 1.440-13.220, $P = 0.010$) (Figure 3C).

There was no significant difference in PFS and OS according to the ROC analysis

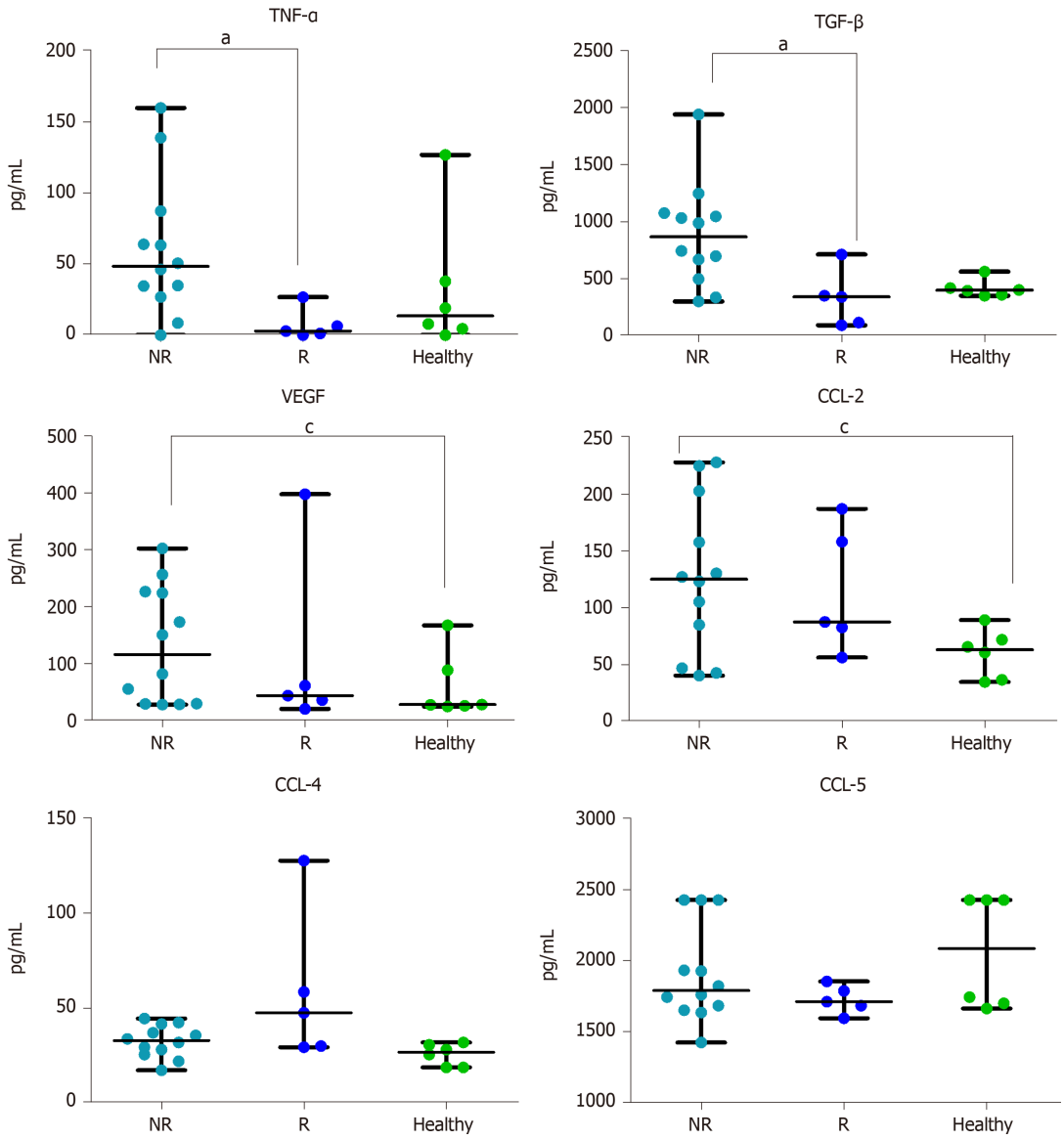


Figure 1 Plasma cytokines levels. Basal evaluation of 6 cytokines in non-responder patients (n = 12), Responder patients (n = 5) and in healthy volunteers (n = 6). Cytokine concentration is expressed in pg/mL. Data are shown as median with range. The difference in median values was computed using the non-parametric Mann Whitney U test. $P < 0.05$ was considered the statistical significance. $^{a}P < 0.05$ NR *vs* R; $^{c}P < 0.05$ NR *vs* Healthy. NR: Non-responder; R: Responder; TNF-α: Tumor necrosis alpha; TGF-β: Transforming growth factor alpha; VEGF: Vascular endothelial growth factor; CCL-2: Chemokine ligand 2; CCL-4: Chemokine ligand 4; CCL-5: Chemokine ligand 5.

for the other cytokines (data not shown).

Moreover, using the univariate Cox analysis, we observed that patients with plasma basal levels of TNF-α ≥ 7.41 pg/mL had a significant increased risk to get progression disease compared to those with plasma basal levels of TNF-α < 7.41 pg/mL (HR: 7.203, 95%CI: 1.531–33.882, P = 0.012) (Figure 4).

DISCUSSION

Our data show that patients that do not benefit from regorafenib might be identified by basal values of TNF-α and TGF-β before treatment.

TNF-α promotes cancer invasion and angiogenesis associated with epithelial-mesenchymal transition through the involvement of canonical NF-κB signalling[15]. Moreover TNF-α is also expressed at higher levels in various pre-neoplastic and tumor tissues. Furthermore, the increased TNF-α expression level in pre-cancerous and tumor cells is associated with the progression of malignant diseases such as chronic lymphocytic leukemia, Barrett's adenocarcinoma, prostate cancer, breast cancer, and cervical carcinoma[16-18].

High plasma levels of TNF-α are associated to an increased risk of recurrence and

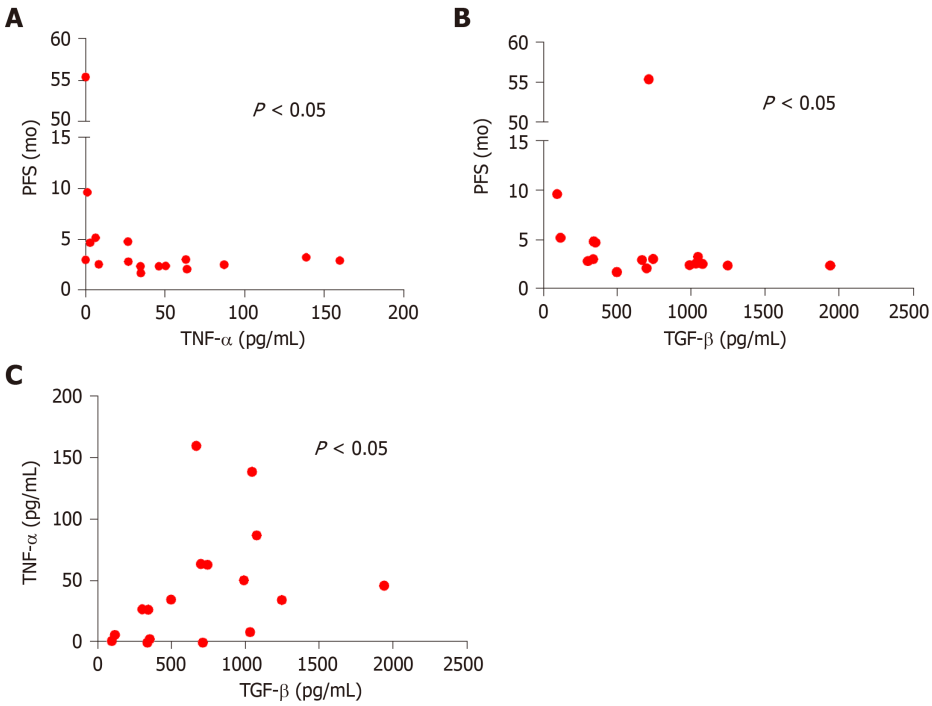


Figure 2 Correlation analysis. A: Correlation between tumor necrosis alpha and progression free survival in all metastatic colorectal cancer (mCRC) patients (n = 17) (r_s = -0.52, P = 0.033); B: Correlation between transforming growth factor alpha and progression free survival in all mCRC patients (n = 17) (r_s = -0.52, P = 0.038); C: Correlation between tumor necrosis alpha and transforming growth factor alpha in all mCRC patients (n = 17) (r_s = 0.53, P = 0.028). P < 0.05 was considered the statistical significance. Each dot represents the value of one patients. Transforming growth factor alpha and tumor necrosis alpha are expressed as a concentration (pg/mL). Progression free survival is expressed in months. PFS: Progression free survival; TNF-α: Tumor necrosis alpha; TGF-β: Transforming growth factor alpha.

mortality in CRC[9]. These findings indicate that TNF-α could be used as an indicator of cancer risk, prognosis and therapy response for cancer patients. Our results support its negative predictive role in regorafenib treatment.

High plasma levels of TGF-β are associated to mRNA over-expression in colon cancer tissues and related to disease progression[8]. Numerous studies also demonstrated that TGF-β production by tumour cells might promote tumor growth and immune escape and enhance angiogenesis[19-21]. Moreover, tumor development removes a cell growth inhibitory signal and increases the amount of TGF-β in the tumor microenvironment[22]. We found that the lower plasmatic levels of TGF-β are associated to longer PFS.

VEGF plays a key role in angiogenic process. Previous studies reported that colon cancer patients have high levels of VEGF compared to a healthy population[23] and that VEGF expression is associated with poor prognosis[24]. Also our results show a higher basal value of VEGF in NR compared to a healthy population.

CCL-2, CCL-4 and CCL-5 are small peptides structurally and functionally similar to growth factors which are able to induce leukocyte migration along a chemical gradient. The complex network of these chemokines and their receptors promotes carcinogenesis and metastasis[25].

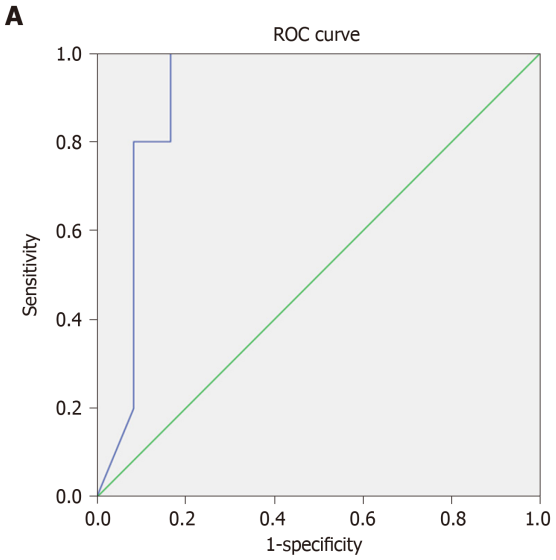
The expression of CCL-2 correlates with lymph node metastasis and predicts the risk of liver metastasis[10]. Indeed, we found that CCL-2 is significantly higher in NR than in healthy controls.

CCL-4 is involved in the recruitment of $CD103^+$-DCs. The failure of Batf3-dependent recruitment $CD103^+$-DCs together with the activation of Wnt/β-catenin pathway is a cause of non-T cell-inflamed tumor development[26]. We found a difference between NR and R, even if no statistical significance was reached.

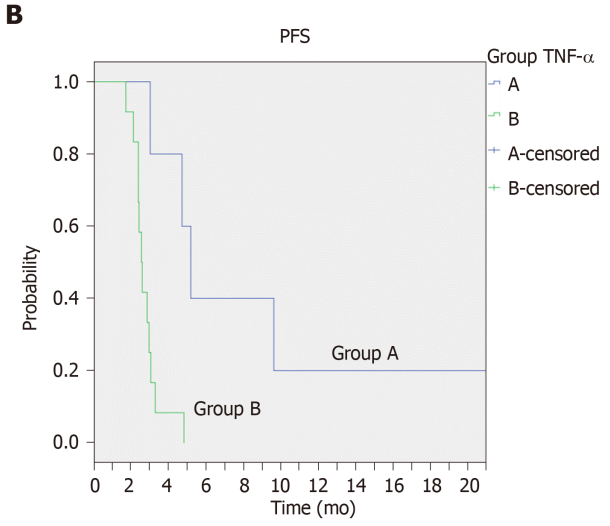
CCL-5 promotes angiogenesis of endothelial cells, chemotaxis and tumor angiogenesis by VEGF production in human cancer cells[12]. Our results do not show any difference in the distribution of this cytokine. Comparing our results with those of the Suenaga *et al*[7] study, the discrepancy in the levels of CCL-5 in the patients could be attributed to the cytokine analysis, which in our case was performed on serum samples instead of plasma samples which may contain platelet contamination.

We found that R mCRC patients are characterized by low basal values of TGF-β, TNF-α, VEGF, CCL-2 and high levels of CCL-4 compared to NR patients, even if the high CCL-4 median value in the R group is due to the very high value of one patient.

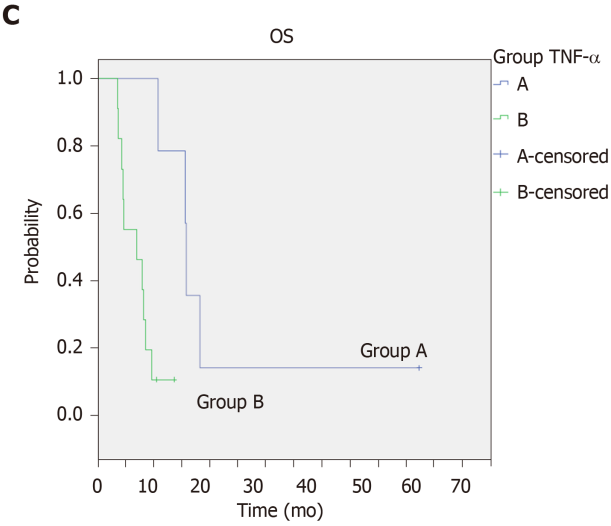
In general, the cytokine profile of R is similar to that of healthy volunteers.



Area under the ROC curve	Standard error	Significance level *P*	95%CI Lower limit	95%CI Upper limit
0.908	0.077	<0.05	0.758	1.000



	TNF-α ≤ Cut-off A	TNF-α > Cut-off B
Median PFS (mo)	5.20	2.60
95%CI	4.198-6.202	2.284-2.850
Long-rank, *P* value	< 0.01	



	TNF-α ≤ Cut-off A	TNF-α > Cut-off B
Median PFS (mo)	16.60	7.30
95%CI	16.171-17.029	1.440-13.220
Long-rank, *P* value	< 0.05	

Figure 3 Receiver operating characteristic curve analysis, progression free survival and overall survival Kaplan-Meier curves. A: Receiver operating characteristic curve with area under the curve (0.908, 95CI: 0.758–1.000, *P* = 0.010) for predicting survival by plasma TNF-α basal levels in patients with metastatic colorectal cancer treated with regorafenib according to the baseline TNF-α levels ≤ (—, *n* = 5) or > (—, *n* = 12) the cut-off value (determined by receiver operating characteristic curve analysis); B: Progression free survival (5.2 *vs* 2.6 mo, Log-rank test, *P* = 0.005); one patient in Group A is not shown because of a graphic choice; C: Overall survival (16.6 *vs* 7.3 mo, Log-rank test, *P* = 0.010). PFS: Progression free survival; OS: Overall survival; ROC: Receiver operating characteristic; TNF-α: Tumor necrosis alpha; TGF-β: Transforming growth factor alpha.

This observation suggests that R have an active immune system and this aspect could be the real difference between R and NR. Of course, our study is hampered by important limitations.

First of all, the number of patients precludes any Cox analysis.

Another limitation is the lack of longitudinal analysis which precludes the possibility to distinguish between the prognostic or predictive role of this signature. However, since the purpose of our research work is to find predictive markers of treatment response, it is reasonable to take in consideration only basal cytokine

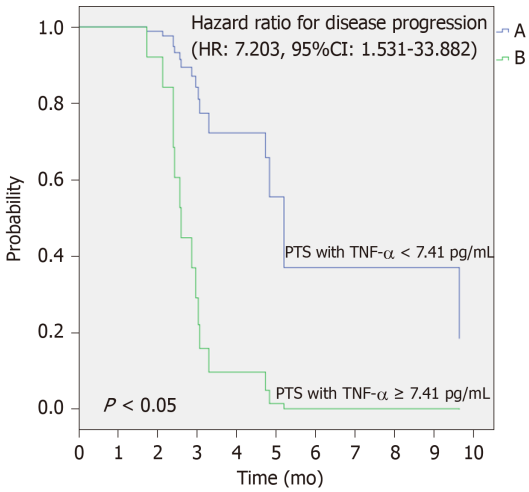


Figure 4 Univariate Cox analysis to predict risk of disease progression. Difference between the two survival curves was assessed by long rank test, the HR with 95%CI was calculated by the Cox regression model (HR = 7.203; 95%CI: 1.531–33.882; *P* = 0.012). PFS: Progression free survival; TNF-α: Tumor necrosis alpha.

values.

We are now leading a new study with the same experimental conditions but a different drug with the aim to verify the prognostic role, rather than predictive, of the proposed signature.

It would also be of interest to explore different tumor types to verify the hypothesis that the true value of our signature is to identify patients with a better prognosis due to a functional immune system.

On this basis, following a prospective study with a greater number of patients, we could identify a potential score, which might select a baseline cytokines profile able to identify NR patients who will not benefit from treatment with regorafenib. Also it might be a useful tool to drive decision-making process in daily clinical practice.

The main limitation of the study is the small population of mCRC patients analyzed. A validation in a larger patient population is strongly recommended.

In addition, the same signature should be evaluated also in patients receiving other treatments, and then the possibility that this could represent a prognostic tool should be considered.

In conclusion, taken together all these observations suggest that patients having high basal levels of TNF-α and TGF-β show a poor prognosis and, probably, will be less respondent to the regorafenib therapy. If our data is confirmed, it will be possible to identify NR mCRC patients in order to avoid ineffective treatments. We are aware that our population is small and data should be verified on larger and independent series of patients. It might also be of interest to extend analysis to other cytokine and cell populations not considered in our study.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer is one of the leading causes of cancer-related death and the third most commonly diagnosed cancer in humans in the world. For many years 5-fluorouracil was the only active drug for treatment of metastatic colorectal cancer (mCRC). The addition of irinotecan and oxaliplatin to 5-fluorouracil increased the median progression free survival (PFS), which further improved with the later addition of target therapies. Regorafenib is a multi-kinase inhibitor targeting VEGFR1-3, TIE2, fibroblast growth factor receptors 1 and platelet-derived growth factor receptors β, c-KIT, RET, c-RAF/RAF-1, BRAF V600E mutant. It can be used after failure of conventional treatment options.

Research motivation

In previous studies, regorafenib monotherapy showed the ability to improve PFS and overall survival in a subset of mCRC patients. However, no appropriate biomarkers are currently available. We analyzed the levels of many cytokines involved in angiogenesis and CRC pathogenesis, in plasma of mCRC patients before treatment with regorafenib. Our purpose was to identify potential biomarkers to select patients most likely to respond to regorafenib.

Research objectives

The aim of our study is to identify biomarkers useful to select mCRC patients for treatment with

regorafenib and, possibly, an immune profile potentially correlated with the clinical outcome.

Research methods
We collected blood samples of mCRC patients before starting regorafenib therapy for the evaluation of circulating TNF-α, TGF-β, VEGF, CCL-2, CCL-4, and CCL-5. The cytokines were measured at baseline using ELISA tests and the clinical outcome of each patient was correlated to the cytokines profile. We also analyzed the same cytokines levels in six healthy volunteers.

Research results
We found higher basal levels of TNF-α, TGF-β, VEGF, CCL-2 and CCL-5 in non-responders (NR; patients showing progression of disease, n = 12) compared to those who respond to therapy (complete response CR, n = 1, partial response PR, n = 1, Stable Disease SD, n = 3), and a reversed trend for CCL-4. Moreover, we found that CCL-2 and VEGF basal levels were significantly higher in NR patients compared to healthy individuals. Furthermore, high values of TGF-β and TNF-α negatively correlated with PFS. We further investigated the possible association between basal cytokine levels and PFS and we found that TNF-α and TGF-β negatively correlated with PFS in the patient cohort. Both these basal cytokines positively correlated between them.

Research conclusions
We realized a cytokine signature which could potentially discriminate between responder and non-responder patients to Regorafenib therapy. If our data is confirmed, it will be possible to drive treatment with regorafenib to patients most likely respond to the drug.

Research perspectives
Our data should be verified on larger and independent series of patients. It might also be of interest to extend analysis to other cytokines and cells population not determined in our study.

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ORIGINAL ARTICLE

Retrospective Study

Impact of preoperative chemoradiotherapy using concurrent S-1 and CPT-11 on long-term clinical outcomes in locally advanced rectal cancer

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Abstract

BACKGROUND

Preoperative chemoradiotherapy regimens using a second drug for locally advanced rectal cancer are still under clinical investigation.

AIM

To investigate the clinical outcomes of patients with locally advanced rectal cancer treated with preoperative chemoradiotherapy using tegafur/gimeracil/oteracil (S-1) plus irinotecan (CPT-11).

METHODS

This was a single-center retrospective study of 82 patients who underwent radical surgery for rectal cancer after chemoradiotherapy with S-1 (80 mg/m^2/d), CPT-11 (60 mg/m^2/d), and radiation (total 45 Gy) between 2009 and 2016. The median follow-up was 51 mo (range: 17–116 mo).

RESULTS

Twenty-nine patients (35.4%) had T3 or T4 rectal cancer with mesorectal fascia invasion, 36 (43.9%) had extramural vascular invasion, 24 (29.8%) had N2 rectal cancer and eight (9.8%) had lateral lymph node swelling. The relative dose

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intensity was 90.1% for S-1 and 92.9% for CPT-11. Seventy-nine patients (96.3%) underwent R0 resection. With regard to pathological response, 13 patients (15.9%) had a pathological complete response and 52 (63.4%) a good response (tumor regression grade 2/3). The 5-year local recurrence-free survival, relapse-free survival and overall survival rates were 90.1%, 72.5% and 91.3%, respectively. We analyzed the risk factors for local recurrence-free survival by Cox regression analysis and none were detected. Previously described risk factors such as T4 stage, mesorectal fascia invasion or lateral lymph node swelling were not detected as negative factors for local recurrence-free survival.

CONCLUSION
We demonstrated good compliance and favorable tumor regression in patients with locally advanced rectal cancer treated with preoperative S-1 and CPT-11.

Key words: Preoperative chemoradiotherapy; Rectal cancer; Irinotecan; Tegafur/gimeracil/oteracil; Neoadjuvant chemoradiotherapy; Radiation therapy

Core tip: Lower advanced rectal cancer located within 8 cm of the anal verge carries a higher risk of local recurrence. The aim of this single-center retrospective study was to assess the clinical outcomes of patients with locally advanced rectal cancer treated preoperative chemoradiotherapy using tegafur/gimeracil/oteracil plus irinotecan. Grade 3 or 4 toxicity was mild and led to good relative dose intensity with on-schedule treatment. Also, we investigated the risk factors for local recurrence-free survival and relapse-free survival. Multivariate analysis detected no factors for local recurrence-free survival. Our study confirmed good compliance and favorable tumor regression.

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INTRODUCTION

In the 2000s, numerous studies were planned to investigate the optimal preoperative treatment strategies for advanced rectal cancer. The National Comprehensive Cancer Network and European Society for Medical Oncology consensus guidelines consider preoperative 5-fluorouracil (5-FU)-based chemoradiotherapy (CRT), 45–50.4 Gy, as standard treatment[1,2]. However, the local recurrence rate remains about 10%, and risk factors for local recurrence include T4 stage, mesorectal fascia invasion (MFI), extramural vascular invasion (EMVI) and lateral lymph node (LLN) swelling[3-6]. Multidisciplinary treatments were planned to overcome this issue, such as extended surgery, higher radiation doses, and concurrent use of second drugs, such as oxaliplatin or irinotecan (CPT-11)[7-11]. With regard to the concurrent use of second drugs, six prospective studies failed to confirm any additional benefit of oxaliplatin, and there was a significant increase in severe toxicity and an insufficient response rate[12-17]. However, several Phase II trials have demonstrated the feasibility, safety and effectiveness of CPT-11 as a second drug, with higher pathological complete response (pCR) rates[7,18-25]. *UGT1A1* polymorphisms that can be used to predict the probability of severe toxicity would be of interest for proper therapeutic management using CPT-11[26]. Therefore, the purpose of this study was to investigate the clinical outcomes of 82 patients with locally advanced rectal cancer, located 8 cm from the anal verge, treated with preoperative CRT using tegafur/gimeracil/oteracil (S-1) plus CPT-11.

MATERIALS AND METHODS

Patients

We included 82 patients with T3-4, N0-2, M0 rectal cancer located within 8 cm of the anal verge who were treated with preoperative CRT using S-1 plus CPT-11 between 2009 and 2016. Prior to preoperative therapy, all patients underwent staging work-ups that included digital rectal examination, measurement of tumor marker levels (carcinoembryonic antigen and carbohydrate antigen 19-9), chest X-ray, abdominal and pelvic computed tomography (CT) and magnetic resonance imaging (MRI). MRI was performed on two occasions, as part of initial staging and following preoperative therapy. Testing for *UGT1A1**6 and *28 polymorphisms under national insurance was finally given approval in November 2008 in Japan, and it became measurable at our institution in March 2009. *UGT1A1* polymorphisms are assessed only in cases in which consent is obtained after consultation with a specialist in hereditary diseases[27]. The protocol for the present study was based on the SAMRAI-1 trial[28].

The patients were divided into two groups in accordance with the European Society for Medical Oncology guidelines to confirm the outcomes for these subgroups[29]: (1) "bad" rectal cancer [T3(b)c/T4 with peritoneal or vaginal involvement only, N1–2, MFI negative]; and (2) "ugly" rectal cancer (T4 with overgrowth to adjacent organs, pelvic side walls or sacrum, LLN positive, MFI positive).

Preoperative CRT protocol

Preoperative CRT consisted of S-1 (Days 1-5, 8-12, 22-26 and 29-33; 80 mg/m^2/d), CPT-11 (Days 1, 8, 22 and 29; 60 mg/m^2/d), and radiation (total 45 Gy, 1.8 Gy/d, 5 d per week for 5 wk). Six to eight weeks after completion of preoperative CRT, the patients were scheduled to undergo radical surgery.

Surgical procedure and pathological assessments

All patients underwent total mesorectal excision or extended total mesorectal excision (total mesorectal excision with adjacent visceral resection) to achieve R0 resection. The surgical procedure included low anterior resection, intersphincteric resection and abdominoperineal resection. Intersphincteric resection was recommended in accordance with tumor stage and location, patient age, and preoperative anal function, and patients who did not meet those criteria were selected for abdominoperineal resection. Diverting ileostomy was routinely constructed for all patients with intestinal continuity. LLN dissection was performed when pretreatment MRI showed that the LLNs had a short-axis diameter > 7 mm. Postoperative complications were assessed according to the Clavien-Dindo classification[30]. Pathological response to CRT was evaluated according to the Japanese Classification of Colorectal Carcinoma of the Japanese Society for Cancer of the Colon and Rectum (8th edition). Grade 0 was defined as no evidence of a therapeutic effect and Grade 3 was pCR[31]. We defined a good response as Grade 2 or 3 and poor response as Grade 0 or 1a/1b.

Toxicity or relative dose intensity of chemotherapy

Hematological and nonhematological toxicity caused by preoperative CRT was evaluated according to the Common Terminology Criteria for Adverse Events, version 4.0[32]. Relative dose intensity was calculated as the ratio of the actual dose to the scheduled dose; S-1 (1600 mg/m^2), CPT-11 (240 mg/m^2) and full irradiation dose (45 Gy). Dose reductions of CPT-11 were not applied to the group of patients with *UGT1A1* mutation.

Patient follow-up

Median follow-up was 51 mo (range, 17-116 mo). Postoperative adjuvant chemotherapy using 5-FU-based chemotherapy was recommended for all patients except those with ypT0/1 stage, high age, comorbidity, postoperative complications, and social factors. Patient surveillance was subsequently performed as follows: chest–abdominal CT every 6 mo, colonoscopy annually, and blood tests (including measurement of carcinoembryonic antigen and carbohydrate antigen 19-9 levels) at 3-mo intervals. Local recurrence was defined as the detection of a recurrent tumor within the pelvis, and recurrence was defined as the presence of recurrent disease outside the pelvis.

Statistical analysis

Local recurrence-free survival (LFS), relapse-free survival (RFS) and overall survival (OS) were estimated using the Kaplan–Meier method and compared using the log-rank test. The χ^2 test was also used to evaluate associations between *UGT1A1*

polymorphisms and toxicity and feasibility of treatment. We further evaluated clinical factors associated with LFS and RFS to determine the optimal clinical criteria of this regimen by Cox proportional hazard regression model. Independent variables with $P < 0.1$ in univariate analysis were entered into a multivariate analysis and $P < 0.05$ was considered statistically significant. Statistical analyses were performed using JMP version 12.0 software (SAS Japan Inc., Tokyo, Japan).

RESULTS

Clinical characteristics

The patients' clinical characteristics are shown in Table 1. Clinical T4 stage was diagnosed in 10 patients (12.2%). Clinical N stage was deemed positive in 46 patients (56.1%). MRI revealed tumor involvement of the MF in 29 patients (35.4%). EMVI was observed in 36 patients (43.9%). According to the risk category of rectal cancer, 50 patients (61.0%) were divided into the bad group and 32 (39.0%) into the ugly group.

Compliance and toxicity

The relative dose intensity was 90.1% for S-1, 92.9% for CPT-11 and 97.6% for RT. Toxicity data are shown in Table 2. Grade 3 or 4 hematological toxicity consisted of leukopenia ($n = 15$; 18.3%), neutropenia ($n = 16$; 19.5%) and febrile neutropenia ($n = 3$; 3.6%). Grade 3 or 4 nonhematological toxicity consisted of diarrhea ($n = 22$; 26.8%). For Grade 3 or 4 hematological toxicity, four of 16 neutropenia patients (25.0%) whose neutrophil count was reduced to < 500 cells/μL received granulocyte colony-stimulating factor. For Grade 3 or 4 nonhematological toxicity, four of 22 diarrhea patients (18.2%) were prescribed loperamide. All patients recovered after these conservative treatments.

UGT1A1 genotype distribution and its association with toxicity profiles

Associations between toxicity/feasibility and *UGT1A1* polymorphisms were investigated (Table 3). Forty-eight of 82 patients (58.5%) were assessed for *UGT1A1* polymorphism, and 25 (52.1%) were wild type and 23 (47.9%) were mutant type. Patients with the mutant type had more Grade 3 or 4 hematological toxicity than those with the wild type had ($P < 0.05$). However, there was no significant difference in the incidence of nonhematological toxicity, including diarrhea, in either genotype ($P = 0.65$). There was no significant difference in CPT-11 dose intensity according to *UGT1A1* polymorphisms despite the significant differences observed in hematological toxicity ($P = 0.26$).

Operative findings and postoperative complications

Thirty-one patients (37.8%) underwent low anterior resection, 43 (52.4%) intersphincteric resection and eight (9.8%) abdominoperineal resection. Five patients (6.1%) underwent combined adjacent organ resection and eight (9.8%) LLN dissection.

The postoperative complications are shown in Table 4. Grade 3 pelvic infection was confirmed in nine patients (11.0%) and five (6.1%) developed Grade 3 ileus. Among the patients undergoing sphincter-preserving surgery, seven (9.5%) had Grade 3 anastomosis leakage. During follow-up, six patients could not undergo stoma takedown because of pelvic infection with anastomotic leakage ($n = 4$) and local recurrence ($n = 2$).

Pathological findings

Pathological findings are listed in Table 5. Thirteen patients (15.9%) achieved complete tumor regression with tumor regression grade 3 (pCR). T downstaging was seen in 41 patients (50.0%) and N downstaging in 36 (43.9%). R0 resection was performed in 79 of 82 patients (96.3%) and R1 resection in three (3.7%), with microscopic residual tumor in the anus levator muscle ($n = 2$) and pelvic plexus on the pelvic sidewall ($n = 1$). No patient had R2 resection. Patients with *UGT1A1* mutations showed a significantly better response to CRT (including CPT-11) than those without mutations (Table 3).

Recurrence and survival

Twenty-six patients (31.7%) received 5-FU-based adjuvant chemotherapy: UFT plus leucovorin ($n = 19$), mFOLFOX6 ($n = 4$), S-1 ($n = 2$) and capecitabine ($n = 1$). The reasons for not receiving adjuvant chemotherapy were: ypT0/1 stage ($n = 18$), high age ($n = 11$), comorbidity ($n = 2$), postoperative complications ($n = 12$), social factors ($n = 6$), and others ($n = 7$).

After a median follow-up of 51 mo, 5-year LFS, 5-year RFS and 5-year OS rates were 90.1%, 72.5% and 91.3%, respectively (Figure 1). Local recurrence was seen in six

Table 1 Patient characteristics

Characteristic	*n* = 82
Age (yr)	
Median (range)	64 (34–79)
Sex	
Male	60 (73.2)
Female	22 (26.8)
Distance from anal verge (cm)	
Median (range)	5.0 (0–8)
Size of tumor (cm)	
Median (range)	4.5 (2–9)
Clinical T stage (before chemoradiotherapy)	
3	72 (87.8)
4	10 (12.2)
Mesorectal fascia invasion	
–	53 (64.6)
+	29 (35.4)
Extramural vascular invasion	
–	46 (56.1)
+	36 (43.9)
Clinical N stage (before chemoradiotherapy)	
–	36 (43.9)
+	46 (56.1)
Subgroup of locally advanced rectal cancer	
Bad	50 (61.0)
Ugly	32 (39.0)
UGT1A1 polymorphism (in 48 patients)	
Wild type	25 (52.1)
Mutant type	23 (47.9)

Data are *n* (%) unless otherwise shown.

patients: LLNs (n = 4) and other sites (n = 2). Distant recurrence was detected in 20 patients: lung (n = 15), liver (n = 6), para-aortic region (n = 2), inguinal region (n = 1) and bone (n = 1). Some patients had overlapping metastases. LFS did not differ significantly between the bad and ugly groups (96.0% *vs* 76.2%; P = 0.10); however, RFS was significantly poorer in the ugly group (38.5% *vs* 87.8% in bad group; P < 0.01).

Risk factors for LFS and RFS

We investigated the risk factors for LFS and RFS (Table 6). Multivariate analysis showed that no risk factors for LFS were detected, including previously described risk factors such as T4 stage, MFI, EMVI and LLN swelling. However, MFI and EMVI were associated with poor RFS for locally advanced rectal cancer (OR: 5.82, 95%CI: 1.68-20.2, P < 0.01; OR: 3.42, 95%CI: 1.02-11.5, P = 0.04).

DISCUSSION

We reported the safety, effectiveness and long-term outcomes of concomitant use of CPT-11 with 5-FU-based CRT for locally advanced rectal cancer. S-1 is an oral anticancer agent containing tegafur (a prodrug of 5-FU) with two modulators, gimeracil and oteracil potassium, which markedly increase the radiosensitivity of cancer cells[33]. CPT-11 augments inhibition of thymidylate synthase – the target enzyme of 5-FU[34]. In addition, 5-FU induces topoisomerase I, and cancer cells overexpressing topoisomerase I show increased chemosensitivity to CPT-11[35]. Such *in vitro* mechanisms are effective in combination with 5-FU as a radiosensitizer for preoperative CRT[7]. Furthermore, *UGT1A1* polymorphisms that can predict the probability of developing potentially severe toxicity during treatment with CPT-11-

Table 2 Acute toxicity according to Common Terminology Criteria for Adverse Events 4.0, on patients receiving chemoradiotherapy, *n* (%)

Toxicity	*n* = 82	
	Any Grade	Grade 3 or 4
Hematological toxicity		
Neutropenia	56 (68.3)	16 (19.5)
Leukopenia	59 (72.0)	15 (18.3)
Febrile neutropenia	3 (3.6)	3 (3.6)
Thrombocytopenia	11 (13.4)	0
Nonhematological toxicity		
Diarrhea	53 (64.6)	22 (26.8)
Anorexia	20 (24.4)	1 (1.2)
Fatigue	15 (18.3)	0
Nausea	13 (15.9)	0

based regimens could be clinical factors in the proper management of treatment[26]. The purpose of this study was to investigate the clinical outcomes of patients with locally advanced rectal cancer treated with preoperative CRT using S-1 plus CPT-11.

Current standard CRT regimens include only 5-FU. However, several clinical trials incorporating a second active systemic agent into conventional CRT regimens have been performed to examine the ability of the regimens to increase pCR rate and improve resectability and locoregional control[6,10]. Two such second drugs, oxaliplatin and CPT-11, have been investigated in clinical trials.

With regard to oxaliplatin, six randomized Phase III studies have compared oxaliplatin-based with 5-FU-based regimens[12-17]. Among these, the STAR-01 (16% both groups), ACCORD 12/0405 (19% *vs* 14%), NSABP R-04 (21% *vs* 19%) and PETACC-6 (15% *vs* 13%) studies reported that there were no substantial improvements in pCR rates, and significantly increased intolerable Grade 3 or 4 toxicity. For this reason, the concomitant use of oxaliplatin in 5-FU-based CRT has not been permitted (Supplementary Table 1). No Phase III studies using CPT-11 have been documented; however, nine Phase II studies (2 randomized controlled trials and 7 single-arm studies) have assessed the usefulness of CPT-11 as a radiosensitizer[7,18-25]. These studies indicated that this CPT-based regimen was promising in terms of pCR rate (range 13.7%-37%). Grade 3 or 4 toxicity was mild and led to good relative dose intensity with on-schedule treatment without dose reduction (Supplementary Table 2).

The most frequent severe toxicity was neutropenia (2.1%-12%) and diarrhea (2.1%-22%). Generally, toxicity was correlated with the dose of chemotherapy. Jung *et al*[25], who used 40 mg/m^2 CPT-11, demonstrated that the rate of Grade 3 or 4 hematological toxicity was 1.4% and the rate of Grade 3 or 4 nonhematological toxicity was 5.7%. Sato *et al*[7], who used 80 mg/m^2 CPT-11, demonstrated that the rate of Grade 3 or 4 hematological toxicity was 6% and the rate of Grade 3 or 4 nonhematological toxicity was 4.5%. These results suggest that concurrent use of second drugs, such as CPT-11 as a radiosensitizer, is well tolerated in terms of toxicity.

UGT1A1 polymorphisms have been confirmed as predictive markers of severe toxicity of CPT-11 in a metastatic setting[26]. Our previous study demonstrated the effectiveness of *UGT1A1* polymorphism in predicting the toxicity of preoperative CRT using CPT-11, although it was only a small retrospective study[36]. Thus, to provide patients with the full benefit of CRT, good tolerance of CPT-11-based regimens for patients with *UGT1A1* mutant type, as well as the prevention and early treatment of severe toxicity, is important. This suggests that drawing definitive conclusions about the role of *UGT1A1* polymorphisms requires a randomized trial, to assess whether genotype-adjusted dose of CPT-11 would help establish a well-tolerated, effective dose for tumor response in patients with wild-type and mutant *UGT1A1*.

The present study included patients with highly advanced rectal cancer: 29 (35.4%) with T4 or T3 with MFI, 36 (43.9%) with EMVI, 24 (29.8%) with N2, and 32 (39.0%) with ugly rectal cancer. Even such highly advanced rectal cancer demonstrated favorable local control. With respect to systemic recurrence, highly advanced rectal cancer has a high recurrence rate, with poor prognosis; therefore, combined use of systemic treatment, mainly including chemotherapy, is important for prolonging survival benefit[37]. Further studies are warranted to examine the additional effect of

Table 3 Associations between toxicity, feasibility and treatment effect and *UGT1A1* polymorphisms, *n* (%)

	Wild type (*n* = 25)	Mutant type (*n* = 23)	*P* value
Toxicity			
Hematological toxicity (Grade 3 or 4)	0	11 (47.8)	< 0.01
Nonhematological toxicity (Grade 3 or 4)	8 (32.0)	6 (26.1)	0.65
Feasibility (%)			
S-1 dose intensity (mean ± SD)	90.9 ± 0.2	89.0 ± 0.2	0.38
CPT-11 dose intensity (mean ± SD)	93.0 ± 0.3	88.2 ± 0.2	0.26
Treatment effect			
Good response	18 (72.0)	22 (95.7)	< 0.01
Poor response	7 (28.0)	1 (4.3)	
Pathological complete response	5 (20.0)	6 (26.1)	0.61

CPT-11: Irinotecan; S-1: Tegafur/gimeracil/oteraci.

CPT-11 on those tumors.

Our study had several limitations. First, it was a small retrospective study performed in a single institution. Second, we excluded atypical rectal cancer, such as mucinous carcinoma caused by anal fistula, which is associated with a poorer response to CRT, because we chose surgery without radiation. Third, we excluded patients with performance status 3/4 or those aged > 80 years who cannot tolerate this regimen owing to comorbidity and old age. Such patients (n = 3) were treated with stoma creation alone. Fourth, the follow-up time was not sufficient to evaluate OS, LFS and RFS. Fifth, *UGT1A1* polymorphism analysis was not performed for all patients receiving preoperative CRT. Finally, we did not study toxicity-based dose-finding methods for S-1 plus CPT-11 preoperative CRT in a Phase I study. Nevertheless, this study demonstrated the safety, effectiveness and long-term oncological outcomes of locally advanced rectal cancer treated with concomitant CPT-11 and 5-FU-based CRT.

In conclusion, our single-center retrospective study confirmed good compliance, favorable tumor regression and feasible oncological outcomes of preoperative CRT using S-1 plus CPT-11, and favorable local control of highly advanced rectal cancer by this regimen.

Table 4 Postoperative surgical complications, *n* (%)

Complication	*n* = 82	
	Any grade	Grade 3
Pelvic infection	15 (18.3)	9 (11.0)
Anastomosis leakage[1]	9 (12.2)	7 (9.5)
Ileus	11 (13.4)	5 (6.1)
Bleeding	1 (1.2)	1 (1.2)
Surgical site infection	2 (2.4)	0
Urinary dysfunction	8 (9.8)	0
Venous thromboembolic event	1 (1.2)	0
Re-operation	0	0

[1]The patients who performed abdominoperineal resection was excluded, and anastomosis leakage rates were calculated. SSI: Surgical site infection; VTE: Venous thromboembolic event.

Table 5 Pathological tumor characteristics, *n* (%)

Pathological tumor characteristics	*n* = 82
ypT stage	
0	13 (15.9)
1	5 (6.1)
2	21 (25.6)
3	25 (42.7)
4	8 (9.8)
ypN stage	
–	65 (79.3)
+	17 (20.7)
yp TNM stage[1]	
0	13 (15.9)
I	20 (24.4)
II	32 (39.0)
III	17 (20.7)
Residual tumor classification	
R0	79 (96.3)
R1	3 (3.7)
R2	0
Histology	
well/moderately differentiated	66 (80.5)
Poorly differentiated/mucinous/signet	16 (19.5)
T downstaging	
–	41 (50.0)
+	41 (50.0)
N downstaging	
–	46 (56.1)
+	36 (43.9)
Tumor regression grade	
1a	18 (22.0)
1b	12 (14.6)
2	39 (47.6)
3	13 (15.9)
Adjuvant chemotherapy	
–	56 (68.3)
+	26 (31.7)

[1]According to the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology. R0: No residual tumor confirmed microscopically; R1: Microscopic tumor residue; R2: Macroscopic tumor residue.

Table 6 Multivariate prognostic analysis for local recurrence-free survival and relapse-free survival

Factors	*n*	LFS						RFS					
		Univariate			Multivariate			Univariate			Multivariate		
		OR	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value
Sex													
Female	22												
Male	60	1.91	0.21-17.7	0.56				1.13	0.36-3.60	0.83			
Location from anal verge (cm)													
≥ 5.0	29												
< 5.0	53	1.10	0.19-6.41	0.91				1.89	0.61-5.89	0.26			
Tumor diameter (cm)													
< 4.5	42												
≥ 4.5	40	2.00	0.86-2.89	0.43				0.63	0.22-1.74	0.37			
cT													
3	72												
4	10	4.25	0.67-27.0	0.10	1.97	0.23-17.1	0.54	3.80	0.97-14.9	0.06	2.05	0.39-10.7	0.39
cN													
−	36												
+	46	1.62	0.28-9.38	0.59				1.25	0.34-2.60	0.90			
Mesorectal fascia invasion													
−	53												
+	29	4.08	0.70-23.8	0.10	2.88	0.39-21.5	0.30	7.31	2.39-22.4	< 0.01	5.82	1.68-20.2	< 0.01
Extramural vascular invasion													
−	46												
+	36	2.75	0.47-15.9	0.24				3.15	1.10-9.03	0.03	3.42	1.02-11.5	0.04
Lateral lymph node (> 7.0 mm)													
−	74												
+	8	5.83	0.88-38.7	0.09	4.71	0.65-34.2	0.12	2.01	0.44-9.29	0.36			
Histology													
Well/moderately differentiated	66												
Poorly differentiated/mucinous/signet	16	0.81	0.09-7.49	0.86				1.55	0.46-7.58	5.15			

CI: Confidence interval; LFS: Local recurrence-free survival; OR: Odds ratio, RFS: Relapse-free survival.

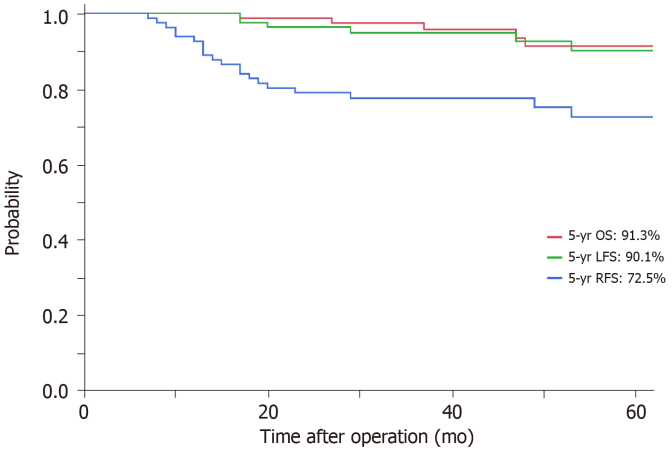

Figure 1 Long-term outcomes of patients with locally advanced rectal cancer treated with preoperative chemoradiotherapy using tegafur/gimeracil/oteracil plus irinotecan.

ARTICLE HIGHLIGHTS

Research background

Prospective studies have investigated the optimal treatment strategies for management of locally advanced rectal cancer, and have concluded that preoperative 5-fluorouracil-based chemoradiotherapy (CRT) at 45–50.4 Gy is a standard treatment. However, local recurrence rate remains about 10%; mainly for highly advanced cases.

Research motivation

Multidisciplinary treatments were planned to overcome highly advanced rectal cancer, such as extended surgery, higher radiation doses, and concurrent use of second drugs, such as oxaliplatin or CPT-11.

Research objectives

The aim of this study was to investigate the safety, therapeutic effect, and outcome of preoperative CRT using S-1 plus irinotecan for locally advanced lower rectal cancer.

Research methods

Between 2009 and 2016, 82 patients underwent total mesorectal excision after preoperative CRT. Preoperative CRT consisted of S-1 (80 mg/m^2/d), CPT-11 (60 mg/m^2/d), and radiation (total 45 Gy). The median follow-up was 51 months (range: 17-116 mo).

Research results

This regimen was well tolerated in terms of toxicity. Associations between toxicity/feasibility and *UGT1A1* polymorphisms were investigated. Compared with patients with wild-type *UGT1A1*, those with mutant type had more Grade 3 or 4 hematological toxicity ($P < 0.05$). With regard to oncological outcome, mesorectal fascia invasion and extramural vascular invasion were associated with poor relapse-free survival for locally advanced rectal cancer. However, Cox regression analysis did not detect any risk factors for local recurrence-free survival.

Research conclusions

This regimen had favorable oncological outcomes for highly advanced rectal cancer.

Research perspectives

This was a small retrospective study performed in a single institution. A randomized multicenter study is needed to investigate the influence of dose setting by *UGT1A1* polymorphism for preoperative CRT using irinotecan.

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ORIGINAL ARTICLE

Retrospective Study

Surgical intervention for malignant bowel obstruction caused by gastrointestinal malignancies

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Abstract

BACKGROUND

Malignant bowel obstruction (MBO) is a common event for end-stage gastrointestinal cancer patients. Previous studies had demonstrated manifestations and clinical management of MBO with mixed malignancies. There still lack reports of the surgical treatment of MBO.

AIM

To analyze the short-term outcomes and prognosis of palliative surgery for MBO caused by gastrointestinal cancer.

METHODS

A retrospective chart review of 61 patients received palliative surgery between January 2016 to October 2018 was performed, of which 31 patients underwent massive debulking surgery (MDS) and 30 underwent ostomy/by-pass surgery (OBS). The 60-d symptom palliation rate, 30-d morbidity and mortality, and overall survival rates were compared between the two groups.

RESULTS

The overall symptom palliation rate was 75.4% (46/61); patients in the MDS group had significantly higher symptom palliation rate than OBS group (90% *vs* 61.2%, $P = 0.016$). Patients with colorectal cancer who were in the MDS group showed significantly higher symptom improvement rates compared to the OBS group (overall, 76.4%; MDS, 61.5%; OBS, 92%; $P = 0.019$). However, patients with gastric cancer did not show a significant difference in symptom palliation rate between the MDS and OBS groups (OBS, 60%; MDS, 80%; $P = 1.0$). The median survival time in the MDS group was significantly longer than in the OBS group (10.9 mo *vs* 5.3 mo, $P = 0.05$).

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CONCLUSION
For patients with MBO caused by peritoneal metastatic colorectal cancer, MDS can improve symptom palliation rates and prolong survival, without increasing mortality and morbidity rates.

Key words: Gastrointestinal neoplasms; Malignant bowel obstruction; Metastasis; Palliative surgery; Prognosis; Quality of life

Core tip: Malignant bowel obstruction (MBO) is a frequent event for patients with end-stage gastrointestinal cancer. There is no consensus on the optimal treatment strategy for improving quality of life and prolonging survival. We performed a retrospective study at a single institution to determine the effects of palliative surgery for MBO in patients with gastrointestinal cancers. In this cohort, we observed higher symptom relief rates and prolonged survival after massive debulking surgery compared with ostomy/by-pass surgery in MBO patients. For select patients with MBO caused by metastatic colorectal cancer, massive debulking surgery can result in higher symptom palliation rates and prolonged survival without increasing mortality and morbidity rates compared with ostomy/by-pass surgery.

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INTRODUCTION

Malignant bowel obstruction (MBO) is a frequent event for patients with end-stage cancer, especially in gastrointestinal (GI) and ovarian cancer[1-3]. It had been reported that 10%-50% of patients with cancer will develop MBO during the preterminal stage[4]. Patients with MBO suffered from an inability to eat, abdominal pain, distention, nausea, and vomiting, resulting in a poor quality of life (QOL), stress, and emotional problems.

The clinical management of MBO requires a specific and individualized approach based on the expected rest time of the disease, objectives of care, and the patient's preferences. The primary treatment objective in patients with MBO is to relieve symptoms, restore food intake, and improve nutrition[5]. The most common treatment options for patients with MBO included nasogastric drainage, total parenteral nutrition, pain control, somatostatin, endoscopically placed stents, and palliative surgery[4]. Due to the heterogeneity of MBO patients, there is no consensus on the optimal treatment strategy for improving QOL and prolonging survival. The decision making of the management of MBO should be individualized[6].

Surgical intervention is usually considered to be the last treatment option that may relieve the symptoms of MBO. However, the decision to proceed with palliative surgery is usually difficult, especially for patients who may only have a few weeks to live. Additionally, MBO patients suffering from malnutrition are not good candidates for surgical intervention because of the high postoperative mortality and morbidity rates. Previous studies included several types of malignancies in their analysis, and most focused on the treatment of ovarian cancer. At present, there is limited evidence on the effectiveness of surgical intervention for MBO[7].

The surgical treatments for MBO include percutaneous venting gastrostomy, stoma diversion and cytoreductive surgery, *etc*.[8-10]. However, some patients with extensive peritoneal metastasis may have multiple obstruction sites, and an ostomy or by-pass surgery may not be feasible. Previous studies have shown that some selected patients with recurrent, unresectable colorectal cancer may benefit from cytoreductive surgery, massive tumor debulking surgery and hyperthermic intraperitoneal chemotherapy[11-14].

We performed a retrospective study at a single institution to determine the effects of palliative surgery for MBO in patients with GI cancers and analyzed symptom

palliation rates, postoperative mortality, complications after surgery, and survival rates.

MATERIALS AND METHODS

Patient selection

Between January 2016 to October 2018, we enrolled 61 patients with MBO caused by peritoneal metastasis of gastric and colorectal cancer underwent surgical treatment at the Peking University Cancer Hospital.

Including criteria for patients are: (1) Clinical evidence of bowel obstruction; (2) Obstruction distal to the Treitz ligament; (3) The presence of primary gastric and colorectal cancer; (4) The absence of curable possibilities; (5) Ineffective conservative treatment for obstruction; (6) Expected survival of more than 2 mo; and (7) Patients and their families willing to undergo surgery[4].

All patients discussed their options with the Multiple Disciplinary Team before the operation, and a treatment plan was developed based on the patients' preferences. The surgical options included ostomy/by-pass surgery (OBS) and massive debulking surgery (MDS).

Indications for OBS: (1) Localized or single-site tumor obstruction; (2) Patients may benefit from further chemotherapy; and (3) Patients and their families do not receive aggressive tumor reductive surgery.

Indications for MDS: (1) Perioperative risk evaluation indicates that patients can tolerate surgery; (2) After imaging and physical examination, it is expected that the obstruction and symptoms can be effectively relieved after operation; (3) Patients can endure at least 2 months of oral feeding and non-obstructive survival; (4) It is expected that at least 2 meters of small intestine will remain after the operation; and (5) Patients and their families had a strong preference for surgery.

Evaluation of surgical effectiveness: (1) Solid food intake and symptom relief rates 60 d after surgery[15]; (2) Postoperative complications and mortality rates within 30 d; and (3) Postoperative survival time.

Statistical analysis

Statistical analysis was performed by using the IBM-SPSS19 software package. The categorical variables were compared by the Pearson's chi-square test. Overall survival rates were analyzed by the Kaplan-Meier method and the difference between the groups were evaluated by a log-rank test. $P < 0.05$ was considered as statistically significant. Significant prognostic factors were analyzed using the Cox proportional hazards regression model to determine the independent prognostic factors of MBO caused by colorectal cancer.

RESULTS

Clinicopathological features

A total of 61 patients with GI cancer were enrolled in this study. Of the 61 patients, 38 were male and 23 were female. Patient ages ranged from 19 to 88 years, with an average age of 59.6 ± 11.5 years and a median age of 61 years. The clinical and pathological data of all the patients in this study are shown in Table 1.

Evaluation of surgical effectiveness and survival analysis

In the MDS group, 3 patients showed no evidence of disease. The overall symptom improvement rate was 75.4% (46/51). The overall symptom improvement rate in the MDS group was significantly higher than in OBS group (90% *vs* 61.3%, respectively, $P = 0.016$). Re-obstruction occurred in 3 patients after the operation, including 2 in the OBS group and 1 in the MDS group.

Ten patients, including 4 patients in OBS group and 6 patients in the MDS group, continued medical treatment and radiotherapy after the operation. By the final follow-up in June 2019, 38 of the 61 patients had died. The median survival time of the whole group was 6.5 mo, and the 6-mo survival rate was 56.5% (Figure 1A). Patients with colorectal cancer had significant longer median survival than gastric cancer (10.6 mo *vs* 1.8 mo, $P = 0.015$, Figure 1B). Patients in MDS group had significant longer median survival than in OBS group (10.9 mo *vs* 5.3 mo, $P = 0.05$, Figure 1C). Patients with improvement of symptoms after operation had significant longer median survival than those without (10.9 mo *vs* 3.9 mo, $P = 0.007$, Figure 1D).

Table 1 Clinicopathological features and survival of 61 malignant bowel obstruction cases

Clinicopathological features	*n* (%)	6-mo OS	Median survival time (mo)	*P* value
Gender				0.923
Male	38 (65.2)	54.7%	6.6	
Female	23 (34.8)	59.2%	6.4	
Age (yr)				0.516
≥ 60	33 (54.1)	50.4%	6.5	
< 60	28 (45.9)	61.5%	10.6	
Primary tumor				0.015
Colorectal	51 (69.6)	83.6%	10.6	
Gastric	10 (30.4)	16.4%	1.8	
Differentiation				0.005
Well + Moderate	41 (67.2)	71.5%	10.9	
Poor	20 (32.8)	26.5%	4.1	
ECOG				0.031
0-1	40 (65.6)	64.4%	10.6	
> 1	21 (34.4)	42.9%	5.1	
Surgical approach				0.053
OBS	31 (50.8)	44.6%	5.3	
MDS	30 (49.2)	68.6%	10.9	
Obstruction				0.25
Single	41 (67.2)	57%	6.6	
Multiple	20 (32.8)	55%	6.1	
Intestinal obstruction				0.084
Yes	31 (50.8)	53.2%	6.1	
No	30 (49.2)	60.1%	10.9	
Colon/rectum obstruction				0.389
Yes	37 (60.7)	57.4%	10.9	
No	24 (39.3)	54.8%	6.5	
Ascites				0.691
Yes	15 (24.6)	41.1%	5.1	
No	46 (75.4)	61.1%	6.6	
Greater omentum metastasis				0.044
Yes	42 (68.9)	51.2%	6.1	
No	19 (31.1)	67.5%	17.3	
Distant metastasis				0.13
Yes	24 (39.3)	50%	5.2	
No	37 (60.7)	60.4%	10.9	
Symptom relief				0.007
Yes	46 (75.4)	63.7%	10.9	
No	15 (24.6)	31.5%	3.9	

OBS: Ostomy/by-pass surgery; MDS: Massive debulking surgery; ECOG: Eastern Cooperative Oncology Group; OS: Overall survival.

Postoperative mortality and morbidity

In this cohort, 32 patients had complications within 30-d of surgery. In the MDS group, 1 patient had secondary surgery due to wound infection.

The overall complication rates in the MDS and OBS groups were 48.4% (15/31) and 56.7% (17/30), respectively. There was no significant difference between the two groups (P = 0.611). The mortality rates within 30 days of the operation in the MDS and OBS groups were 19.4% (6/31) and 6.7% (2/30), respectively. There was no significant difference between the two groups (P = 0.255, Table 2).

Because of the short survival time of patients with gastric cancer, we conducted a univariate analysis of MBO in patients with colorectal cancer, and found that surgical approach [hazard ratio (HR) = 2.301, 95%CI: 0.999-5.299, P = 0.05], differentiation (HR

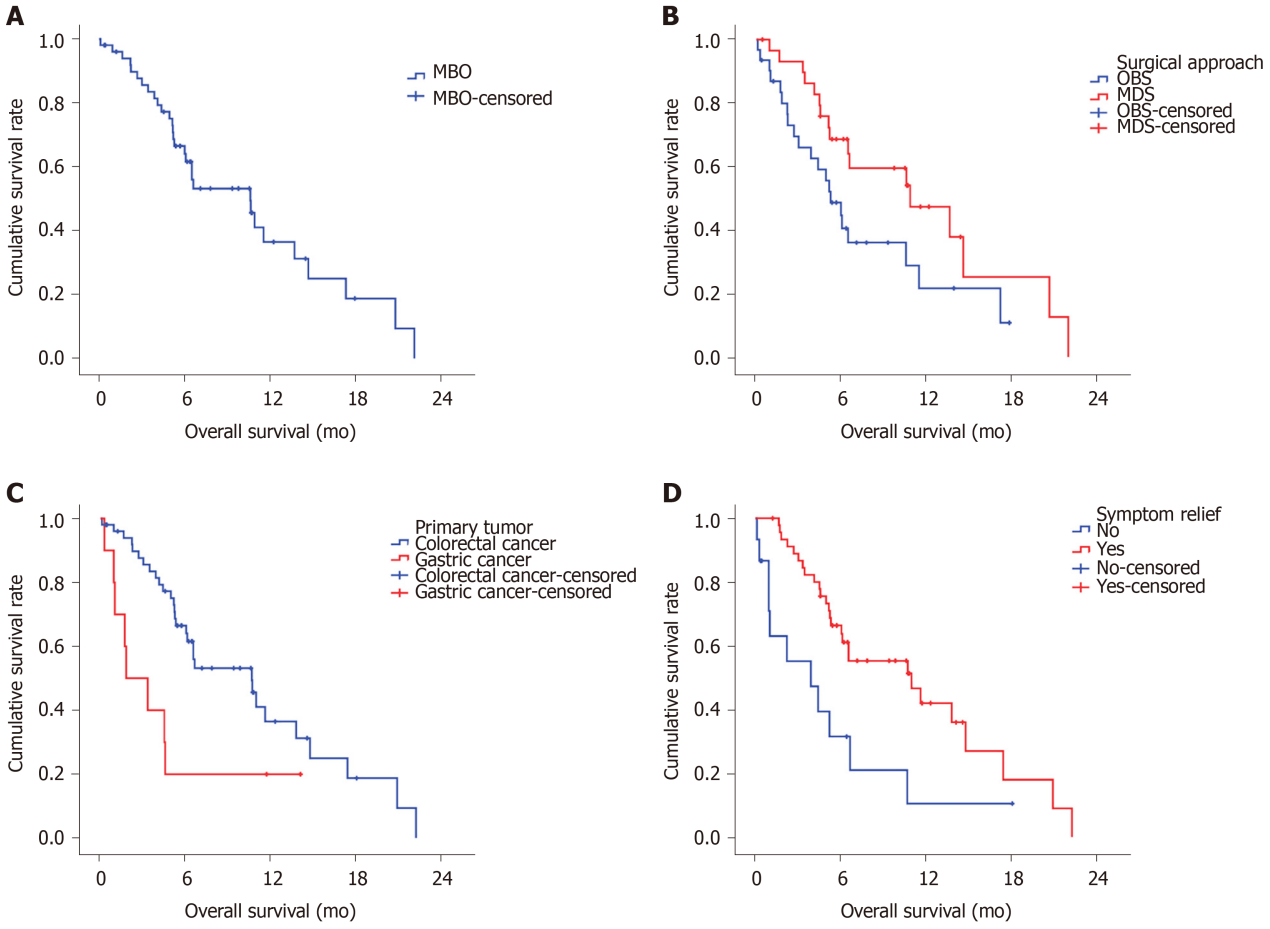

Figure 1 Kaplan-Meier curves of overall survival for patients. A: Overall survival for patients with 61 malignant bowel obstruction patients (median survival time of 6.5 mo); B: Overall survival for patients of ostomy/by-pass surgery group and massive debulking surgery group; C: Overall survival for patients with colorectal cancer and gastric cancer; D: Overall survival for patients of improvement of symptoms after operation. MBO: Malignant bowel obstruction; MDS: Massive debulking surgery; OBS: Ostomy/by-pass surgery.

= 8.509, 95%CI: 2.455-26.448, *P*= 0.001), greater omentum metastasis (HR = 7.718, 95%CI: 2.224-26.782, *P* = 0.001) and distant organ metastasis (HR = 2.375, 95%CI: 1.022-5.253, *P* = 0.044, Table 3) were independent prognostic factors for MBO caused by metastatic colorectal cancer (mCRC).

DISCUSSION

MBO patients represent a complex and heterogeneous population, and there are no evidence-based guidelines to help with the decision-making process for clinical management of the disease. Previous studies have reported on the effectiveness of palliative surgery for MBO, most of which focused on gynecologic and GI cancer populations[17-19]. However, there is little data on palliative surgery for MBO in colorectal cancer patients and most of the literature that included colorectal cancer combined several types of malignancies.

In this cohort study focused on GI cancer, we observed higher symptom relief rates and prolonged survival after MDS compared with OBS in MBO patients. For select patients with MBO caused by peritoneal mCRC, MDS can result in higher symptom palliation rates and prolonged survival without increasing mortality and morbidity rates compared with OBS. However, because of the short survival time of GI cancer patients, no surgical intervention which may cause a patient to spend the remainder of their life in the hospital should be seriously considered.

The priority of care for inoperable and conservative MBO patients is to control their symptoms and improve their QOL. Medical treatment combining total parenteral nutrition, opioids, antiemetics, and somatostatin drugs is usually considered to be the preferred treatment[20]. The primary goals in palliative surgery are symptom relief and the restoration of oral feeding. Single site obstructions can be resolved by endoscopic treatment and ostomy[21,22]. However, some patients with complete obstructions may

Table 2 Sixty-one cases of complications after surgery, *n* (%)

Complications	OBS group (*n* = 31)	MDS group (*n* = 30)
Pulmonary infection	6 (19.4)	4 (13.3)
Abdominal infection	2 (6.5)	4 (13.3)
Incision infection	3 (9.7)	4 (13.3)
Urinary tract infection	1 (3.2)	-
Diarrhea	-	1 (3.3)
Arrhythmia	1 (3.2)	-
Hemorrhage	1 (3.2)	4 (13.3)
Cerebral infarction	-	2 (6.7)
Short intestine	-	2 (6.7)
Necrosis of ostomy mucosa	2 (6.5)	-
Clavien-Dindo classification[16]		
I	-	1(3.3)
II	9 (29)	13 (43.3)
III	-	-
IV	-	1 (2.3)
V	6 (19.4)	2 (6.7)

OBS: Ostomy/by-pass surgery; MDS: Massive debulking surgery.

have multiple sites that are occlusive, and therefore, tumor reduction surgery becomes a necessity.

The overall symptom relief rate of all the patients included in this study was 76.1%. The MDS group achieved a higher symptom relief rate than the OBS group (91.3% *vs* 60.9%, respectively), which was higher than that reported in the literature[15]. The re-obstruction rate was 6.5% in this group, which was lower than the 9% reported in the literature[23]. In this group, although the obstructions were removed, the symptom improvement rate in the OBS group was not high, and 40% of these patients still needed parenteral nutritional support.

Previous studies reported that the median survival for MBO patients treated with conservative care was no longer than 4–5 wk[24]. For some MBO patients, surgical intervention can prolong survival[1,4,25-27]. In this study, the overall median survival time was 6.5 mo. The patients who underwent MDS had a longer survival time than those who underwent OBS (median survival time was 11.5 mo *vs* 5.1 mo, respectively), and a longer survival time than those reported in previous literature (4-9 mo)[28,29].

In this cohort, the patients that showed significant symptom improvement and good oral food intake had significantly longer survival times than those who did not. This indicates that palliative surgery, although aimed at relieving symptoms, has also prolonged survival, likely due to the improvement in nutrition and subsequent systemic treatment. These results were in accordance with the literature[26]. However, due to the high heterogeneity of patients with malignant intestinal obstructions, it is usually difficult for surgeons to determine the best surgical approach before the operation is performed[5].

Although MDS and OBS can improve the QOL and potentially the survival time, these benefits are accompanied by high mortality and complication rates. In this study, the 30-d postoperative complication rates were as high as 54.3% and 13%, respectively, which are similar to those reported in previous literature (6%-40%) and 5%-15%, respectively[5,18,28,30]. Notably, patients undergoing OBS had comparable complication rate and mortality after surgery as patients in the MDS group, suggesting that the short-term prognosis of patients cannot be overlooked.

Much of the literature defines surgical benefit as at least 60 d of survival after the operation. In this cohort, the median survival of gastric cancer patients was only 1.8 mo, suggesting surgery should not be routinely undertaken in patients with end-stage gastric cancer. However, MBO patients with colorectal cancer may benefit from surgical intervention especially for select patients that can withstand treatment with MDS. Previous studies have suggested candidate prognostic indicators that a patient has a low likelihood of benefiting from surgery for MBO. Age, surgical treatment, ascites, low albumin, hypoproteinemia, and primary tumors are reported to be the main prognostic factors. The number of combined risk factors determines survival

Table 3 Multivariable analysis prognostic factors of 51 malignant bowel obstruction colorectal cancer patients

Variables	HR	95%CI	*P* value
Surgical approach (OBS *vs* MDS)	2.301	0.999-5.299	0.05
Greater omentum metastasis (Yes *vs* No)	7.718	2.224-26.782	0.001
Differentiation (Poor *vs* Well+ Moderate)	8.059	2.455-26.448	0.001
Distant organ metastasis (Yes *vs* No)	2.375	1.022-5.253	0.044

OBS: Ostomy/by-pass surgery; MDS: Massive debulking surgery; HR: Hazard ratio.

rates and the incidence rates of short-term complications after surgery[6,18,27,28,31]. Before any palliative surgical intervention is performed, the feasibility of surgery and the probability that a patient will benefit not only from an improvement in QOL but in terms of survival should be taken into consideration[32]. In this cohort, survival analysis showed that the primary tumor site, differentiation, the surgical approach, the ECOG score and greater omentum metastasis were the prognostic factors for MBO patients with GI cancer. Whereas in mCRC, differentiation, surgical approach, greater omentum metastasis and distant organ metastasis were the most important independent prognostic factors.

The limitations of this study were the small sample size and the high heterogeneity of the patients. We lacked the data on the use of medical antineoplastic drugs and the patient *KARS*/*BRAF* status. There may also be selection bias that affects the conclusions. Although symptom relief and survival benefit were observed in this group, the risk of serious complications and mortality cannot be ignored. Surgeons should fully weigh the life-expectancy, potential benefits and risks, and the QOL of patients after the operation. The aim of surgical and other approaches must be considered and prioritized based on each patient's and family's goals and preferences. Although surgeons usually actively treat postoperative complications, it is may be unacceptable for some patients with end-stage diseases spending the rest life in hospital.

Because of the patient heterogeneity, it seems impossible to conduct a random study of malignant intestinal obstruction. In the future, registry studies may be needed to provide high-level evidence about the treatment of MBO and the QOL of patients should be taken into account.

In conclusion, because of the short expected-survival time for patients with gastric cancer and MBO, the high perioperative complication and mortality rates should be seriously considered before any surgical intervention is performed even for an ostomy. For select patients with MBO caused by peritoneal mCRC, MDS may result in high symptom palliation rates and prolonged survival compared with OBS, without increasing mortality and morbidity rates.

ARTICLE HIGHLIGHTS

Research background

Malignant bowel obstruction (MBO) is a frequent event for end-stage malignant cancers. There is no consensus on the optimal treatment strategy for improving quality of life and prolonging survival. There were fewer studies focused on the surgical intervention of MBO with gastrointestinal (GI) cancers.

Research motivation

We wanted to investigate the effects of palliative surgery for MBO in patients with GI cancers in order to guide treatment.

Research objectives

To define the surgical outcome difference between massive debulking surgery (MDS) and ostomy/by-pass surgery (OBS) for MBO patients with GI cancer.

Research methods

MBO patients with GI cancer receive palliative surgery were included MDS group and OBS group. This study mainly investigated the difference of short outcome and survival between the two groups.

Research results

This study reported that patients in the MDS group had significantly higher symptom palliation

rate than OBS group, and the median survival time in the MDS group was significantly longer than in the OBS group.

Research conclusions

Massive debulking surgery can significantly improve symptom and prolong survival for MBO patients with colorectal cancer, without increasing mortality and morbidity rates compared with ostomy/by-pass surgery. However, MDS had no such advantage in gastric cancer.

Research perspectives

The treatment of MBO remained controversial and no well-evidenced. This small sample study demonstrates the effectiveness, safety and survival benefit of massive debulking surgery in colorectal cancer patients with MBO. It is difficult to carry out large sample randomized controlled study. In the future, it is necessary to establish a large sample registration study.

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ORIGINAL ARTICLE

Observational Study

FOLFOXIRI *vs* FOLFIRINOX as first-line chemotherapy in patients with advanced pancreatic cancer: A population-based cohort study

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Abstract

BACKGROUND

FOLFIRINOX regimen is the first-line reference chemotherapy (L1) in advanced pancreatic ductal adenocarcinoma (aPDAC). FOLFOXIRI, a schedule with a lower dose of irinotecan and no bolus 5-fluorouracil, has demonstrated efficacy and feasibility in colorectal cancer.

AIM

To investigate the potential clinical value of FOLFOXIRI in patients with aPDAC in routine clinical practice.

all authors read and approved the final manuscript.

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METHODS
Analyses were derived from all consecutive aPDAC patients treated in L1 between January 2011 and December 2017 in two French institutions, with either FOLFOXIRI (n = 165) or FOLFIRINOX (n = 124) regimens. FOLFOXIRI consisted of irinotecan (165 mg/m^2), oxaliplatin (85 mg/m^2), leucovorin (200 mg/m^2) and 5-fluorouracil (3200 mg/m^2 as a 48-h continuous infusion) every 2 wk. Ninety-six pairs of patients were selected through propensity score matching, and clinical outcomes of the two treatment regimens were compared.

RESULTS
Median overall survival was 11.1 mo in the FOLFOXIRI and 11.6 mo in the FOLFIRINOX cohorts, respectively. After propensity score matching, survival rates remained similar between the two regimens in terms of overall survival (hazard ratio = 1.22; P = 0.219) and progression-free survival (hazard ratio = 1.27; P = 0.120). The objective response rate was 37.1% in the FOLFOXIRI group *vs* 47.8% in the FOLFIRINOX group (P = 0.187). Grade 3/4 toxicities occurred in 28.7% of patients in the FOLFOXIRI cohort *vs* 19.5% in the FOLFIRINOX cohort (P = 0.079). FOLFOXIRI was associated with a higher incidence of grade 3/4 digestive adverse events. Hematopoietic growth factors were used after each chemotherapy cycle and the low hematological toxicity rates were below 5% with both regimens.

CONCLUSION
FOLFOXIRI is feasible in L1 in patients with aPDAC but does not confer any therapeutic benefit as compared with FOLFIRINOX. The low hematological toxicity rates strengthened the relevance of primary prophylaxis with hematopoietic growth factors.

Key words: Advanced pancreatic cancer; First-line chemotherapy; FOLFOXIRI; FOLFIRINOX; Propensity score; Cohort study

Core tip: This is the first study to compare FOLFOXIRI and FOLFIRINOX regimens head-to-head, to assess whether FOLFOXIRI contributes to a better balance in the toxicity/efficacy ratio in advanced pancreatic ductal adenocarcinoma. These findings do not suggest any therapeutic benefit of FOLFOXIRI compared to FOLFIRINOX in first-line chemotherapy. These results show that additional evaluation is not warranted in future clinical trials. FOLFIRINOX chemotherapy remains the standard of care first-line therapy in metastatic pancreatic ductal adenocarcinoma. Interestingly, the low hematological toxicity rates in both regimens underscore the relevance of prophylactic administration of hematopoietic growth factors in routine use after each polychemotherapy cycle.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) carries a poor prognosis, with a 5-year overall survival (OS) rate of only 8%-9% for all stages taken together[1]. Pancreatic cancer is expected to become the second leading cause of cancer death in the United States and Europe by 2030[2,3]. This poor prognosis is mainly due to late diagnosis, with only 20% of patients with PDAC eligible for surgery. Complete surgical resection of localized PDAC followed by 6 mo of adjuvant chemotherapy is the only recognized

standard of care that has been shown to improve patient survival, with a median OS up to 54.4 mo with modified FOLFIRINOX [5-fluorouracil (5-FU), irinotecan, and oxaliplatin][4]. Furthermore, more than 80% of cases are diagnosed at an advanced, unresectable stage, with almost 50% of patients presenting with metastatic disease and almost 30% with locoregional extension[1]. In addition, it has been shown that most patients who undergo surgery develop further tumor recurrence[4].

Advanced or recurrent PDAC remains a challenging, non-curable disease, for which therapeutic options are still limited and mainly rely on supportive care and systemic chemotherapy to improve patient OS and health-related quality of life (HRQoL)[5]. Up to 2010, gemcitabine was the only standard of care as first-line chemotherapy (L1) in patients with metastatic PDAC[6]. Over the last decade, incremental progress has been achieved in the landscape of advanced PDAC (aPDAC) management with the approval of two active cytotoxic combinations, namely FOLFIRINOX, and gemcitabine plus *nab*-paclitaxel regimens[7,8]. FOLFIRINOX polychemotherapy became a reference regimen in this setting, based on the results of the PRODIGE 4/ACCORD 11 phase III trial[7]. This study demonstrated the superiority of FOLFIRINOX over gemcitabine monotherapy in terms of OS (median: 11.1 *vs* 6.8 mo; $P < 0.001$) and progression-free survival (PFS; median: 6.4 mo *vs* 3.3 mo; $P < 0.001$) in 342 selected patients with metastatic PDAC, age < 76 years, Eastern Cooperative Oncology Group performance status 0-1, and normal bilirubin level (< 1.5 times the upper limit of normal). The objective response rate for FOLFIRINOX was 31.6% *vs* 9.4% for gemcitabine ($P < 0.001$). FOLFIRINOX consisted of oxaliplatin (85 mg/m^2), irinotecan (180 mg/m^2), leucovorin (400 mg/m^2), and 5-FU (400 mg/m^2 administered by intravenous bolus, followed by 2400 mg/m^2 given as a 46-h continuous infusion), every 2 wk[7].

The HRQoL of patients was significantly better with FOLFIRINOX as compared with gemcitabine, except for diarrhea[9]. A higher incidence of adverse events was observed with the FOLFIRINOX group, including grade 3 or 4 neutropenia (45.7%), febrile neutropenia (5.4%) and diarrhea (12.7%)[7]. A retrospective study evaluated a "modified FOLFIRINOX" regimen without the bolus of 5-FU, and administration of hematopoietic growth factors to all patients with aPDAC[10]. This study showed a better safety profile (grade 3 or 4 neutropenia 3%), and maintained efficacy, with a response rate of 30% and a median OS of 16.4 mo (9 mo for metastatic disease). However, the incidence of severe diarrhea remained high, at 13%[10].

FOLFOXIRI, another modified schedule was developed based on the experience of the Gruppo Oncologico Nord Ovest (GONO) in metastatic colorectal cancers[11] to limit digestive and hematological toxicities. This regimen consisted of a lower dose of irinotecan (165 mg/m^2), no bolus of 5-FU, and an increase in continuous intravenous 5-FU infusion at 3200 mg/m^2, while oxaliplatin and leucovorin remained unchanged. To the best of our knowledge, to date, the FOLFOXIRI and FOLFIRINOX regimens have never been compared head-to-head. In this exploratory population-based cohort study, we aimed to compare clinical outcomes, in terms of safety and efficacy, between the FOLFOXIRI and FOLFIRINOX regimens, in patients with aPDAC in routine clinical practice.

MATERIALS AND METHODS

Patients

All consecutive patients with histologically proven aPDAC (*i.e.*, metastatic, locally advanced, or recurrent after surgery) who were treated in L1 in two French institutions were included. Patients treated with FOLFOXIRI were enrolled at Besancon University Hospital, between January 2011 and December 2015, whereas, the FOLFIRINOX group comprised patients who received this standard regimen at Lille University Hospital, between January 2011 and December 2017. Patients were prospectively identified through the chemotherapy prescribing software used at Besancon (Bonnes Pratiques de la Chimiothérapie - BPC®, SQLI) and Lille University Hospitals (CHIMIO®, Computer Engineering). Patients with early postoperative tumor relapse (*i.e.*, within 6 mo after the last administration of the adjuvant chemotherapy) were excluded. All therapeutic decisions were discussed and validated during digestive oncology-dedicated multidisciplinary meetings. Computed tomography-scan assessment was performed every 3 mo.

The database was registered and declared to the National French Commission for bioinformatics data and patient liberty (CNIL; No. of CNIL declaration: 1906173 v 0). The study followed standard procedures in France, with approval by the relevant institutional review boards. All patients with cancer signed a general informed consent at the time of their first visit to both Medical Oncology Departments. This

consent allows the use of their clinical and biological data in the cohort study. No additional specific informed consent for this study was deemed necessary. Demographics, cancer history, pathological, clinical, biological, and radiological parameters at chemotherapy initiation, as well as treatment outcomes, were retrospectively collected from medical records. The database was locked on April 23, 2019.

Treatment regimens

FOLFIRINOX was administered according to the standard schedule validated by the PRODIGE 4/ACCORD 11 study. This regimen consisted of a combination of oxaliplatin (85 mg/m^2, over 2 h), followed by leucovorin (400 mg/m^2, over 2 h), with the addition through a Y-connector, after 30 min, of irinotecan (180 mg/m^2, over 90 min), followed by 5-FU (400 mg/m^2) by intravenous bolus, on Day 1. Then, a continuous intravenous infusion of 5-FU (2400 mg/m^2) was administered over 46 h starting on Day 1[7]. FOLFOXIRI consisted of the same molecules with a reduced dose of irinotecan and no bolus 5-FU, according to the GONO regimen used in metastatic colorectal cancer: Irinotecan (165 mg/m^2, over 1 h), followed by oxaliplatin (85 mg/m^2) and leucovorin (200 mg/m^2) concomitantly over 2 h through a Yconnector, on Day 1; and followed by a continuous intravenous infusion of 5-FU (3200 mg/m^2) over 48 h starting on Day 1[11]. These two treatments were administered every 2 wk until disease progression or unacceptable toxicity. Hematopoietic growth factors were systematically used after each chemotherapy cycle in at least 80% of cases in the FOLFIRINOX group and for all patients enrolled in the FOLFOXIRI group.

Statistical analysis

Median value (interquartile range) and frequency (percentage) were provided for the description of continuous and categorical variables, respectively. Medians and proportions were compared between the FOLFIRINOX and FOLFOXIRI groups using Wilcoxon–Mann–Whitney and chi-square tests (or Fisher's exact test, if appropriate), respectively. OS was calculated from the date of the first administration of L1 to the date of death from any cause. Survival data were censored at the last follow-up. PFS was calculated from the date of the first administration of L1 to the date of progression or death from any cause, or the date of the last follow-up, at which point data were censored. OS and PFS were estimated using the Kaplan-Meier method and described using median or rate at specific time points with 95% confidence intervals (CIs), and compared using the log-rank test. Follow-up time was calculated using a reverse Kaplan-Meier estimation when feasible[12]. Objective tumor response was determined according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 criteria[13]. Toxicity was evaluated according to the National Cancer Institute Common Terminology Criteria[14].

The primary analysis was conducted using data from the total population, and compared characteristics and outcomes between the FOLFIRINOX and FOLFOXIRI groups. A propensity score approach was then applied to deal with potential heterogeneity in baseline characteristics between the two administered regimens in L1. Two methods were used to address the potential confounding effect of the unbalanced factors: First, the inverse probability of treatment weighting (IPTW) and second, propensity score matching[15]. Propensity score construction was based on probability estimation with a nonparsimonious multivariable logistic regression model including the main parameters distributed unequally between the FOLFIRINOX and FOLFOXIRI groups. Hazard ratios (HRs) and 95%CIs were estimated using the IPTW Cox proportional hazards model. Accuracy of the model was verified by testing discrimination and calibration. Discrimination of the IPTW Cox model was assessed by the area under the curve, and calibration by the Hosmer–Lemeshow goodness-of-fit test. Propensity score matching, based on the caliper method with a ratio of 1:1, was performed to generate two samples with well-balanced characteristics. Sensitivity analysis to determine the reliability and the robustness of the primary analysis was performed in the subgroup of patients with metastatic disease. All analyses were performed using SAS software version 9.4 (SAS Institute, Cary NC, USA). $P < 0.05$ were considered statistically significant, and all tests were two-sided.

RESULTS

Patient characteristics

A total of 289 patients with aPDAC treated in L1 were included in this study. Of these patients, 124 received the FOLFIRINOX regimen and 165 received FOLFOXIRI

chemotherapy. Patient characteristics of the two treatment groups are described and compared in Table 1. The two cohorts displayed similar characteristics, except for primary tumor site (located in the pancreatic head in 43.1% in the FOLFIRINOX group *vs* 56.7% in the FOLFOXIRI group; $P = 0.022$), histological grade, stage at chemotherapy initiation (88.7% had metastatic stage in the FOLFIRINOX group *vs* 63.6% in the FOLFOXIRI group; $P < 0.001$), and pain (corresponding to the prescription of morphine). Of note, patients in the FOLFIRINOX group had a significantly higher albumin level (39.1 g/L *vs* 35.0 g/L; $P < 0.001$), and an increased number of metastatic sites ($P < 0.001$) than those in the FOLFOXIRI group (Table 1).

Outcomes of the overall population

After a median follow-up of 30.8 mo (95%CI: 23.0-NA) and 61.4 mo (95%CI: 43.2-87.9), median OS was 11.6 mo (95%CI: 10.8-15.5) and 11.1 mo (95%CI: 9.8-13.1) in the FOLFIRINOX and FOLFOXIRI groups, respectively (HR = 1.12; 95%CI: 0.86-1.46; $P = 0.391$; Figure 1A). Median PFS was 5.8 mo (95%CI: 3.9-6.9) in the FOLFOXIRI group and 6.7 mo (95%CI: 6.0-7.8) in the FOLFIRINOX group (HR = 1.14; 95%CI: 0.89 to 1.46; $P = 0.298$; Figure 1B). OS rates at 12, 18, and 24 mo were 49.6%, 36.4%, and 28.3%, respectively, in the FOLFIRINOX group as compared with 45.1%, 30.9%, and 21.2%, respectively, in the FOLFOXIRI group.

Detailed outcomes data are summarized in Table 2. The objective response rate was 47.8% in the FOLFIRINOX group, compared to 37.1% in the FOLFOXIRI group ($P = 0.187$), while disease-control rates were 75.7% and 66.7%, respectively ($P = 0.124$). The number of cycles was significantly higher in the FOLFIRINOX group (11.0 *vs* 7.0 cycles; $P = 0.027$). Maintenance chemotherapy was administered in 45.2% of patients in the FOLFIRINOX group and in 37.6% of those in the FOLFOXIRI group ($P = 0.194$). The median maintenance time was 2.8 mo (95%CI: 1.0-4.4) in the FOLFIRINOX group *vs* 2.7 mo (95%CI: 1.9-5.8) in the FOLFOXIRI group ($P = 0.421$). Second-line chemotherapy was administered to 91 (73.4%) patients in the FOLFIRINOX group and 117 (70.9%) patients in the FOLFOXIRI group ($P = 0.643$). No treatment-related deaths were observed. Grade 3 or 4 toxicities occurred in 19.5% of patients in the FOLFIRINOX group as compared with 28.7% in the FOLFOXIRI group, but this difference did not reach statistical significance ($P = 0.079$). In the FOLFOXIRI group, grade 3 or 4 hematological adverse events of any type were observed in 3.1% of patients. FOLFOXIRI was associated with a higher incidence of grade 3 or 4 digestive adverse events as compared to the FOLFIRINOX group (12.8% *vs* 4.2%, respectively).

Propensity score approach

Potential biases identified during the description of the total cohort were minimized by the use of a propensity score estimated by an unconditional multivariable logistic regression model. Histological grade and albumin level were not selected in the propensity score process due to the high rate of missing data (Supplementary Table 1). Thus, primary tumor site, stage at diagnosis, stage at chemotherapy initiation, number of metastatic sites, lymph node and liver metastases, and pain were included in the propensity score (Supplementary Table 2). The model exhibited excellent discrimination with an area under the curve of 0.73 (Supplementary Figure 1) and a good calibration ($P = 0.840$, Hosmer–Lemeshow goodnessoffit test). For each patient, a propensity score value was then calculated based on the multivariable model (Supplementary Figure 2). In the IPTW analysis, the L1 regimen was not significantly associated with either OS (282 patients, 236 events; HR = 1.19; 95%CI: 0.91-1.54; $P= 0.202$) or PFS (281 patients, 256 events; HR = 1.25; 95%CI: 0.98-1.60; $P= 0.077$). Patients treated with the FOLFOXIRI regimen were then matched considering their nearest neighbor, with a caliper of 0.10 and a ratio of 1:1, with patients in the FOLFIRINOX group.

Patient characteristics and outcomes in the propensity scorematched population

After propensity score matching, 96 patients in each group (79.0% and 59.3% in the FOLFIRINOX and FOLFOXIRI groups, respectively) were successfully matched. There were no statistically significant differences between the two matched groups in baseline characteristics: Primary tumor site ($P = 0.385$), stage at chemotherapy initiation ($P = 0.439$), number of metastatic sites ($P = 0.724$), lymph node metastases ($P = 0.817$), liver metastases ($P = 0.385$), and pain ($P = 0.877$). Patients in the FOLFIRINOX group were characterized by tumors with a more differentiated histological grade ($P= 0.011$) and a higher albumin level ($P = 0.001$) (Table 3). After a median follow-up of 43.2 mo (95%CI: 31.0-61.4) in the matched groups, survival rates for patients remained similar between the two regimens in terms of OS (HR= 1.22; 95%CI: 0.89-1.67; $P = 0.219$; Figure 1C) and PFS (HR = 1.27; 95%CI: 0.94-1.71; $P = 0.120$; Figure 1D). There was no statistically significant difference in objective response ($P = 0.079$), maintenance chemotherapy ($P = 0.553$), or second-line administration rates (P

Table 1 Patient characteristics of the overall population according to first-line chemotherapy, *n* (%)

Characteristics	FOLFIRINOX (*n* = 124)	FOLFOXIRI (*n* = 165)	*P* value
Demographic parameters			
Age, median [IQR], yr	60.2 [53.0-65.9]	62.5 [54.6-67.5]	0.174
Missing	1	0	
Gender			0.989
Male	73 (58.9)	97 (58.8)	
Female	51 (41.1)	68 (41.2)	
Familial history of cancer			0.195
No	32 (45.1)	89 (54.3)	
Yes	39 (54.9)	75 (45.7)	
Missing	53	1	
Personal history of cancer			0.219
No	110 (90.2)	139 (85.3)	
Yes	12 (9.8)	24 (14.7)	
Missing	2	2	
Pathological parameters			
Stage at diagnosis			< 0.001
Localized	20 (16.1)	13 (7.9)	
Locally advanced	18 (14.5)	60 (36.3)	
Metastatic	86 (69.4)	92 (55.8)	
Primary tumor site			0.022
Head	53 (43.1)	93 (56.7)	
Body and/or tail	70 (56.9)	71 (43.3)	
Missing	1	1	
Histological grade			0.014
Well or moderately differentiated	45 (83.3)	39 (62.9)	
Poorly differentiated or undifferentiated	9 (16.7)	23 (37.1)	
Missing	70	103	
Tumor extension			
Stage at chemotherapy initiation			< 0.001
Locally advanced	14 (11.3)	60 (36.4)	
Metastatic	110 (88.7)	105 (63.6)	
Number of metastatic sites			< 0.001
0	14 (11.3)	60 (36.6)	
1	69 (55.7)	74 (44.9)	
≥ 2	41 (33.0)	31 (18.8)	
Lymph node metastases			< 0.001
No	95 (76.6)	153 (92.7)	
Yes	29 (23.4)	12 (7.3)	
Liver metastases			< 0.001
No	37 (29.8)	84 (50.9)	
Yes	87 (70.2)	81 (49.1)	
Peritoneal metastases			0.124
No	109 (87.9)	134 (81.2)	
Yes	15 (12.1)	31 (18.8)	
Lung metastases			0.133
No	102 (82.3)	146 (88.5)	
Yes	22 (17.7)	19 (11.5)	
Other metastases			0.060
No	116 (93.6)	162 (98.2)	
Yes	8 (6.4)	3 (1.8)	
Clinical parameters			
Performance status (WHO)			0.185

0	42 (35.0)	54 (32.7)	
1	66 (55.0)	103 (62.4)	
≥ 2	12 (10.0)	8 (4.9)	
Missing	4	0	
Body mass index, kg/m^2	23.9 [20.8-27.4]	23.0 [20.8-25.6]	0.114
Missing	9	0	
Pain			< 0.001
No	90 (73.8)	83 (50.9)	
Yes	32 (26.2)	80 (49.1)	
Missing	2	2	
Jaundice			0.266
No	114 (93.4)	148 (89.7)	
Yes	8 (6.6)	17 (10.3)	
Missing	2	0	
Ascites			1.000
No	117 (95.9)	157 (96.3)	
Yes	5 (4.1)	6 (3.7)	
Missing	2	2	
Biological parameters			
Albumin, median [IQR], g/L	39.1 [37.0-43.0]	35.0 [29.0-39.0]	< 0.001
Missing	48	80	
Lymphocytes, median [IQR], mm^3			0.408
< 1000	10 (15.9)	12 (11.4)	
≥ 1000	53 (84.1)	93 (88.6)	
Missing	19	102	
Neutrophil-to-lymphocyte ratio, median [IQR]			0.103
< 5	82 (78.1)	42 (66.7)	
≥ 5	23 (21.9)	21 (33.3)	
Missing	19	102	
CA19-9, median [IQR], UI/mL	885.0 [79.0-4756.0]	650.0 [138.0-5300.0]	0.669
Missing	3	34	
Previous treatment			
Primary tumor resection			0.277
Yes	18 (14.5)	17 (10.3)	
No	106 (85.5)	148 (89.7)	
Adjuvant chemotherapy			0.865
Yes	12 (9.7)	15 (9.1)	
No	112 (90.3)	150 (90.9)	
Median follow-up time (95%CI), mo	30.8 [23.0-NA]	61.4 [43.2-87.9]	

χ^2 tests or Fisher's exact tests were used to compare proportions, and Wilcoxon tests were used to compare continuous variables between the FOLFIRINOX and FOLFOXIRI groups. All statistical tests were two-sided. CA19-9: Carbohydrate Antigen 19-9; IQR: Interquartile range; WHO: World Health Organization.

= 0.636). Grade 3 or 4 toxicities remained unchanged between the two treatments (20% in the FOLFIRINOX group *vs* 29.2% in the FOLFOXIRI group, P = 0.148). The incidence of grade 3 or 4 digestive adverse events remained higher in the FOLFOXIRI group (9.4% *vs* 5.6%) (Table 2).

Sensitivity analyses in the subgroup of patients with metastatic disease

Sensitivity analysis was performed exclusively including the metastatic population from the two treatment groups, corresponding to 110 patients in the FOLFIRINOX group and 105 patients in the FOLFOXIRI group. Patient characteristics are detailed in Supplementary Table 3. As observed for the overall population, characteristics were similar, except for histological grade, lymph node metastases, pain, and albumin level (39 g/L in the FOLFIRINOX group *vs* 34.6 g/L in the FOLFOXIRI group, P < 0.001). Patients in the FOLFIRINOX group displayed significantly more peritoneal metastases (86.4% *vs* 70.5%, P = 0.005) and a lower neutrophil-to-lymphocyte ratio (P = 0.010), compared to those in the FOLFOXIRI group (Supplementary Table 3).

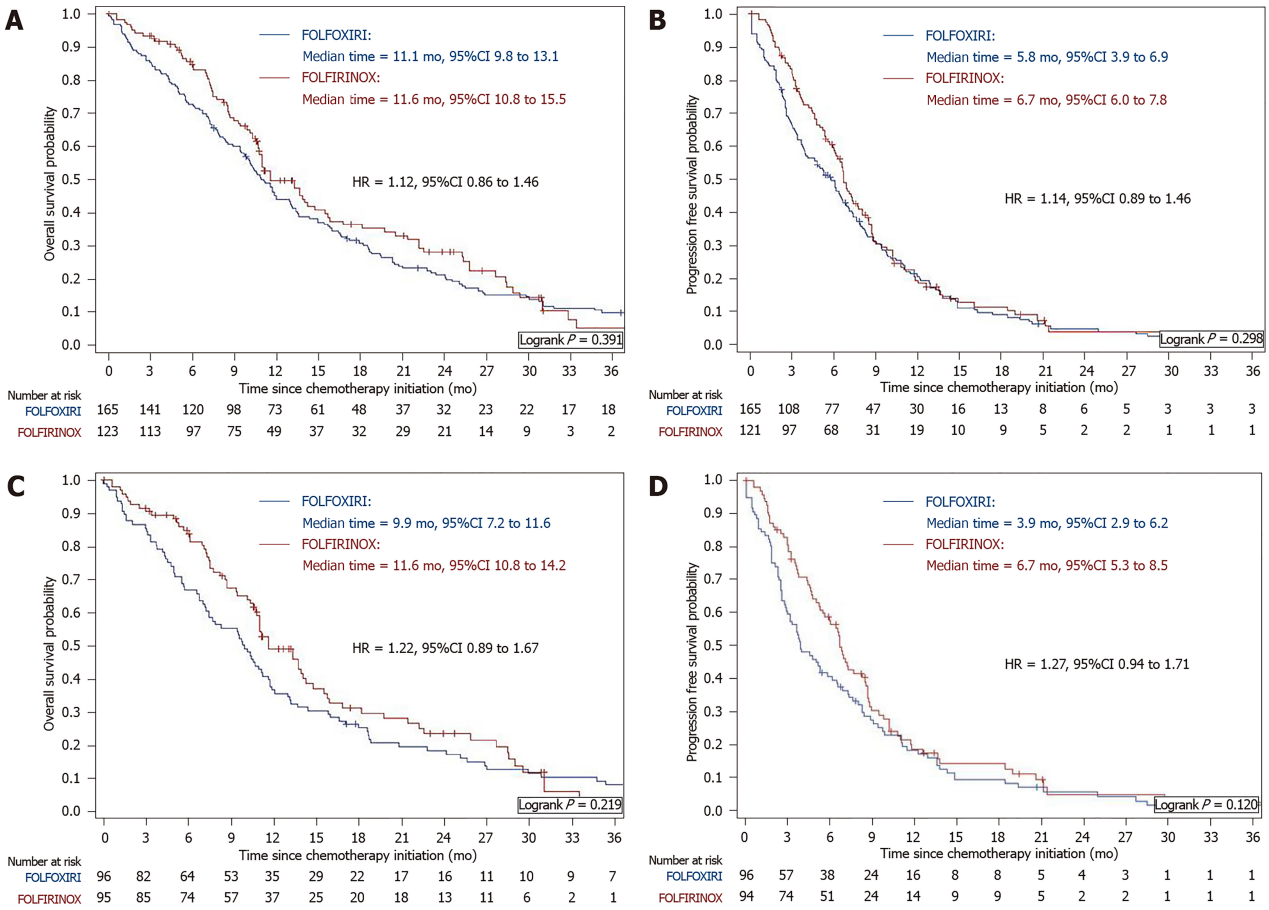

Figure 1 Kaplan-Meier curves of overall survival and progression-free survival for the FOLFIRINOX and FOLFOXIRI groups in the overall population and the propensity scorematched population. A: Overall survival in the whole population; B: Progression-free survival in the whole population; C: Overall survival in the propensity score-matched population; D: Progression-free survival in the propensity score-matched population. $P < 0.05$ from the log-rank test was considered statistically significant, and all tests were two-sided. CI: Confidence interval; HR: Hazard ratio.

The median duration of follow-up was 26.7 mo (95%CI: 23.0-31.1) in the FOLFIRINOX group compared to 44.2 mo (95%CI: 36.7-71.5) in the FOLFOXIRI group. Median OS was significantly longer in the FOLFIRINOX group (13.3 mo; 95%CI: 10.7-15.5) compared to the FOLFOXIRI group (8.5 mo; 95%CI: 6.7-10.2) (HR= 1.44; 95%CI 1.07-1.94; $P = 0.017$; Supplementary Figure 3A). Similarly, patients treated with the FOLFIRINOX regimen had more favorable PFS (6.7 mo; 95%CI: 5.7-7.8; *vs* 3.9 mo; 95%CI: 2.9-6.1, respectively), but the difference was not statistically significant (HR= 1.30; 95%CI: 0.97-1.72; $P = 0.073$; Supplementary Figure 3B). Both cohorts exhibited other similarities in outcomes, which are summarized in Supplementary Table 4.

Furthermore, propensity score analysis was performed in metastatic patients (Supplementary Tables 5 and 6, Supplementary Figures 4 and 5). In the IPTW analysis, the L1 regimen was not significantly associated with either OS (122 patients, 100 events; HR = 1.08; 95%CI: 0.73-1.60; $P = 0.703$) or PFS (122 patients, 113 events; HR = 1.06; 95%CI: 0.73-1.55; $P = 0.746$). After propensity score matching (Supplementary Table 7), survival rates for patients were similar between the two regimens in terms of OS (HR = 0.94; 95%CI: 0.54-1.61; $P = 0.810$; Supplementary Figure 6A) and PFS (HR = 0.94; 95%CI: 0.55-1.61; $P = 0.827$; Supplementary Figure 6B). Moreover, no difference in objective response ($P = 0.317$), maintenance chemotherapy ($P = 1.000$), or second-line administration rates ($P = 1.000$) was observed. Treatment-related grade 3 or 4 adverse events, including digestive and hematological adverse events, were similar between the two propensity score-matched treatment groups ($P = 0.362$) (Supplementary Table 8).

DISCUSSION

This is the first study to show, in a head-to-head comparison, that FOLFOXIRI is feasible as L1 in patients with aPDAC but does not confer any therapeutic benefit as

Table 2 Outcomes in the overall population and propensity score-matched population according to first-line chemotherapy, *n* (%)

Outcomes	Overall population			Propensity score-matched population		
	FOLFIRINOX (*n* = 124)	FOLFOXIRI (*n* = 165)	*P* value	FOLFIRINOX (*n* = 96)	FOLFOXIRI (*n* = 96)	*P* value
Number of cycles, median [IQR]	11.0 [6.0-13.0]	7.0 [4.0-13.0]	0.027	11.0 [6.0-14.0]	7.0 [4.0-14.5]	0.164
Missing	1	0		1	0	
RECIST best response			0.187			0.079
Complete or partial response	53 (47.8)	49 (37.1)		41 (47.7)	25 (32.5)	
Stability	31 (27.9)	39 (29.6)		24 (27.9)	22 (28.5)	
Progression	27 (24.3)	44 (33.3)		21 (24.4)	30 (39.0)	
Missing	13	33		108	19	
Toxicity of grade 3 or 4			0.079			0.148
No	95 (80.5)	117 (71.3)		72 (80.0)	68 (70.8)	
Yes	23 (19.5)	47 (28.7)		18 (20.0)	28 (29.2)	
Digestive	5 (4.2)	21 (12.8)		5 (5.6)	9 (9.4)	
Hematology	1 (0.9)	5 (3.1)		1 (1.0)	4 (4.2)	
Neurology	9 (7.6)	14 (8.5)		6 (6.7)	9 (9.4)	
Other	8 (6.8)	7 (4.3)		6 (6.7)	6 (6.2)	
Missing	6	1		6	0	
Reason for discontinuation			0.291			0.441
Progression	83 (68.0)	108 (65.5)		63 (67.0)	67 (69.8)	
Toxicity	12 (9.9)	10 (6.0)		12 (12.8)	7 (7.3)	
Other	27 (22.1)	47 (28.5)		19 (20.2)	22 (22.9)	
Missing	2	0		2	0	
Maintenance			0.194			0.553
Yes	56 (45.2)	62 (37.6)		39 (40.6)	35 (36.5)	
No	68 (54.8)	103 (64.4)		57 (59.4)	61 (63.5)	
Second-line chemotherapy administration			0.643			0.636
Yes	91 (73.4)	117 (70.9)		69 (71.9)	66 (68.8)	
No	33 (26.6)	48 (29.1)		27 (28.1)	30 (31.2)	

χ^2 tests or Fisher's exact tests were used to compare proportions, and Wilcoxon tests were used to compare continuous variables between the FOLFIRINOX and FOLFOXIRI groups. All statistical tests were two-sided. IQR: Interquartile range.

compared with FOLFIRINOX. PDAC is a highly aggressive cancer, and chemotherapy remains the cornerstone of advanced disease therapy. Preclinical studies showed synergistic activity between oxaliplatin, irinotecan and 5-FU[16-18], and phase II/III trials confirmed the antitumor activity of the FOLFIRINOX combination in metastatic pancreatic cancers[7,19]. Although FOLFIRINOX is a first-line option for patients with metastatic PDAC, the significant adverse event rate limits its administration in full doses. However, its substantial benefit in terms of survival rates and HRQoL encourages the assessment of a modified schedule that will likely yield a much-needed improvement in the balance of toxicity *vs* efficacy in this setting.

FOLFOXIRI, a triplet-chemotherapy regimen with a lower dose of irinotecan and no bolus of 5-FU, has already demonstrated its efficacy and good tolerance and is validated in metastatic colorectal cancer[11,20]. Vivaldi *et al*[21] evaluated FOLFOXIRI in pancreatic cancer in an observational cohort study of 137 patients, of whom 59.1% had metastatic disease. They reported that FOLFOXIRI improved patient survival rates with a median OS of 12 mo for the overall population and 10.8 mo for the metastatic patients. The objective response rate was 38.6% in the whole population and 35.8% in patients with metastatic PDAC. Moreover, the schedule showed a good tolerance profile with the occurrence of grade 3 diarrhea in only 8%, and febrile neutropenia (grade 3 or 4 neutropenia 35.7%) in < 1%. These results are in line with those observed in our study. Nevertheless, FOLFOXIRI was never compared head-to-head to the standard FOLFIRINOX in this setting.

In our study, the two regimens were compared for the first time in routine clinical practice, taking into account a large number of variables. Due to the design of this

Table 3 Patient characteristics in the propensity score-matched population according to first-line chemotherapy, *n* (%)

Characteristics	FOLFIRINOX (*n* = 96)	FOLFOXIRI (*n* = 96)	*P* value
Demographic parameters			
Age, median [IQR], years	59.9 [53.0-66.9]	63.1 [55.2-67.1]	0.221
Missing	1	0	
Gender			0.661
Male	54 (56.3)	57 (59.4)	
Female	42 (43.7)	39 (40.6)	
Familial history of cancer			0.806
No	31 (51.7)	51 (53.7)	
Yes	29 (48.3)	44 (46.3)	
Missing	36	1	
Personal history of cancer			0.824
No	83 (88.3)	82 (87.2)	
Yes	11 (11.7)	12 (12.8)	
Missing	2	2	
Pathologic parameters			
Stage at diagnosis			0.598
Localized	14 (14.6)	12 (12.5)	
Locally advanced	13 (13.5)	18 (18.87	
Metastatic	69 (71.9)	66 (68.8)	
Primary tumor site			0.385
Head	41 (42.7)	47 (49.0)	
Body and/or tail	55 (57.3)	49 (51.0)	
Histological grade			0.011
Well or moderately differentiated	36 (83.7)	24 (58.5)	
Poorly differentiated or undifferentiated	7 (16.3)	17 (41.5)	
Missing			
Tumor extension			
Stage at chemotherapy initiation			0.439
Locally advanced	14 (14.6)	18 (18.2)	
Metastatic	82 (85.4)	78 (81.3)	
Number of metastatic sites			0.724
0	14 (14.6)	18 (18.8)	
1	57 (59.4)	53 (55.2)	
≥ 2	25 (26.0)	25 (26.0)	
Lymph node metastases			0.817
No	86 (89.6)	85 (88.5)	
Yes	10 (10.4)	11 (11.5)	
Liver metastases			0.274
No	26 (27.1)	33 (34.4)	
Yes	70 (72.9)	63 (65.6)	
Peritoneal metastases			0.059
No	84 (87.5)	74 (77.1)	
Yes	12 (12.5)	22 (22.9)	
Lung metastases			0.845
No	81 (84.4)	80 (83.3)	
Yes	15 (15.6)	16 (16.7)	
Other metastases			0.279
No	90 (93.8)	94 (97.9)	
Yes	6 (6.2)	2 (2.1)	
Clinical parameters			
Performance status (WHO)			0.165
0	36 (39.1)	35 (36.5)	

1	49 (53.3)	59 (61.5)	
≥ 2	7 (7.6)	2 (2.0)	
Missing	4	0	
Body mass index, kg/m^2	23.9 [20.7-27.1]	23.0 [21.2-25.0]	0.285
Missing	5	0	
Pain			0.877
No	65 (67.7)	66 (68.8)	
Yes	31 (32.3)	30 (31.2)	
Jaundice			0.637
No	87 (91.6)	86 (89.6)	
Yes	8 (8.4)	10 (10.4)	
Missing	1	0	
Ascites			1.000
No	92 (96.8)	92 (95.8)	
Yes	3 (3.2)	4 (4.2)	
Missing	1	0	
Biological parameters			
Albumin, median [IQR], g/L	39.6 [37.0-43.0]	34.6 [29.0-40.0]	0.001
Missing	40	51	
Lymphocytes, median [IQR], mm^3			0.157
< 1000	9 (11.2)	8 (21.0)	
≥ 1000	71 (88.8)	30 (79.0)	
Missing	16	58	
Neutrophil-to-lymphocyte ratio, median [IQR]			0.072
< 5	63 (78.8)	24 (63.2)	
≥ 5	17 (21.2)	14 (36.8)	
Missing	16	58	
CA19-9, median [IQR], UI/mL	857.5 [69.0-6210.0]	726.5 [152.0-5507.0]	0.500
Missing	2	20	
Previous treatment			
Primary tumor resection			0.284
Yes	10 (10.4)	15 (15.6)	
No	86 (89.6)	81 (84.4)	
Adjuvant chemotherapy			0.091
Yes	6 (6.2)	13 (13.5)	
No	90 (93.8)	83 (86.5)	

χ^2 tests or Fisher's exact tests were used to compare proportions, and Wilcoxon tests were used to compare continuous variables between the FOLFIRINOX and FOLFOXIRI groups. All statistical tests were two-sided. CA19-9: Carbohydrate Antigen 19-9; IQR: Interquartile range; WHO: World Health Organization.

exploratory study, patients were included in an observational cohort and the treatment regimens were not randomized. In addition to its retrospective nature, further limitations of the present study warrant discussion. Patients were treated in two centers, although both were high-volume units with similar clinical practices. Of note, patients with a different tumor extension were included, with locally advanced and metastatic stages. Thus, sensitivity analyses were performed exclusively in the metastatic population. Computed tomography-scan assessment of tumor response according to RECIST criteria was not performed centrally. This bias could explain a trend towards better tumor response in the FOLFIRINOX group. Additional variables, particularly febrile neutropenia, biological or HRQoL data, could not be evaluated in our study due to the retrospective design of the data collection, with a high rate of missing patient information.

To overcome these limitations, FOLFOXIRI and FOLFIRINOX were compared from a large population-based cohort of prospectively included patients with aPDAC. Most importantly, we used a rigorous methodological framework and applied a propensity score approach to take into account the potential heterogeneity in baseline characteristics between the two populations. Moreover, two different methods, namely the IPTW Cox model and propensity score matching, demonstrated the

satisfactory performance and validity of the analysis. The reproducibility obtained with the sensitivity analysis in the metastatic cohort strengthened the observed results.

The present study showed that the FOLFOXIRI and FOLFIRINOX L1 regimens were similar in terms of efficacy. Median OS and PFS were comparable between the two schedules, and similar to survival rates reported by Conroy *et al*[7] in FOLFIRINOX-treated patients. The objective response rate with the FOLFOXIRI regimen observed in our cohort (37.1%) was similar to that reported in the GONO study in FOLFOXIRI-treated patients (38.6%)[21]. No differences in response rates between the FOLFOXIRI and FOLFIRINOX regimens in our unselected population were detected. Nevertheless, they were higher than those reported in the randomized phase III trial (31.6%)[7]. The methodological approach used in our study showed that FOLFOXIRI does not provide an improved efficacy compared to FOLFIRINOX.

The FOLFOXIRI regimen was associated with an increased risk of the occurrence of grade 3 or 4 digestive toxicities (12.8%), including diarrhea, nausea/vomiting, and stomatitis. Of note, this incidence was similar to the gastrointestinal safety profile reported by Vivaldi *et al*[21] with FOLFOXIRI chemotherapy. Interestingly, hematological toxicities were very low in both regimens in our study, compared to FOLFIRINOX in the PRODIGE 4/ACCORD 11 study[7]. A primary prophylactic administration of hematopoietic growth factors contributed to the reduction of grade 3 or 4 neutropenia[22,23], and thus, routine use after each polychemotherapy cycle has been adopted in some institutions.

Many combinations with "modified FOLFIRINOX" chemotherapy have been evaluated, and the schedules used to deliver this polychemotherapy are heterogeneous[24,25]. Dose reductions of single or multiple agents differ among studies compared to the standard schedule[24]. An optimal relative dose intensity for FOLFIRINOX was determined by Lee *et al*[23] to balance toxicity and efficacy, suggesting that a decrease of 30% in chemotherapy dosages preserve tumor response. In addition, two meta-analyses suggested that dosage attenuation improves tolerance while preserving survival benefits (overall response rates: 32% with "modified FOLFIRINOX" *vs* 33% with full doses; $P = 0.879$)[24,25]. A modified-dose regimen decreased the frequency of hematological and digestive adverse events and cycles reported, while making it possible to maintain dose-dense chemotherapy and treatment activity[26]. In the adjuvant setting, a modified FOLFIRINOX with no bolus of 5-FU and irinotecan at a dose of 150 mg/m^2 significantly increased survival compared to gemcitabine for PDAC[4]. These dose adjustments were also effective in the neoadjuvant setting for patients with locally advanced or borderline PDAC. Of note, resection was performed in more than half of the patients, and with R0 resection in 86.4% of cases[27].

In previous retrospective and single-arm phase II studies, the bolus of 5-FU was more frequently discontinued in the "modified FOLFIRINOX" combination[10,28-31]. Infusion of 5-FU is preferred for the treatment of colorectal cancer over bolus 5-FU. In this setting, the omission of the bolus of 5-FU has been shown to improve the safety profile, while significantly decreasing hematological toxicity[32]. A dose reduction of irinotecan (130-135 mg/m^2, 150 mg/m^2, or 165 mg/m^2) was evaluated in previous studies[28-31,33-35]. The addition of irinotecan in FOLFOXIRI chemotherapy increased digestive toxicity occurrence, notably nausea/vomiting and diarrhea, compared to the doublet-chemotherapy (FOLFOX) in metastatic colorectal cancer[36]. In aPDAC, a 25% reduction of irinotecan compared to full dose has been associated with a decrease in diarrhea (3.1% *vs* 12.5%, respectively) and vomiting (0% *vs* 23.5%, respectively)[35].

Dihydropyrimidine dehydrogenase and uridine diphosphate glucuronosyltransferase (UGT) 1A1 are two key enzymes involved in the catabolic pathways of 5-FU and irinotecan, respectively. Their deficiency related to genetic polymorphisms, leads to increased exposure to the cytotoxic agents with a higher risk of adverse events. Indeed, variants of UGT1A1 have been reported to increase the risk of grade 3 or 4 hematological toxicity and diarrhea[37,38]. A study that evaluated the FOLFIRINOX regimen in pancreatic cancer, reported a significantly higher incidence of diarrhea among patients with UGT1A1 heterozygous type (UGT1A1 −/*6 and UGT1A1 −/*28) compared to those with UGT1A1 wild-type (−/−). However, for patients who received the "modified FOLFIRINOX", there was no observed difference in the frequency of adverse events due to UGT1A1 status[39]. Furthermore, studies have indicated an association between polymorphisms of the dihydropyrimidine dehydrogenase gene encoding (DPYD) and 5-FU-induced toxicity[40]. Currently, DPYD genotype or phenotype-based dose reduction improves the safety of patients receiving fluoropyrimidine treatment and is recommended[41,42]. Preemptive screening of DPYD and UGT1A1 variants could identify patients at risk of clinically relevant adverse events, to improve FOLFIRINOX administration[43]. In an era of personalized medicine, a "genotype-guided" approach could help to individualize the dose to

optimize efficacy, limit toxicity and guarantee HRQoL.

In conclusion, FOLFOXIRI is feasible in L1 in patients with aPDAC, but does not appear to confer any therapeutic benefit as compared with the FOLFIRINOX regimen. FOLFOXIRI was associated with a higher incidence of grade 3 or 4 digestive adverse events compared to FOLFIRINOX. A major difference in hematological toxicities was observed between our cohort and the PRODIGE 4/ACCORD 11 trial[7], underlining the relevance of prophylactic administration of hematopoietic growth factors in routine clinical practice. These results show that additional evaluation is not warranted in future clinical trials. FOLFIRINOX chemotherapy remains the standard of care in L1 in metastatic PDAC.

ARTICLE HIGHLIGHTS

Research background

The FOLFIRINOX regimen is the first-line reference chemotherapy (L1) in advanced pancreatic ductal adenocarcinoma (PDAC). FOLFOXIRI might contribute to a better balance in the toxicity/efficacy ratio in this setting.

Research motivation

FOLFOXIRI has demonstrated efficacy and feasibility in colorectal cancer.

Research objectives

To investigate the potential clinical value of FOLFOXIRI in patients with advanced PDAC (aPDAC) in routine clinical practice.

Research methods

This exploratory study compared clinical outcomes between the two treatments in the overall population and after propensity score matching.

Research results

All consecutive aPDAC patients treated in L1 with FOLFOXIRI (n = 165) or FOLFIRINOX (n = 124) regimens were included. Median overall survival was 11.1 mo in the FOLFOXIRI cohort and 11.6 mo in the FOLFIRINOX cohort. After propensity score matching, survival rates remained similar between the regimens in terms of overall survival and progression-free survival. FOLFOXIRI was associated with a higher incidence of grade 3/4 digestive adverse events. The low hematological toxicity rates in both regimens underline the relevance of primary prophylaxis with hematopoietic growth factors.

Research conclusions

FOLFOXIRI is feasible in L1 in patients with aPDAC but does not confer any therapeutic benefit as compared with FOLFIRINOX.

Research perspectives

These results suggest that further evaluation of FOLFOXIRI in future clinical trials is not warranted. FOLFIRINOX chemotherapy remains the standard of care in L1 in metastatic PDAC.

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ORIGINAL ARTICLE

Observational Study

Clinical outcomes of patients with duodenal adenocarcinoma and intestinal-type papilla of Vater adenocarcinoma

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Abstract

BACKGROUND

Duodenal adenocarcinoma (DA) and intestinal-type papilla of Vater adenocarcinoma (it-PVA) are rare malignancies of the gastrointestinal tract. Current therapeutic options are translated nowadays from treatment strategies for patients with colorectal cancer due to histopathological similarities.

AIM

To retrospectively investigate the clinical outcome of patients with DA and it-PVA.

METHODS

All patients with DA and it-PVA diagnosed between 2000 and 2017 were

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included at two academic centers in the Netherlands. All patients with histopathologically-confirmed DA or it-PVA were eligible for inclusion. Clinical outcome was compared between DA and it-PVA per disease stage. In the subgroup of stage IV disease, survival after local treatment of oligometastases was compared with systemic therapy or supportive care.

RESULTS

In total, 155 patients with DA and it-PVA were included. Patients with it-PVA more often presented with stage I disease, while DA was more often diagnosed at stage IV ($P < 0.001$). Of all patients, 79% were treated with curative intent. The median survival was 39 mo, and no difference in survival was found for patients with DA and it-PVA after stratification for disease stage. Seven (23%) of 31 patients with synchronous stage IV disease underwent resection of the primary tumor, combined with local treatment of oligometastases. Local treatment of metastases was associated with an overall survival of 37 mo, compared to 14 and 6 mo for systemic therapy and supportive care, respectively.

CONCLUSION

Survival of patients with DA and it-PVA is comparable per disease stage. These results suggest a potential benefit for local treatment strategies in selected patients with oligometastases, although additional prospective studies are needed.

Key words: Duodenal adenocarcinoma; Papilla of Vater adenocarcinoma; Clinical outcomes; Local treatment; Metastases; Survival

Core tip: This study demonstrates the clinical outcome for duodenal adenocarcinoma and intestinal-type papilla of Vater adenocarcinoma, which are rare tumor types of the gastrointestinal tract. The overall survival is comparable per disease stage, resulting in a median survival of 39 mo. Most patients (79%) are treated with curative intent by surgical resection of the tumor. For patients with metastatic disease, local treatment of metastases was associated with a better overall survival compared to systemic treatment or supportive care. Future prospective studies are needed to confirm this survival benefit.

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INTRODUCTION

Duodenal adenocarcinoma (DA) is a rare malignancy of the gastrointestinal tract with an incidence of approximately 0.5 per 100000 persons, and it constitutes less than 5% of all gastrointestinal tumors[1-3]. Despite its rarity, the incidence of DA has increased over the last years[3,4]. Papilla of Vater adenocarcinoma (PVA) also develops as a primary tumor in the duodenal wall[5]. The papilla of Vater has an ambiguous position in the duodenum, since tumors originating from the papilla can be classified as either an intestinal- or pancreaticobiliary-type based on their histological differentiation[6]. While the outcome of pancreaticobiliary-type PVA resembles pancreatic or bile duct cancer, the clinical outcome of patients with intestinal-type PVA (it-PVA) is comparable to DA[7,8]. Interestingly, it-PVA carcinomas and DA show considerable overlap in molecular features and clinical behavior, underlining the rationale for similar treatment strategies[9,10]. However, comparisons between clinical characteristics and outcome of these tumor types are sparse.

Surgical resection of the primary tumor is the preferred treatment option for patients with localized tumors[11,12]. However, no practical guidelines exist for patients with metastatic disease stages. Recent studies have identified histopathological and

molecular biological similarities between small bowel adenocarcinomas, including DA, it-PVA and colorectal cancer (CRC)[2,13]. Therefore, treatment protocols for patients with DA and it-PVA are increasingly based on combined multimodality therapy regimens that are already established and validated for patients with CRC[14,15].

Optimal curative therapy for patients with metastatic CRC comprises local treatment of oligometastases, alone or combined with (induction) chemotherapy[16-18]. The benefit of these approaches has not been investigated for patients with DA and it-PVA. Based on the comparability with CRC, our clinical practice was changed towards local treatment for all consecutive patients with DA and it-PVA with oligometastases of the liver and lymph nodes. These patients were considered for resection of the primary tumor, combined with local treatment of oligometastases, according to the guidelines for patients with CRC. This study aims to investigate the clinical outcomes of patients with DA and it-PVA, and evaluate the effect of the introduction of this new way of treatment in these selected patients with metastatic disease by comparing this regimen to systemic treatment and supportive care.

MATERIALS AND METHODS

Study population and inclusion criteria

This retrospective case series analysis included all consecutive patients with DA or PVA treated at one of the locations of the Cancer Center Amsterdam, Amsterdam UMC (VUMC and AMC, Amsterdam) from 2000 to 2017. To assess all eligible patients, a systematic search was performed in the automated pathology database (PALGA), in which all histopathologically-confirmed diagnoses by either biopsy or resection are documented. All patients ≥ 18 years with histopathologically-confirmed intestinal-type adenocarcinoma of the duodenum or intestinal-type PVA were eligible for inclusion. Histopathological confirmation was based on the written pathology report, and no additional staining was performed if histological differentiation was unspecified, omitting these patients for further analysis[19,20]. Patients with tumors other than intestinal-type adenocarcinoma or metastases from other primary tumors were excluded, as well as patients diagnosed with a malignancy within 5 years prior to or after diagnosis. The medical ethics committees of both medical centers approved of this retrospective multicenter study (#2017.215 and #W17_399), in accordance with the Declaration of Helsinki.

Local treatment of oligometastases

At location VUMC, the clinical treatment of patients with oligometastases of DA or it-PVA changed into applying the local treatment of metastases. Consecutive patients presenting at location VUMC with synchronous liver or lymph node metastases were discussed at a multidisciplinary meeting, with experience in the management of hepatobiliary disease, for consideration of the resection of the primary tumor combined with local treatment of metastases, *i.e.*, resection or ablation. Patients were considered if they had sufficient performance status, and in case local therapy was technically feasible, according to the guidelines for local treatment of CRC liver metastases[21]. Briefly, the following criteria were applied to select candidate patients for the local treatment of oligometastases: (1) Patients with synchronous oligometastases of the liver, with the following criteria: (a) The aim of liver resection was to achieve a complete resection with negative resection margins, and leave sufficient liver function; (b) Patients were considered for ablative therapy if deemed unfit for surgery, or if unfavorable location of the metastases for surgery was present, or insufficient liver remnants was expected after resection[22]; and (2) Patients with synchronous lymph node metastases for which resection of the metastases was feasible during resection of the primary tumor. When repeat metastases occurred after the local treatment of liver metastases, local treatment was reconsidered in one patient.

Consecutive patients presenting at the AMC location were also discussed at a dedicated multidisciplinary meeting, and received standard of care for metastatic disease, *i.e.*, systemic therapy or supportive care. Patients treated with local therapy of metastases were compared to the standard of care for metastatic disease applied at the AMC location, and a historic cohort concerning all patients treated before the introduction of this novel strategy at the VUMC location. To optimally compare the effect of local metastases treatment, a subgroup analysis was performed on patients with synchronous metastases confined to the liver. Patients with synchronous liver metastases who received systemic treatment or supportive care were retrospectively reviewed by a surgeon, who had been blinded, for the outcome of previous feasibility

of local therapy application based on location, number, and size of metastases.

Collection of data

All relevant data were retrospectively collected, including age, sex, ASA-score, site of primary tumor, disease stage (AJCC staging system, 8th edition)[23], tumor size, histopathological subtype, location and number of metastases, and treatment, including type of surgical resection, local treatment of metastases and systemic therapy, including specified treatment regimens. The involvement of lymph nodes was either classified as regional (N1/2) or distant (M1), depending on the location of lymph node involvement according to the AJCC staging system. Follow-up of patients included clinical assessment and diagnostic imaging every 6 mo, or based on clinical symptoms. Overall survival was calculated from the date of diagnosis until the date of death, or date of last follow-up. Surgery-related deaths, defined as patient death within 30 d after resection of the primary tumor or a palliative bypass procedure, were excluded for survival analysis.

Statistical analysis

Descriptive statistics were reported for demographics and clinicopathological characteristics. Continuous variables are presented as median [interquartile range (IQR)], and comparisons between groups were analyzed using the Student's *t* test or one-way ANOVA, as appropriate. Categorical variables are presented as frequencies (percentages), and were analyzed using the χ^2-test. Overall survival was calculated using the Kaplan-Meier method, and statistical significance for survival was assessed using the log-rank test. $P < 0.05$ was considered statistically significant. Statistical analyses were computed using SPSS® version 25 (IBM, New York, United States).

RESULTS

Study population

A total of 155 consecutive patients were identified; 99 consecutive patients with DA and 56 patients with it-PVA. Patient characteristics are summarized in Table 1. Baseline characteristics were comparable between DA and it-PVA. Patients with it-PVA presented more often with stage I disease compared to patients with DA (32% *vs* 7%, respectively), while DA was more often diagnosed at stage IV disease (29% *vs* 9% for it-PVA, $P < 0.001$). Treatment with curative intent was performed in 122 (79%) of the included patients. Among these, 90% of the patients with it-PVA were treated with surgery alone. Curative resection combined with either (neo) adjuvant therapy (28%) or local treatment of metastasis (11%) was more common in patients with DA compared to it-PVA ($P = 0.003$). In patients with metastases, the use of palliative chemotherapy did not significantly differ between patients with DA (90%) and it-PVA (100%, $P = 0.848$).

Of all patients, 86 (55%) were followed until death, and the median follow-up period was 55 mo (IQR 22-82 mo) for patients still alive at the end of follow-up. Median survival for the entire cohort was 39 mo, and the 1-year, 3-year and 5-year overall survival (OS) rates were 72%, 38% and 23%, respectively. There was no significant difference in OS between patients with DA and it-PVA after adjusting for disease stage (Figure 1). The median OS for stage I-II was 113 mo and 133 mo ($P = 0.841$), OS for stage III was 50 mo and 49 mo ($P = 0.927$) and OS for stage IV was 14 mo and 13 mo ($P = 0.676$) for DA and it-PVA, respectively.

Survival of patients with metastases

To investigate the outcomes of patients with metastatic disease, 34 patients with synchronous metastatic DA and it-PVA at initial presentation were eligible for analyses. Three patients were excluded due to surgery-related deaths, which included two patients after resection of the primary tumor and one patient after a palliative bypass procedure. Subsequently, 31 patients were divided retrospectively into three treatment modality groups: local treatment of oligometastases ($n = 7$), systemic treatment ($n = 20$), or supportive care ($n = 4$, Table 2). All patients selected for local treatment presented with synchronous oligometastases, and they all underwent resection of the primary tumor, while none of the patients received induction chemotherapy prior to resection or adjuvant therapy. In addition, these patients underwent synchronous metastasectomy ($n = 5$), ablation of liver metastases ($n = 1$), or a combination of metastasectomy and ablation ($n = 1$). One patient in this group underwent an additional resection of metachronous liver metastases, and three patients received palliative chemotherapy for recurrence of metastases. In all stage IV patients, capecitabine combined with oxaliplatin (CAPOX) was most commonly administered as systemic therapy ($n = 13$). Other administrated regimens are reported

Table 1 General characteristics of all patients with duodenal adenocarcinoma and intestinal-type papilla of Vater adenocarcinoma included in this study

	Total, *n* = 155	Duodenum, *n* = 99	Papilla, *n* = 56	*P* value
Age				0.237
Age, median [IQR]	64 [58-73]	63 [56-72]	66 [58-74]	
Sex (%)				0.742
Male	94 (60.6)	61 (61.6)	33 (58.9)	
ASA (%)				0.852
ASA I	19 (12.3)	12 (12.1)	7 (12.5)	
ASA II	83 (53.5)	49 (49.5)	34 (60.7)	
ASA III	31 (20.0)	19 (19.2)	12 (21.4)	
ASA IV	1 (0.6)	1 (1.0)	0 (0.0)	
NR	21 (13.5)	18 (18.2)	3 (5.4)	
TNM stage (%)				< 0.001
Stage I	25 (16.1)	7 (7.1)	18 (32.1)	
Stage II	26 (16.8)	20 (20.2)	6 (10.7)	
Stage III	65 (41.9)	39 (39.4)	26 (46.4)	
Stage IV	34 (21.9)	29 (29.3)	5 (8.9)	
NR	5 (3.2)	4 (4.0)	1 (1.8)	
Treatment (%)				
Curative intent	122 (78.7)	71 (71.7)	51 (91.1)	0.003
Surgery	89 (73.0)	43 (60.6)	46 (90.2)	
Neoadjuvant + surgery	3 (2.5)	3 (4.2)	0 (0.0)	
Surgery + adjuvant therapy	21 (17.2)	17 (23.9)	4 (7.8)	
Primary resection + treatment metastasis	9 (7.4)	8 (11.3)	1 (2.0)	
Palliative treatment	23 (14.8)	20 (20.2)	3 (5.4)	0.848
Chemotherapy	21 (91.3)	18 (90.0)	3 (100.0)	
Radiotherapy	1 (4.3)	1 (5.0)	0 (0.0)	
Chemotherapy + radiotherapy	1 (4.3)	1 (5.0)	0 (0.0)	
Best-supportive care	8 (5.2)	7 (7.1)	1 (1.8)	
NR	2 (1.3)	1 (1.0)	1 (1.8)	

Duodenum: Duodenal adenocarcinoma; Papilla: Intestinal-type papilla of Vater Adenocarcinoma; ASA: American Society of Anesthesiologists physical status classification system (ASA-score); TNM: disease stage according to the American Joint Committee on Cancer staging system (AJCC 7th edition); IQR: Interquartile range; NR: Not reported.

in Table 2. One patient also received radiotherapy in addition to systemic therapy. The supportive care group was defined as patients who did not receive any type of treatment for the primary tumor or metastases. A palliative bypass procedure was performed in 11 patients of the systemic treatment group, and in three patients of the supportive care group. The median OS was 37 mo in the patient group receiving local treatment of metastases, *vs* 14 mo for the patient group receiving systemic treatment and 6 mo for patients receiving supportive care, after a median follow-up of 23 mo (IQR 15–34 mo) for patients who were still alive. The local treatment of metastases was associated with a better survival, with five of seven patients still living after 2 years (Figure 2A). Subgroup analysis of all patients, with metastases confined to the liver and retrospectively deemed eligible for local treatment, confirmed these results, including four patients who remained alive after almost 2 years following the local treatment of metastases (Figure 2B).

DISCUSSION

This study demonstrates a comparable outcome for patients with DA and it-PVA per disease stage, with a median survival of 39 mo. A potential benefit was found for the local treatment of oligometastases in selected patients with liver or lymph node metastases in DA. The survival of patients who received local treatment of metastases was longer than in patients receiving systemic therapy or supportive care. This was also true after subgroup analysis of patients with synchronous metastases confined to

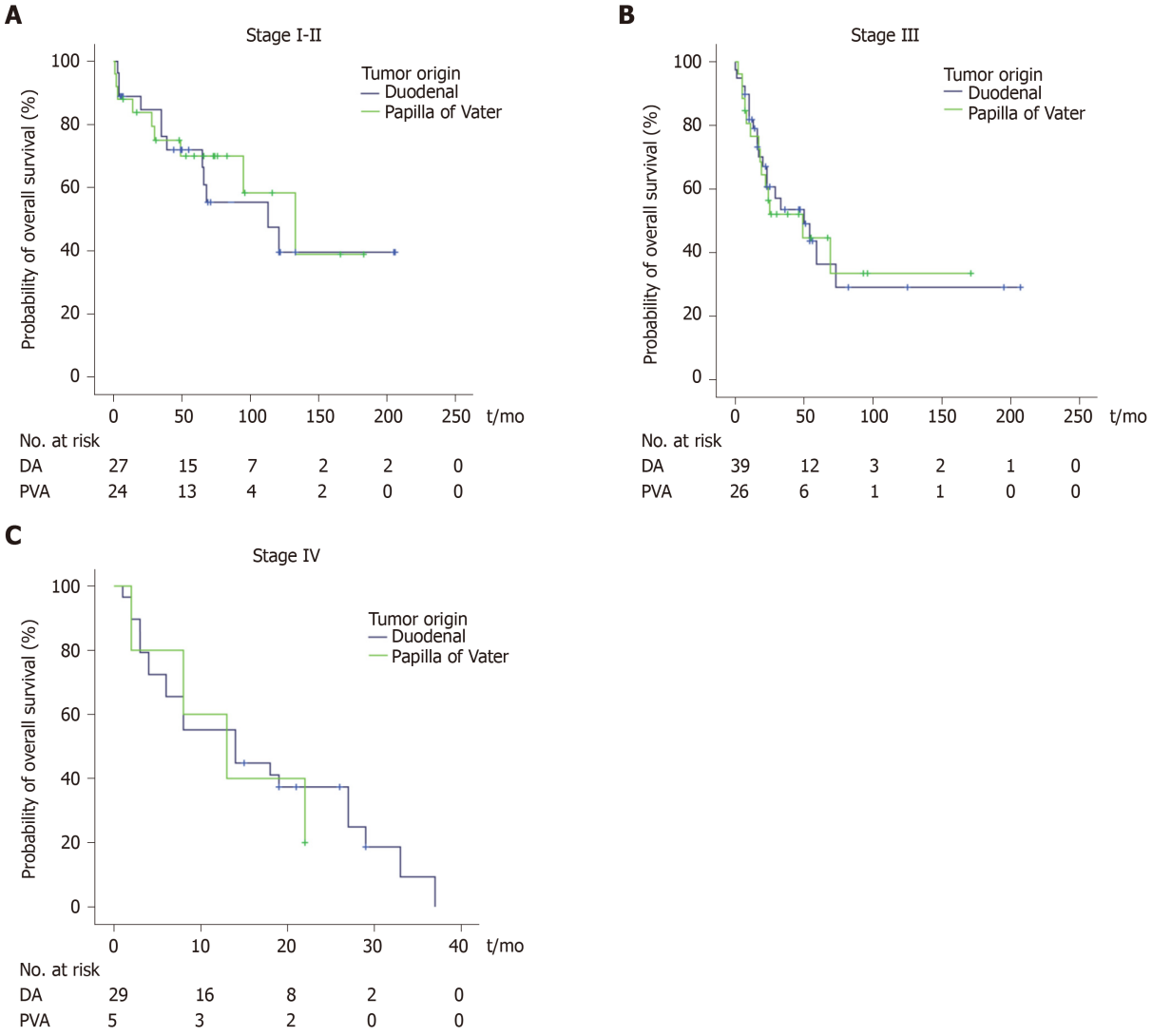

Figure 1 Survival per disease stage is similar for duodenal adenocarcinoma and intestinal-type papilla of Vater adenocarcinoma. Survival specified for duodenal adenocarcinoma and intestinal-type papilla of Vater adenocarcinoma. A: Stage I-II (P = 0.841); B: Stage III (P = 0.927); C: Stage IV (P = 0.676). Kaplan-Meier curves are shown. No. at risk: Number at risk.

the liver. Eventually, only patients with DA received local treatment of metastases in this study, hampering translation of these results to patients with it-PVA. However, since molecular biology and survival per disease stage have been shown to be comparable for patients with DA and it-PVA, further investigation of local treatment strategies in patients with it-PVA is justifiable.

The current study provides the largest reported series comparing OS for DA and it-PVA. Both tumor types demonstrated comparable survival rates after stratification for disease stage, consistent with previous studies[7,8,24]. Of note, patients with it-PVA were more often diagnosed at early disease stages, which is likely due to earlier clinical presentation with tumor-related symptoms, such as jaundice[25]. Clinical presentation of patients with DA might be less specific, as 25%-43% of patients may be asymptomatic at diagnosis[12,26,27]. Patients with DA were more frequently diagnosed with metastatic disease. This might not represent the true incidence of stage IV it-PVA, since histopathological differentiation has often been inconclusive. Moreover, these tumors might easily be mistaken for other tumors of the periampullary area, especially when no surgical resection specimens are available[28]. This could have resulted in an underestimation of the incidence of stage IV it-PVA. The resemblance of DA and it-PVA based on biological similarity and clinical behavior underlines the importance of accurate histopathological classification and corresponding treatment approaches[29,30].

A recent study investigating the outcomes of patients with small bowel adenocarcinomas, including DA, reported similar results of enhanced survival after the local treatment of metastases[26]. However, the current study expands this knowledge, with a unique focus on DA and it-PVA. The small bowel adenocar-

Table 2 General characteristics of all patients with stage IV duodenal adenocarcinoma and intestinal-type papilla of Vater adenocarcinoma, *n* (%)

	Total, *n* = 31	Local treatment of metastases, *n* = 7	Systemic treatment, *n* = 20	Supportive care, *n* = 4
Age				
Age, median [IQR]	63 [58-71]	69 [59-73]	62 [52-73]	63 [58-67]
Sex				
Male	17	5	11	1
Origin				
Duodenum	27	7	17	3
Papilla (intestinal-type)	4	0	3	1
ASA				
ASA I	3	1	2	0
ASA II	13	4	8	1
ASA III	4	1	1	2
NR	11	1	9	1
Metastatic site				
Liver	13	5	7	1
Lymphatic	4	2	2	0
Lung	2	0	2	0
Peritoneal	9	0	6	3
Lung + liver or lymphatic	3	0	3	0
Number of liver oligometastases[1] (%)				
Number, median [IQR]	2 [1-7]	2 [1-3]	4 [1-15]	3
Local treatment of liver metastases				
Metastasectomy	5 (14.7)	5	N/A	N/A
Ablation	1 (2.9)	1	N/A	N/A
Metastasectomy + ablation	1 (2.9)	1	N/A	N/A
Systemic therapy				
5-FU-LV	1 (2.9)	0	1	0
Capecitabine	5 (14.7)	0	5	0
CAPOX	13 (41.9)	2	11	0
EOX	1 (2.9)	0	1	0
FOLFOX	1 (2.9)	1	0	0
FOLFOX + radiotherapy	1 (2.9)	0	1	0
No chemotherapy	6 (19.4)	3	0	3
Unspecified chemotherapy	1 (2.9)	0	1	0
NR	2 (6.5)	1	0	1

[1]Only patients with oligometastases of the liver. 5-FU-LV: 5-fluorouracil and leucovorin; CAPOX: Capecitabine and oxaliplatin; EOX: Epirubicine, oxaliplatin and capecitabine; FOLFOX: 5-fluorouracil and oxaliplatin; IQR: Interquartile range; NR: Not reported; N/A: Not applicable.

cinomas also include tumors originating from the jejunum and ileum. Controversy exists regarding the association between primary tumor location within the small bowel and prognosis. Previously, a higher incidence and worse outcome for DA compared to jejunal tumors has been reported, although this was not confirmed by others[4,31-34]. The duodenum might be more susceptible to carcinogenesis, due to its proximity near the ampulla, and pancreaticobiliary excretions[34]. In the current study, only patients with DA and it-PVA were included to minimize the possible bias of tumor location. Several clinically-relevant factors have not been sufficiently considered in previous studies, such as the number of metastases, specifications of local treatment regimens, and the type of chemotherapy administered to patients, making the results presented in this study more transparent[26].

Molecular characterization and histopathology demonstrated the resemblance of DA to CRC[9,13]. Therefore, resection of oligometastases in patients with DA and it-PVA is an attractive therapeutic option to explore. Interestingly, the benefit of local treatment of liver metastases in CRC is solely based on historical cohort studies, which demonstrated enhanced survival benefit. Furthermore, local treatment of

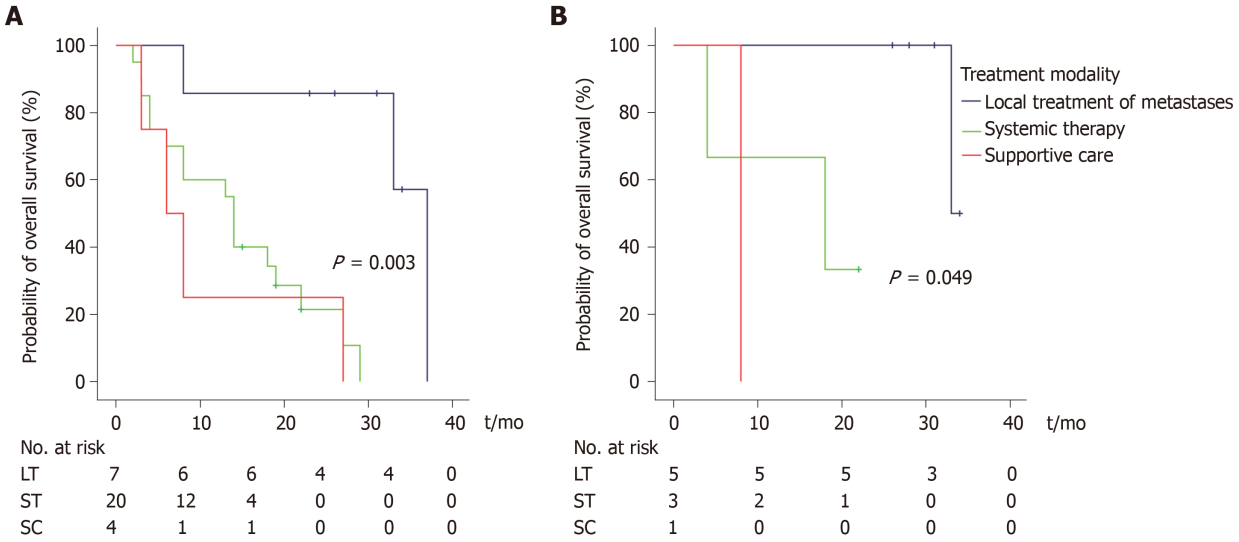

Figure 2 Overall survival for patients with stage IV duodenal adenocarcinoma and intestinal-type papilla of Vater adenocarcinoma compared based on treatment modality. A: Overall survival of patients treated with local therapy of metastases compared with systemic therapy or supportive care (all stage IV patients were included); B: Subgroup analysis of patients with liver metastases eligible for local treatment only. Kaplan-Meier curves are shown per treatment group. LT: Local treatment of metastases; ST: Systemic therapy; SC: Supportive care; No. at risk: Number at risk.

oligometastases confined to the liver is currently the first choice of treatment in CRC[35-38]. Although many therapeutic options remain to be fully explored in carefully selected patient groups, the results of this first study demonstrate the feasibility of applying local treatment of oligometastases in selected patients with DA. Nevertheless, patient-related factors, such as the physical condition of the patient, tumor size, tumor location, and extent of metastases, could still withhold aggressive treatment interventions.

The limitations of this study include those intrinsic to the retrospective design and the small sample size, which restricted statistical analysis, although this is one of the largest series to report outcomes for DA and it-PVA[11]. Ultimately, multicenter, prospective studies that include larger numbers of patients could provide more insight into the outcome specified per treatment modality (*e.g.*, resection, ablation, and chemotherapeutic regimen). The small number of patients impeded adequate stratification for clinicopathological and prognostic factors, such as tumor markers, tumor grade, and distribution of metastases[26,31,39]. Thus, the presented results are based on selected patients who may have a favorable prognostic biology, and these results hold an overall bias.

Despite the small study size and limited evidence, we advocate further studies to investigate the true merits of more aggressive and intensified treatment modalities in selected patients with oligometastases from DA and it-PVA[40]. In the future, the selection of patients could also be based on novel insights into tumor biology, the biological behavior of the tumor, and response to induction chemotherapy. However, based on evidence from this study, the local treatment of oligometastases deserves consideration by a dedicated multidisciplinary team in an attempt to optimally utilize available treatment possibilities, and may help to further enhance survival in patients with DA.

ARTICLE HIGHLIGHTS

Research background

Duodenal adenocarcinoma (DA) and intestinal-type papilla of Vater adenocarcinoma (it-PVA) are rare malignancies of the gastrointestinal tract. No practical guidelines exist for patients with metastatic disease stages. Current treatment protocols are increasingly based on treatment strategies for patients with colorectal cancer.

Research motivation

The clinical outcomes of patients with DA and it-PVA are unclear. In addition, the benefit of local treatment of oligometastases, alone or combined with chemotherapy, has not been investigated for these patients.

Research objectives

This study aims to investigate the clinical outcomes of patients with DA and it-PVA, specified

per disease stage. The outcome after treatment of oligometastases in selected patients with DA and it-PVA is evaluated.

Research methods
All patients with DA and it-PVA diagnosed between 2000 and 2017 were included. All patients with histopathologically-confirmed DA or it-PVA were eligible for inclusion. Clinical outcome was compared between DA and it-PVA per disease stage. In the subgroup of stage IV disease, survival after the local treatment of oligometastases was compared with systemic therapy or supportive care.

Research results
No difference in survival was found for patients with DA and it-PVA stratified for disease stage. Seven (23%) of 31 patients with synchronous stage IV disease underwent resection of the primary tumor, combined with local treatment of oligometastases. Local treatment of metastases was associated with an overall survival of 37 mo, compared to 14 and 6 mo for systemic therapy and supportive care, respectively.

Research conclusions
Survival of patients with DA and it-PVA is comparable per disease stage. A potential benefit of local treatment strategies in selected patients with oligometastases was found.

Research perspectives
Multicenter, prospective studies, including larger numbers of patients, are needed to provide more insight into the outcome specified per treatment modality, and the true merits of more aggressive and intensified treatment modalities in selected patients with oligometastases.

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CASE REPORT

Utility of positron emission tomography-computed tomography scan in detecting residual hepatocellular carcinoma post treatment: Series of case reports

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Abstract

BACKGROUND

Multi-phase computed tomography (CT) or magnetic resonance imaging (MRI) has been the standard of care for hepatocellular carcinoma (HCC) diagnosis for years.

CASE SUMMARY

We report a case series of four patients in whom positron emission tomography-computed tomography (PET-CT) scan complemented the conventional CT/MRI scans in evaluating treatment response. In these four cases the conventional multi-phase CT and MRI failed to identify residual HCC disease post-treatment, while PET-CT complemented and aided in treatment response evaluation. In each case, the addition of PET-CT identified and located residual HCC disease, allowed retreatment, and altered medical management.

CONCLUSION

This case series suggests that PET-CT should perhaps play a role in the HCC management algorithm, in addition to the conventional contrast-enhanced multi-phase scans.

Key words: Hepatocellular carcinoma; Positron emission tomography; Contrast-enhanced multiphase scan; Cirrhosis; Residual cancer; Treatment response evaluation; Case series

Core tip: This is a case series of four hepatocellular carcinoma patients who had undergone locoregional therapies. The conventional multi-phase computed tomography

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and magnetic resonance imaging scans failed to identify residual hepatocellular carcinoma disease post-treatment, while positron emission tomography-computed tomography scan complemented in treatment response evaluation by identifying and locating residual disease, allowing retreatment.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a well-known complication of chronic liver disease and cirrhosis. It has remained as one of the leading causes of death worldwide[1], responsible for nearly 746000 deaths in 2012[1]. It is the second most common cause of death from cancer globally[1,2]. The incidence of HCC in the United States has been rising in the past four decades[3-5]. Multi-phase computed tomography (CT) or magnetic resonance imaging (MRI) has been the standard of care for HCC diagnosis for years[6]. HCC lesions are known to display arterial enhancement and delayed washout on multi-phase CT or MRI[7]. These contrast-enhanced multi-phase cross-sectional imaging modalities have also been utilized for follow-up on known cases of HCC, especially in determining the response to treatment[8]. Positron emission tomography (PET) scan has been considered unreliable as an imaging modality for HCC diagnosis and for treatment response follow-up due to its lack of sensitivity[9,10]. Many HCC tumors do not show up on PET scan[11]. This case series intends to describe cases in which PET scan complemented the conventional multi-phase CT or MRI in evaluating treatment response.

CASE PRESENTATION

Chief complaints

(1) Case 1: A 62-year-old male with known hepatitis C cirrhosis self-referred to our liver center for further management; (2) Case 2: A 69-year-old male with cryptogenic cirrhosis was referred to our liver center with a 3.3 cm liver lesion in segment 6/7 that appeared to be hypodense without enhancement on a multi-phase CT scan; (3) Case 3: A 62-year-old male with compensated cirrhosis secondary to chronic hepatitis C was referred to our center with HCC tumors based on outside MRI; and (4) Case 4: A 75-year-old female with chronic hepatitis C and compensated cirrhosis was referred to our center due to two HCC tumors, 8.4 cm and 1.2 cm based on multi-phase MRI.

History of present illness

(1) Case 1: The patient was discovered to have HCC upon routine surveillance multi-phase CT, with original tumor burden of 4.2 cm in segment 3 cm and 2.6 cm in segment 5/6. He then received multiple trans-arterial chemo-embolization (TACE) treatments to both lobes of the liver; (2) Case 2: Our multi-disciplinary liver tumor board subsequently reviewed the outside CT scan and confirmed the findings of a non-enhancing hypodense liver lesion. Alpha fetoprotein (AFP) was less than 10 ng/mL. The tumor board recommended a biopsy, which revealed poorly differentiated HCC, based on histological characteristics and immunohistochemical staining. The patient underwent TACE; (3) Case 3: The patient was subsequently treated for HCC tumors (2.1 cm in segment 8, and 1.8 cm and 1.2 cm in segment 6) with two TACE treatments and one microwave ablation (MWA). He was also listed for liver transplant, and a PET/CT scan was done to rule out lung metastasis. He had had tuberculosis, successfully treated many years ago; a chest CT had shown a cavitary lesion within some infiltrate in the right upper lung; and (4) Case 4: The patient's AFP remained normal in the single digit (ng/mL) at baseline. She subsequently underwent TACE and proton treatments as recommended by our tumor board. The patient underwent multi-phase MRI for monitoring treatment response every three to four months subsequently, and was deemed in complete response for more than two years after the second treatment with proton. She also underwent

hepatitis C treatment successfully and achieved sustained virologic response with negative viral titer more than two years from the end of treatment.

History of past illness

(1) Case 1: Negative for diabetes or cardiac disease; (2) Case 2: Diabetes mellitus type 2, atrial fibrillation, skin cancer, esophageal varices; (3) Case 3: Tuberculosis; and (4) Case 4: Diabetes mellitus type 2, and atrial fibrillation.

Personal and family history

(1) Non-contributory (Case 1, 3, 4); and (2) He was exposed to agent orange in the early 1970s (in his 20s) (Case 2).

Physical examination

Anicteric; abdomen soft, non-distended, and non-tender to palpation; liver not palpable; no asterixis (Case 1, 2, 3, 4).

Laboratory examinations

(1) Case 1: AFP rose to 7344 ng/mL approximately 16 mo after presentation; it raised concerns of extrahepatic metastasis, though recent bone scan and chest CT were both negative; (2) Case 2: The patient's AFP remained low throughout his course, 9.4 ng/mL at the time of the PET/CT scan; his total bilirubin was mildly elevated 2.3 mg/dL while his albumin remained normal, 3.8 g/dL; international normalized ratio (INR) was 1.2; (3) Case 3: The patient's AFP remained normal throughout his course, 3.8 ng/mL at the time of the PET/CT scan; his albumin remained normal, 3.8 g/dL, while his total bilirubin was slightly elevated 1.5 mg/dL; INR remained normal 1.1; and (4) Case 4: AFP started to increase about 31 mo after presentation, to 24.7 ng/mL, and later to 75 ng/mL in month 36. Total bilirubin had remained normal 0.3 mg/dL, and so had albumin 3.7 g/dL.

Imaging examinations

Case 1: A PET-CT scan was done to search for metastasis, and it revealed three foci of increased fludeoxyglucose (FDG) activity within the treated area of segment 3 (Figure 1A), while showing no FDG activity in the treated area of segment 5/6; no metastasis was identified. A repeat multi-phase MRI was done concurrently, and it failed to reveal any arterial enhancement in the liver (Figure 1B); (2) Case 2: The one-month post-TACE multi-phase CT scan was again inconclusive, showing a 4.2 cm hypodense lesion (Figure 2A) similar to the pre-TACE CT scan. A PET-CT scan was performed, revealing an FDG avid uptake of 3.5 cm × 3.2 cm in measurement at the same area of the liver (Figure 2B) previously biopsied and treated, consistent with residual HCC; (3) Case 3: The PET/CT scan 16 wk post MWA incidentally showed a small site of localized metabolic activity corresponding to a low-density lesion adjacent to a larger right hepatic lobe mass which demonstrated absent metabolic activity, consistent with residual HCC in the treated segment-6 lesion (Figure 3A); no FDG activity in the lungs or elsewhere. A multi-phase MRI a few weeks prior had revealed focal bleed at the periphery of the treatment zone post MWA without evidence of any viable tumor (Figure 3B); and (4) Case: A multi-phase MRI in month 36 was negative for any arterial enhancement, but concurrently a PET-CT scan in month 36 revealed positive FDG uptake at the periphery of the treated lesion in segment 2/3 (Figure 4).

FINAL DIAGNOSIS

Recurrent HCC post locoregional therapies (Case 1, 2, 3, 4).

TREATMENT

Case 1

The patient then underwent more TACE treatments. Both multi-phase MRI scans and PET-CT scans were utilized to monitor treatment response. PET-CT scans subsequently showed residual disease in the left lobe. Treatment modality was changed to proton after the fourth TACE to the segment-3 HCC, approximately 29 mo after presentation.

Case 2

The patient underwent and completed a course of proton treatment consisted of 15 fractions.

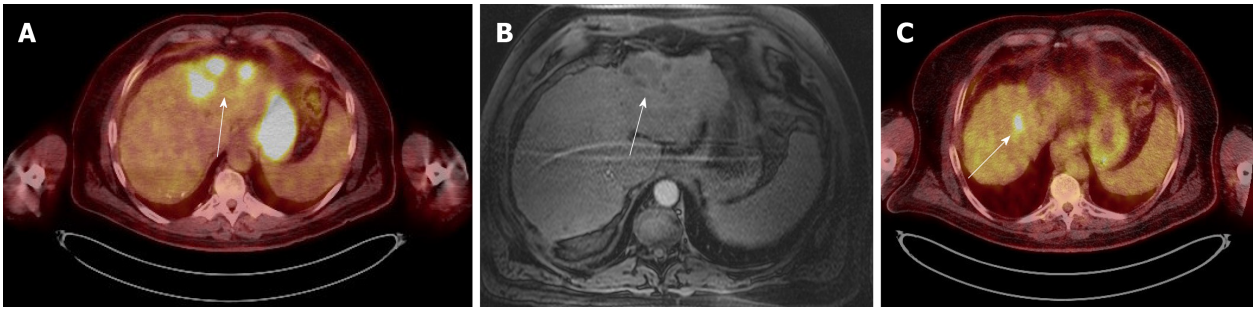

Figure 1 Case 1. A: Positron emission tomography scan: the white arrow shows the area of multiple foci of fludeoxyglucose uptake in the treated area; B: Multi-phase magnetic resonance imaging scan: it shows no arterial enhancement in the same area during the arterial phase; C: Positron emission tomography scan: the arrow indicates an area of fludeoxyglucose uptake, indicating another residual tumor.

Case 3
The patient underwent third TACE approximately five months after the MWA.

Case 4
The patient opted out of recommended laparoscopic ablation, citing her advanced age and the invasiveness of the proposed procedure. She later elected to start nivolumab infusion.

OUTCOME AND FOLLOW-UP

Case 1
The patient's AFP responded from 3841 ng/mL before the proton treatment to 7 ng/mL after proton. He remained in complete response based on both multi-phase MRI and PET-CT scans every three months until approximately 41 mo after presentation when a new focus of FDG uptake was seen in the dome; a concurrent multi-phase MRI again failed to reveal any arterial enhancement. The dome lesion was treated with proton. Both PET-CT and multi-phase MRI three months post-proton showed the dome lesion well treated, but there was a recurrent HCC focus with arterial enhancement and washout, as well as FDG uptake (Figure 1C), at the previously treated area in segment 6. The patient received proton treatment to segment 6 approximately 48 mo after presentation. The PET-CT scans aided in detecting HCC for this patient and allowed appropriate treatments to prolong his survival. He was followed at our center for a total of 52 mo.

Case 2
The patient was followed up at our center for a total 9 mo. After the proton therapy, he decided to follow up with another institution closer to his residence.

Case 3
Both multi-phase MRI and PET-CT scans one-month post-TACE showed no residual HCC in the liver. The patient was followed up at our center for a total of 37 mo.

Case 4
The patient has tolerated nivolumab infusion well for 14 mo, currently on 2 mg/kg every 2 wk. She has been followed at our center for a total of 58 mo.

DISCUSSION

We have described a series of four cases in which the conventional multi-phase CT and MRI failed to identify residual HCC disease post-treatment, while the FDG PET-CT scan aided in evaluating treatment response (Table 1). In all these cases, FDG PET-CT scans detected residual HCC tumors in treatment zone status post locoregional therapy while the contrast-enhanced multiphase scans could not, and these allowed for timely treatment and meaningful survival.

Cirrhosis occasionally could alter the vasculature and distort the manifestation of arterial enhancement and delayed washout in HCC tumors *via* multi-phase CT or MRI, thereby decreasing the sensitivity and specificity of these contrast-enhanced imaging modalities[6,12], not to mention when these HCC tumors have been treated

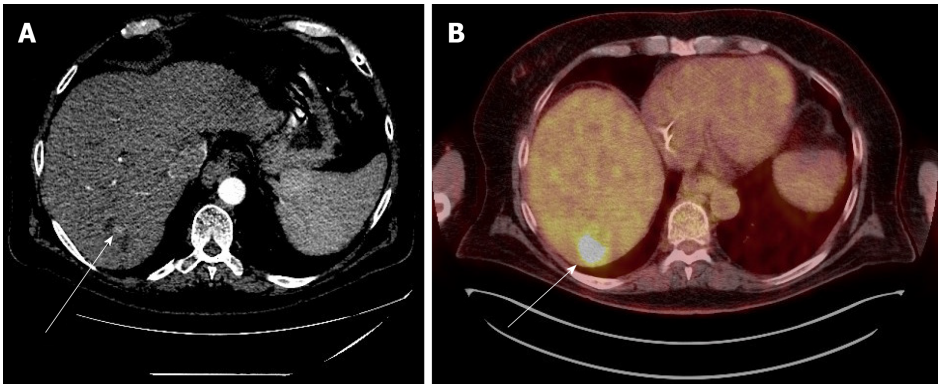

Figure 2 Case 2. A: Multi-phase computed tomography scan: a hypodense area, indicated by the white arrow, in the liver during the arterial phase post trans-arterial chemo-embolization treatment; B: Positron emission tomography scan: in the same area, there is avid fludeoxyglucose uptake, indicating residual tumor.

with locoregional therapies or even adjuvant systemic therapy (Case 1). In our case series PET-CT scans appeared very useful when the AFP was elevated and the contrast-enhanced scans did not reveal any pathognomonic findings in treated tumors. The utility and strengths of PET-CT scans are likely underestimated since it is not part of the standard of care in screening for and monitoring HCC, even in the latest United States guidelines[13,14]; it is certainly not part of our institution's protocol yet. There have been several studies describing the efficacy of combining the traditional 18F-FDG isotope with another isotope, 11C-acetate, in the utility of PET-CT scan in the detection of HCC[15-19]. This dual-tracer approach appears to be quite promising in complementing multi-phase CT or MRI scans, as well as FDG PET-CT scan.

CONCLUSION

PET-CT scans can be very helpful in select HCC cases for monitoring of treatment response, especially when contrast-enhanced multi-phase scans fail to identify pathognomonic findings of residual HCC tumors. A prospective study comparing the addition of dual-tracer PET-CT scan to the conventional multi-phase CT or MRI, *vs* multi-phase CT or MRI alone in detecting HCC tumors, is needed to improve the evaluation of treatment response in this disease.

Table 1 How positron emission tomography-computed tomography altered medical management in our cases

Case	Indication for PET-CT	How PET-CT changed management
Case 1	To rule out metastatic HCC while AFP in the 7000s; chest CT and bone scan had been negative	The PET-CT scans successfully detected residual HCC in the treated areas in both lobes and allowed for appropriate treatments to prolong his survival by at least 36 mo; multiple multi-phase MRI scans failed to do so. Subsequently PET-CT scan subsequently detected a new HCC lesion when MRI did not
Case 2	To evaluate treatment response in a biopsy-proven hepatocellular carcinoma mixed with poorly differentiated carcinoma, which had had atypical characteristics on multi-phase CTs (MRI was contraindicated due to his pacemaker)	The PET-CT scan successfully revealed residual carcinoma and allowed for further treatment in prolonging survival
Case 3	To rule out metastatic HCC disease to the lungs in which anatomy had been distorted due to prior Tb infection	The PET-CT scan successfully detected residual HCC while a multi-phase MRI failed to do so. The PET-CT scan subsequently detected a metastatic focus to the bone and averted liver transplant
Case 4	To aid in the investigation of rising AFP in a treated HCC patient when multi-phase MRI scans had been negative	The PET-CT scan identified a recurrent HCC focus in the periphery of a previously treated HCC tumor location

PET-CT: Positron emission tomography-computed tomography; AFP: Alpha fetoprotein; HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging.

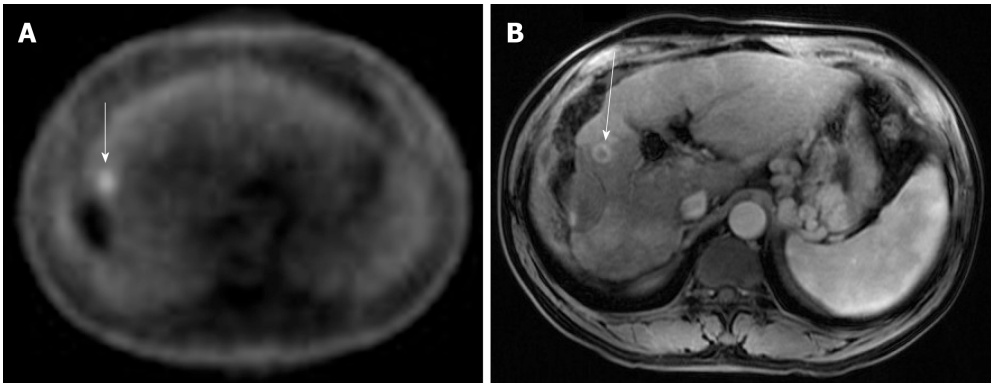

Figure 3 Case 3. A: Positron emission tomography scan: the white arrow indicates a small focus of fludeoxyglucose uptake adjacent to the treatment zone showing absent metabolic activity; B: Multi-phase magnetic resonance imaging: the white arrow points to focal bleed at the periphery of the treatment zone post microwave ablation without evidence of any viable tumor during the arterial phase.

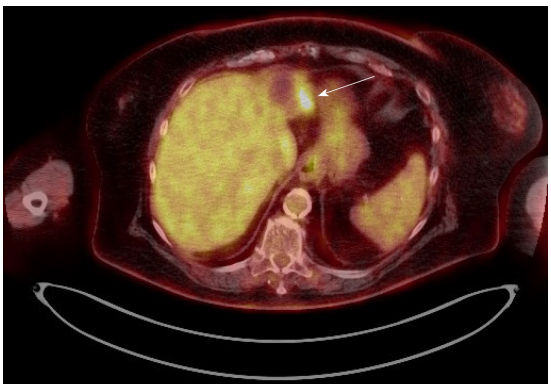

Figure 4 Case 4. Positron emission tomography scan: the white arrow points to the fludeoxyglucose uptake at the periphery of a treated lesion.

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