ISSN 1948-5204 (online)

World Journal of *Gastrointestinal Oncology*

World J Gastrointest Oncol 2020 February 15; 12(2): 124-247

Published by Baishideng Publishing Group Inc

World Journal of Gastrointestinal Oncology

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Monthly Volume 12 Number 2 February 15, 2020

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World Journal of Gastrointestinal Oncology

Volume 12 Number 2 February 15, 2020

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INDEXING/ABSTRACTING

The *WJGO* is now indexed in Science Citation Index Expanded (also known as SciSearch®), PubMed, and PubMed Central. The 2019 edition of Journal Citation Reports® cites the 2018 impact factor for *WJGO* as 2.758 (5-year impact factor: 3.220), ranking *WJGO* as 52 among 84 journals in gastroenterology and hepatology (quartile in category Q3), and 131 among 229 journals in oncology (quartile in category Q3).

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Gastrointestinal Oncology

ISSN
ISSN 1948-5204 (online)

LAUNCH DATE
February 15, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Monjur Ahmed, Rosa M Jimenez Rodriguez, Pashtoon Kasi

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-5204/editorialboard.htm

EDITORIAL OFFICE
Jin-Lei Wang, Director

PUBLICATION DATE
February 15, 2020

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2020 February 15; 12(2): 124-148

DOI: 10.4251/wjgo.v12.i2.124

ISSN 1948-5204 (online)

REVIEW

Biomarkers for detecting colorectal cancer non-invasively: DNA, RNA or proteins?

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Author contributions: Loktionov A is responsible for all work related to the preparation of this paper; he designed the paper structure, performed the literature search, analysed the literature data, prepared and contributed one figure and five tables and wrote the paper.

Conflict-of-interest statement: Alexandre Loktionov holds posts of CEO and Scientific Director at DiagNodus Ltd.

Manuscript source: Invited manuscript

Received: September 13, 2019
Peer-review started: September 13, 2019
First decision: October 18, 2019
Revised: October 30, 2019
Accepted: November 29, 2019
Article in press: November 29, 2019

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Abstract

Colorectal cancer (CRC) is a global problem affecting millions of people worldwide. This disease is unique because of its slow progress that makes it preventable and often curable. CRC symptoms usually emerge only at advanced stages of the disease, consequently its early detection can be achieved only through active population screening, which markedly reduces mortality due to this cancer. CRC screening tests that employ non-invasively detectable biomarkers are currently being actively developed and, in most cases, samples of either stool or blood are used. However, alternative biological substances that can be collected non-invasively (colorectal mucus, urine, saliva, exhaled air) have now emerged as new sources of diagnostic biomarkers. The main categories of currently explored CRC biomarkers are: (1) Proteins (comprising widely used haemoglobin); (2) DNA (including mutations and methylation markers); (3) RNA (in particular microRNAs); (4) Low molecular weight metabolites (comprising volatile organic compounds) detectable by metabolomic techniques; and (5) Shifts in gut microbiome composition. Numerous tests for early CRC detection employing such non-invasive biomarkers have been proposed and clinically studied. While some of these studies generated promising early results, very few of the proposed tests have been transformed into clinically validated diagnostic/screening techniques. Such DNA-based tests as Food and Drug Administration-approved multitarget stool test (marketed as Cologuard®) or blood test for methylated septin 9 (marketed as Epi proColon® 2.0 CE) show good diagnostic performance but remain too expensive and technically complex to become effective CRC screening tools. It can be concluded that, despite its deficiencies, the protein (haemoglobin) detection-based faecal immunochemical test (FIT) today presents the most cost-effective option for non-invasive CRC screening. The combination of non-invasive FIT and confirmatory invasive colonoscopy is the current strategy of choice for CRC screening. However, continuing intense research in the area promises the emergence of new superior non-invasive CRC screening tests that will allow the development of improved disease prevention strategies.

Published online: February 15, 2020

P-Reviewer: Cao ZF, Fan RY, Kadiyska T, Shenoy S, Yamada SL
S-Editor: Dou Y
L-Editor: Webster JR
E-Editor: Liu MY

Key words: Colorectal cancer screening; Biomarkers; Non-invasive testing; Stool; Colorectal mucus; Blood

Core tip: Numerous biomarkers detectable in non-invasively collected samples of stool, colorectal mucus, blood, urine, saliva and exhaled air have been investigated to develop new tests for colorectal cancer (CRC) early detection and screening. Promising results are often reported, but it is difficult to achieve the right balance between technical complexity, cost and diagnostic performance of the new tests. Today the combination of non-invasive faecal immunochemical test and confirmatory invasive colonoscopy remains the CRC screening strategy of choice. However, on-going intense research promises the emergence of new superior non-invasive screening tests that will allow the development of improved prevention strategies for these malignancies.

Citation: Loktionov A. Biomarkers for detecting colorectal cancer non-invasively: DNA, RNA or proteins? *World J Gastrointest Oncol* 2020; 12(2): 124-148
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/124.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.124

INTRODUCTION

Colorectal cancer (CRC) is currently the third most frequently diagnosed cancer worldwide. The global incidence for 2018 is estimated at 1801000 new cases, and the number of CRC-related deaths for this period is 861700[1]. Although the highest CRC incidence continues to be observed in economically developed Western countries, it is now rapidly increasing in other parts of the world[2]. Sporadic CRC development can take decades and is in most cases characterised by a slow progression from aberrant crypt formation in the colonic mucosa to benign polyps that may give rise to early cancer, then gradually evolving to invasive and metastasising advanced neoplasms (Figure 1)[2-4]. These pathogenetic features make CRC one of the most preventable and often curable malignancies. However, disease curability entirely depends on its early detection, which is not straightforward as clinical symptoms usually emerge only when CRC is already advanced. The latter factor warrants the necessity of active population screening for CRC, and it has been well proven that screening saves lives[2].

Full colonoscopy is regarded as the gold standard diagnostic technique for colorectal tumour detection[5], and it has become a very popular method for primary CRC screening[6-8] in the United States. One apparent reason for this trend is that diagnostic colonoscopy is usually combined with the simultaneous removal of detected polyps and functions as both a diagnostic and preventive procedure clearly reducing mortality from CRC[9]. Nonetheless, colonoscopy is an expensive and invasive technique that requires unpleasant bowel preparation and occasionally causes serious complications[10]. Moreover, its sensitivity is not perfect, with polyps sometimes missed[11], the latter problem often depending on the operator's skills[12]. Although colonoscopy as the final (confirmatory) diagnostic step is undisputable, its use in primary CRC screening remains questionable as indiscriminate application of this method inevitably results in frequent negative outcomes and a large health economic burden[13]. In theory, the global introduction of non-invasive tests employing biomarker analysis to select patients that really require endoscopy could dramatically reduce the numbers of unnecessary colonoscopies. Unfortunately, none of the existing non-invasive tests successfully combine high diagnostic sensitivity and specificity with technical simplicity and low cost, the key characteristics of an ideal screening modality. This paper provides a brief overview of the current state of the area encompassing biomarker-based non-invasive tests for CRC detection.

SOURCES OF MATERIAL FOR NON-INVASIVE CRC BIOMARKER TESTING

CRC development is an extraordinarily complex process driven by multiple genetic,

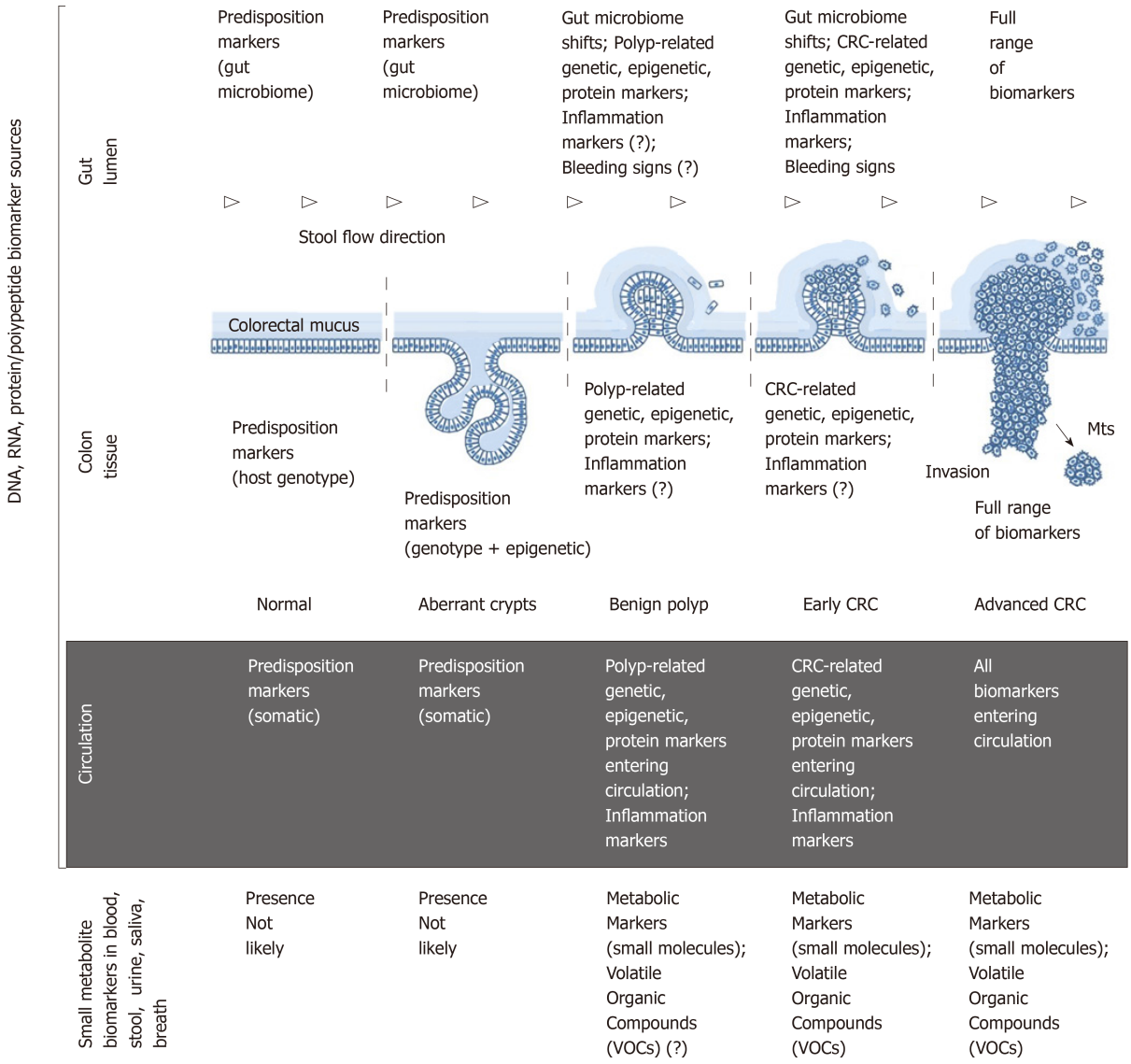

Figure 1 Colorectal cancer pathogenesis and sources of potential diagnostic biomarkers at different stages of colorectal cancer development. CRC: Colorectal cancer.

epigenetic, metabolic and immune alterations at the host level and influenced by numerous environmental factors[4,14,15]. Despite intense research, precise mechanisms of CRC development remain largely obscure[4,14,15]. Genome-targeting investigations, especially genome-wide association studies, have revealed a highly complex pathogenetic landscape comprising multiple alternative cascades of molecular events that may eventually result in cancer[4,16]. This complexity leads some investigators to a hardly satisfactory conclusion that "each patient's CRC is genetically and epigenetically unique"[4]. Nevertheless, colorectal tumours frequently have common molecular patterns that are diagnostically relevant and will be considered below.

The series of morphological events accompanying CRC development is presented in Figure 1. This sequence involves numerous associations with various types of biomolecules that can be characterised as biomarkers. The ideal biomarkers for CRC can be defined as substances that satisfy the following criteria: "(1) Are measured easily and inexpensively to identify a patient's cancer; (2) Identify a patient's prognosis to improve treatment outcome; and (3) Predict a patient's response to a specific treatment"[15]. This paper is focused only on the first category, *i.e.*, diagnostic biomarkers of CRC that can be sampled and tested non-invasively.

Figure 1 outlines the main sources of CRC biomarkers in relation to disease stages. From the morphological point of view, it is obvious that (1) colon tissue; (2) gut lumen; (3) blood/lymph circulation are the main sources of CRC-associated DNA, RNA and protein/polypeptide biomarkers associated with the host; (4) moreover, specific pattern shifts in small metabolite molecules derived from CRC-affected metabolic pathways constitute an additional group of post-metabolic markers that can

be analysed by metabolomics techniques[17,18]; and (5) CRC-associated gut microbiome changes[19] deserve to be considered as a separate category of diagnostic markers of non-human origin.

Normal and neoplastic colon tissue

Colonic epithelium is the site of neoplastic growth initiation. After that CRC progresses within the colonic wall until advanced stages of the disease, hence pre-malignant and malignant colon tissues are certainly the richest biomarker sources[4]. However, invasive biopsies are required for sampling tissue. Therefore, CRC markers detectable in tissue samples are not discussed here.

Gut lumen

Colonic epithelium is the key element of the gastrointestinal barrier between host tissues and microbiota-rich colon contents. Until recently it was presumed that all host cells exfoliated or migrated from the surface of the colonic epithelium were immediately incorporated in the faecal matter. According to this simplistic notion, it seemed to be logical that analysing naturally excreted stool samples constitutes the only perfectly non-invasive approach to investigating CRC biomarkers. It should, however, be stressed that stool is a complex mixture of microbiota-dominated faecal matter and occasional fragments of colorectal mucus secreted by goblet cells of the colonic epithelium. While the prevailing faecal component of stool entirely belongs to the environment, colorectal mucus is host-derived. The two-layered structure and functional significance of the mucus overlaying colonic epithelium have been elucidated only during the last decade[20,21], and it is now clear that colorectal mucus rather than faecal matter is the main receptacle of all cells and biomolecules released from either normal or malignant epithelium[22,23]. Intrarectal collection of colorectal mucus had demonstrated high informativeness of this substance[22,23], which was shown to accept CRC-generated malignant colonocytes exfoliated from tumour surface and transport them distally alongside stool flow without incorporating them into faeces (Figure 1)[20,23]. Biomarker-rich colorectal mucus essentially serves as a border between well oxygenated colonic epithelium and anaerobic gut lumen. Our group has recently developed a simple technique for non-invasive sampling of this mucus[24-26], the analysis of which may constitute a very convenient alternative to stool-based tests.

Blood/lymph circulation

Blood-derived biomarker analysis is another area of significant interest in the context of CRC detection since blood collection is regarded as a practically non-invasive procedure. It is evident that a wide range of CRC-associated biomarkers can be detected in the circulating blood and lymph of patients with these malignancies, but lymph collection cannot be performed with minimal invasiveness. For this reason, only biomarkers measurable in blood will be discussed below. In the modern literature the term "liquid biopsy" is often applied to this group of biomarker-based techniques[27]. Nevertheless, despite the easiness of blood sampling and the availability of numerous analytical techniques for biomarker detection in human plasma or serum, the presence of cancer biomarkers in blood may or may not be associated with CRC. Malignancies of other sites should always be excluded if this approach is considered for CRC screening.

Post-metabolic biomarkers

The use of metabolomics for revealing CRC-specific changes in patterns of low molecular weight metabolites has recently become another area of active exploration[28]. This new approach can potentially employ a wider range of biological samples comprising blood, stool, colorectal mucus, urine, saliva and exhaled breath, thus bringing about additional diagnostic options.

Gut microbiome changes associated with CRC

Recent research has revealed that specific changes in gut microbiome composition may be associated with the development of CRC[19]. In this context stool samples are usually investigated quantitatively for the presence of particular types of bacteria.

The limited choice of sample sources for non-invasive testing creates obvious problems. Collecting gut-derived samples looks preferable, but stool samples, albeit containing cells and molecules originating from the colonic mucosa (*i.e.*, colorectal mucus fragments), are usually dominated by the presence of abundant microbiota-rich faecal matter that often interferes with analytical procedures employed for host-related biomarker detection. A recently described analysis of non-invasively collected colorectal mucus presents a very interesting alternative; however, this approach is new and requires further testing. On the other hand, blood collection is very

straightforward and easy to standardise, but molecular changes detectable in blood (or plasma/serum) samples are not necessarily gut-specific. Finally, although the use of easily collectable materials (urine, saliva or exhaled air) is extremely attractive, the presence of CRC-specific biomarkers in such samples remains to be adequately explored. The sources of biological material characterised above may contain several types of diagnostic biomarkers that are discussed in the next section.

BIOMARKERS ASSOCIATED WITH CRC DEVELOPMENT

The story of non-invasively detectable CRC markers started due to a 1967 publication by Greegor, describing his observation of the frequent presence of occult blood in stool samples collected from patients with CRC[29]. That important discovery resulted in the development and prolonged use of the haemoglobin-recognising faecal occult blood test (FOBT) as the only non-invasive test for CRC detection. The situation had changed considerably in 1992, when a publication by Sidransky *et al*[30] described K-ras gene mutation detection in stool samples obtained from CRC patients and shifted the focus of attention to molecular markers. The area of CRC biomarker research has since exponentially expanded with thousands of papers published, but many initially promising findings failed to transform into clinically relevant diagnostic approaches. The purpose of this paper is to briefly outline the present status of non-invasive biomarkers proposed for detecting asymptomatic CRC. Only the most impressive and clinically relevant observations related to the main groups of these biomarkers (proteins/polypeptides, DNA, RNA, small metabolites, microbiome changes) are highlighted in the text below. However, numerous other markers that demonstrated promise in the context of CRC detection are presented in comprehensive Tables 1, 2, 3, 4 and 5. As it was impossible to cover all relevant studies, restrictions had to be applied when the Tables were prepared. Publications describing very small studies or reporting negative results were omitted. Likewise, only papers related to CRC, but not adenoma detection, were included since in most cases diagnostic sensitivity of biomarker tests for adenomatous polyps correlates with that for CRC. In addition, the necessity of non-invasive detection of colorectal polyps is still a debatable question, as the proportion of adenomas likely to progress to malignancy is relatively small, whereas the vast majority of these lesions (especially small polyps) never give rise to CRC[134,135].

Protein markers

Protein biomarkers considered in CRC early detection and screening are listed in Table 1. Historically, the use of haemoglobin detection in stool for non-invasive CRC detection can be regarded as the most popular approach in terms of population screening. Indeed, the traditional guaiac FOBT was almost exclusively employed for this purpose for several decades, and was attractive due to its simplicity and low cost. Although this test has insufficient sensitivity, it can be credited for saving many human lives[2,136,137]. Nevertheless, the outdated FOBT is now being replaced by the faecal immunochemical test (FIT) characterised by a much higher sensitivity. In a recent comprehensive review on FIT, Gies *et al*[31] discussed numerous studies of varying sizes and reported sensitivities between 66% and 74% and specificity levels between 84% and 95% when numbers of analysed CRC cases and controls were over 50. Table 1 also shows that M2-pyruvate kinase (M2-PK) is a relatively well-studied stool marker of CRC[32,33]; however, FIT performs better and remains considerably more popular. Other stool tests, including metalloproteinase 9 (MMP9)[34] and multimarker protein panels (see Table 1) have been investigated, but these tests have not been clinically accepted so far. It is also intriguing that in a recent small study, our group compared 24 protein biomarkers in non-invasively collected samples of colorectal mucus and concluded that haemoglobin, tissue inhibitor of metalloproteinase 1, M2-PK, peptidyl arginine deiminase 1, C-reactive protein and MMP9 could reliably detect CRC[138].

Blood (or plasma/serum) testing for CRC-associated proteins has been employed by many research groups (Table 1), but most of those studies produced relatively modest results. Among single protein markers detectable in the serum only CA11-19 marker protein[36], cysteine-rich 61 protein of the CCN family (Cyr 61)[38], B6-integrin[39] and trefoil factor 3 (TFF3)[36] can be regarded as promising. A number of protein panels were also examined; however, analysing multiple proteins is usually more technically complex and expensive. Impressive test sensitivity and specificity values (98.7% and 94.8%, respectively) were reported for combined testing for lectins DC-SIGN and DC-SIGNR by Jiang *et al*[42] in 2014, but these results remain to be confirmed in larger studies. Although blood collection is simple and easy to standardise, protein

Table 1 Non-invasive protein (including cytokine) biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s)	Sensitivity (or its range)	Specificity (or its range)	Ref.
Screening (reviewed)	Stool	Protein	Haemoglobin (FIT)	66.0%-74.0%	84.0%-95.0%	[31]
Case-control (reviewed)	Stool	Protein	M2-PK	68.0%-93.0%	70.0%-97.5%	[32,33]
Case-control	Stool	Protein	MMP 9	89.30%	91.20%	[34]
Case-control	Stool	Protein panel	Complement C3, Lactotransferrin, Haemoglobin subunit α1 and Haptoglobin	71.00%	95.00%	[35]
Case-control	Serum	Protein	CA11-19	98.00%	84.00%	[36]
Case-control	Serum	Protein (cytokine)	MIC-1 (GDF15)	43.80%	96.70%	[36]
Case-control	Serum	Protein (cytokine)	IL-6	28.0%-89.5%	46.0%-94.0%	[37]
Case-control	Serum	Protein (cytokine)	IL-8	70.00%	91.00%	[36]
Case-control	Serum	Protein (cytokine)	Growth-related gene product β1	56.10%	95.30%	[36]
Case-control	Serum	Protein	Cyr61	83.00%	97.00%	[38]
Case-control	Serum	Protein	B6-integrin	69.80%	100.00%	[39]
Case-control (reviewed)	Serum	Protein	TIMP-1	52.0%-85.0%	60.0%-95.0%	[40]
Case-control	Serum	Protein	RBP4	74.90%	81.70%	[36]
Case-control	Serum	Protein	THBS2	64.90%	87.10%	[36]
Case-control	Serum	Protein	TFF3	74.20%	94.80%	[36]
Case-control	Serum	Protein	COL3A1	98.80%	69.10%	[36]
Case-control	Serum	Protein	COL10A1	63.00%	85.00%	[36]
Case-control	Serum	Protein	AZGP1	55.80%	85.00%	[36]
Case-control	Serum	Protein	Angiopoietin-2	79.30%	82.40%	[36]
Case-control	Serum	Protein	Kininogen	63.60%	65.90%	[36]
Case-control	Plasma	Protein	Melanotransferrin	48.20%	92.50%	[36]
Case-control	Serum	Protein panel	RBP4 and CEA	80.80%	91.20%	[41]
Case-control	Serum	Protein panel	TFF3 and CEA	89.40%	87.80%	[42]
Case-control	Serum	Protein panel	sDC-SIGN and sDC-SIGNR	98.70%	94.80%	[43]
Case-control	Serum	Protein panel	IGFBP-3 and CEA	75.00%	90.00%	[36]
Case-control	Serum	Protein panel	AZGP1, CEA and CA19-9	67.50%	82.50%	[36]
Case-control	Serum	Protein panel	IGFBP2, DKK3 and PKM2	73.00%	95.00%	[44]
Case-control	Plasma	Protein panel	BAG4, IL6ST, VWF, EGFR and CD44	73.00%	90.00%	[45]
Case-control, prospective	Serum	Protein panel	CEA, hs-CRP, CYFra21-1 and Ferritin	60.0%-70.0%	81.0%-89.0%	

FIT: Faecal immunochemical test.

biomarkers of CRC present in stool or colorectal mucus currently look more diagnostically reliable than those detectable in blood.

An additional advantage of using protein biomarkers for CRC detection is defined by the fact that their immunochemical detection can be easily presented as point of care (POC) tests, which are already available for FIT[139].

DNA and mRNA markers

This sub-section briefly discusses studies on CRC detection using DNA and mRNA markers that are listed in Table 2.

Gene mutations, especially those of *K-Ras* and *APC* genes, were the first CRC-associated genetic markers assessed with the purpose of developing new non-invasive modalities for CRC early detection and screening. Regrettably, it soon became clear

Table 2 Non-invasive DNA, messenger RNA and long non-coding RNA biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s)	Sensitivity (or its range)	Specificity (or its range)	Ref.
Screening	Stool	DNA mutation panel	3 *K-ras* mutations, 10 *APC* mutations, 8 *p53* mutations, microsatellite instability marker BAT-26 and long DNA marker	51.60%	94.40%	[46]
Case-control	Stool	Panel including DNA mutation, DNA methylation, DNA amount and protein testing	*K-ras* mutation, methylation of *Vimentin* (*VIM*), *BMP3*, *NDRG4* and *TFPI2* genes, DNA measurement by *β-actin* assessment and HemoQuant test for haemoglobin	78.0%-85.0%	85.0%-90.0%	[47]
Screening	Stool	Panel including DNA mutation, DNA methylation, DNA amount and protein testing	*K-ras* mutation, *BMP3* and *NDRG4* promoter methylation, DNA measurement by *β-actin* assessment and test for haemoglobin (FIT)	92.30%	86.60%	[48]
Case-control	Stool	Methylated DNA	*BMP3* gene	51.0%-84.0%	90.0%-100.0%	[49]
Case-control	Stool	Methylated DNA	*CDKN2A* gene	20.0%-40.0%	84.0%-100.0%	[49]
Case-control	Stool	Methylated DNA	*ECAD* gene	65.20%	88.00%	[49]
Case-control	Stool	Methylated DNA	*FBN1* gene	72.00%	93.30%	[49]
Case-control	Stool	Methylated DNA	*GATA 4/5* gene promoter	42.9%-71.0%	84.0%-95.0%	[49,50]
Case-control	Stool	Methylated DNA	*HLTF* gene	20.0%-37.5%	90.0%-92.6%	[49]
Case-control	Stool	Methylated DNA	*HIC1* gene	42.30%	98.00%	[49]
Case-control	Stool	Methylated DNA	*HPP1* gene	71.20%	57.10%	[49]
Case-control	Stool	Methylated DNA	*ING1b* gene	73.70%	95.00%	[49]
Case-control	Stool	Methylated DNA	*ITGA4* gene	40.00%	96.80%	[49]
Case-control	Stool	Methylated DNA	*MGMT* gene	33.9-55.1%	52.0%-100.0%	[49]
Case-control	Stool	Methylated DNA	*NDRG4* gene promoter	53.0%-92.0%	89.1%-100.0%	[49-51]
Case-control	Stool	Methylated DNA	*P16INK4A* gene	71.70%	86.00%	[49]
Case-control	Stool	Methylated DNA	*PHACTR3* gene	55.0%-66.0%	95.0%-100.0%	[49]
Case-control	Stool	Methylated DNA	*RASSF2* gene	45.30%	94.70%	[49]
Case-control	Stool	Methylated DNA	*SDC2* gene	81.10%	93.30%	[52]
Case-control	Stool	Methylated DNA	*SEPT9* gene	20.0%-84.8%	80.0%-94.5%	[49]
Case-control	Stool	Methylated DNA	*SFRP1* gene	26.4%-89.0%	86.0%-95.5%	[49]
Case-control	Stool	Methylated DNA	*SFRP2* gene	32.1%-94.2%	54.0%-100.0%	[49,51]
Case-control	Stool	Methylated DNA	*SPG20* gene	80.2%-89.0%	99.0%-100.0%	[49,51]
Case-control	Stool	Methylated DNA	*SNCA* gene	83.90%	75.00%	[49]
Case-control	Stool	Methylated DNA	*TFPI2* gene	63.3%-92.0%	79.0%-100.0%	[49-51]
Case-control	Stool	Methylated DNA	*TP53* gene	56.30%	100.00%	[49]
Case-control	Stool	Methylated DNA	*Vimentin* (*VIM*) gene	32.6%-86.0%	82.0%-100.0%	[49-51]
Case-control	Stool	Methylated DNA	*WIF1* gene	19.3%-60.4%	96.7%-99.4%	[49]
Case-control	Stool	Methylated DNA	*XAF1* gene	55.90%	52.00%	[49]
Case-control	Stool	Methylated DNA panel	*BMP3* and *NDRG4* genes	98.00%	90.00%	[49]
Case-control	Stool	Methylated DNA panel	*MGMT* and *XAF1* genes	73.50%	52.00%	[49]
Case-control	Stool	Methylated DNA panel	*MGMT-B* and *SFRP2* genes	88.30%	91.20%	[49]
Case-control	Stool	Methylated DNA panel	*RASSF1A* and *SFRP2* genes	75.00%	89.40%	[51]

Case-control	Stool	Methylated DNA panel	*SNCA* and *FNB1* genes	84.30%	93.30%	[53]
Case-control	Stool	Methylated DNA panel	*Vimentin (VIM)* and *SFRP2* genes	92.50%	91.20%	[53]
Case-control	Stool	Methylated DNA panel	*AGTR1*, *WNT2* and *SLIT2* genes	74.0%-78.0%	88.0%-89.0%	[49,50]
Case-control	Stool	Methylated DNA panel	*ECAD*, *MGMT* and *P16INK4A* genes	72.00%	88.00%	[49]
Case-control	Stool	Methylated DNA panel	*ITGA4*, *SFRP2* and *P16INK4A* genes	70.00%	96.80%	[49]
Case-control	Stool	Methylated DNA panel	*MGMT*, *CDKN2A* and *hMTH1* genes	55.00%	63.00%	[49]
Case-control	Stool	Methylated DNA panel	*MGMT*, *MLH1* and *Vimentin (VIM)* genes	75.00%	86.50%	[49,51]
Case-control	Stool	Methylated DNA panel	*SFRP2*, *HPP1* and *MGMT* genes	93.70%	77.10%	[49]
Case-control	Stool	Methylated DNA panel	*WIF-1*, *ALX-4* and *Vimentin (VIM)* genes	25.00%	98.00%	[49]
Case-control	Stool	Methylated DNA panel	*Vimentin (VIM)*, *OMSR* and *TFPI2* genes	86.70%	87.60%	[49]
Case-control	Stool	Methylated DNA panel	*SFRP2*, *GATA4/5*, *NRDG4* and *Vimentin (VIM)* genes	96.40%	65.00%	[49]
Case-control	Stool	Human DNA content	Total human DNA content	66.00%	89.80%	[54]
Case-control	Bowel Lavage Fluid	Methylated DNA panel	*miR-124-3*, *LOC386758* and *SFRP1* genes	82.00%	79.00%	[55]
Case-control	Intrarectally collected colorectal mucus	Human DNA content	Total human DNA content	60.40%	94.80%	[56]
Case-control	Serum/plasma	Methylated DNA	*ALX4* gene	23.0%-90.7%	72.5%-100.0%	[57]
Case-control	Serum/plasma	Methylated DNA	*APC* gene	57.0%-86.5%	86.0%-92.1%	[57]
Case-control	Plasma	Methylated DNA	*CDH1 (E-cadherin)* gene	60.00%	84.00%	[55]
Case-control	Serum/plasma	Methylated DNA	*SDC2* gene	87.0%-90.7%	72.5%-95.2%	[36,57]
Case-control	Serum/plasma	Methylated DNA	*SEPT9* gene	47.1-95.6%	81.0%-96.7%	[36,57-62]
Case-control	Serum/plasma	Methylated DNA	*SFRP2* gene	54.0%-69.4%	40.0%-98.7%	[57,63]
Case-control	Plasma	Methylated DNA	*THBD (Thrombomodulin)* gene	70.70%	80.30%	[51]
Case-control	Serum/plasma	Methylated DNA	*TPEF* gene	65.0%-81.0%	69.0%-90.0%	[57]
Case-control	Serum/plasma	Methylated DNA	*VIM (Vimentin)* gene	59.0%-90.7%	72.5%-93.0%	[57]
Case-control	Plasma	Hypomethylated DNA	LINE-1 transposable DNA element	65.80%	90.00%	[36]
Case-control	Serum/plasma	Methylated DNA panel	*IKFZ* and *BCAT1* genes	62.1%-95.0%	92.0%-95.0%	[36,57]
Case-control	Serum	Methylated DNA panel	*SEPT9* and *SDC2* genes	86.50%	92.10%	[64]
Case-control	Serum/plasma	Methylated DNA panel	*APC*, *MGMT*, *RASSF2A* and *WIF-1* genes	86.50%	92.10%	[57]
Case-control	Plasma	Methylated DNA panel	*ALX4*, *BMP3*, *NPTX2*, *RARB*, *SDC2*, *SEPT9* and *VIM* genes	90.70%	72.50%	[63]
Case-control	Serum	ALU115 DNA content	Free ALU115 DNA content	69.20%	99.10%	[36]
Case-control	Serum	DNA integrity	ALU247/115 DNA integrity index	73.10%	97.30%	[36]
Case-control	Serum	Free DNA content	ALU-based cell-free DNA	64.50%	98.90%	[36]

Case-control	Whole blood	mRNA expression	*TSPAN8* gene	83.60%	58.20%	[36]
Case-control	Whole blood	mRNA expression	*LGALS* gene	82.10%	61.20%	[36]
Case-control	Whole blood	mRNA expression	*COL1A2* gene	73.10%	59.70%	[36]
Case-control	Whole blood	mRNA expression	*CEACAM6* gene	65.70%	61.20%	[36]
Case-control	Whole blood or serum	mRNA expression	*SALL4* gene	85.9%-96.1%	85.7%-95.0%	[65,66]
Case-control	Whole blood	mRNA expression panel	*TSPAN8* and *LGALS4* genes	92.50%	67.20%	[36]
Case-control (CRC and high-risk adenomas in the case group)	Whole blood	mRNA expression panel	*LGALS4, CEACAM6, TSPAN8* and *Col1A2* genes	75.00%	87.00%	[67]
Case-control	Whole blood	mRNA expression panel	*CEA, EpCAM, CK19, MUC1, EGFR* and *C-Met* genes	87.00%	85.00%	[68]
Case-control	Whole blood	Long non-coding RNA expression	NEAT1 variant 1	69.00%	79%	[36]
Case-control	Whole blood	Long non-coding RNA expression	NEAT1 variant 2	70.00%	96.00%	[36]
Case-control	Serum	Long non-coding RNA expression	BLACAT1	83.30%	76.70%	[69]
Case-control	Plasma	Long non-coding RNA expression panel	ATB and CCAT1	82.00%	75.00%	[70]
Case-control	Plasma	Long non-coding RNA expression panel	91H, PVT-1 and MEG3	82.80%	78.60%	[71]
Case-control	Serum	Long non-coding RNA expression panel	LOC285194, RP11-462C24.1 and Nbla12061	68.30%	86.90%	[72]

FIT: Faecal immunochemical test; CRC: Colorectal cancer.

that using gene mutations alone does not achieve satisfactory levels of diagnostic sensitivity. One demonstrative study evaluating this approach in a representative colonoscopy screening group concluded that the sensitivity of a panel comprising 21 DNA alterations (point mutations in *K-ras*, *APC* and *p53* genes, microsatellite instability marker BAT-26 deletions and long DNA assay) was only slightly above 50%[46].

The relatively disappointing diagnostic performance of mutation-based assays stimulated the search for CRC-related epigenetic changes, in particular aberrant hypermethylation of CpG islands usually located in gene promoter regions[140]. Gene-specific DNA methylation in stool was extensively investigated (Table 2), and several genes, including *BMP3*, *NDGR4*, *septin 9* (*SEPT9*), *SFRP2, SPG20, TFPI2* and *vimentin* (*VIM*) were shown to have diagnostic sensitivities between 50% and 92% at specificities between 80% and 100% for CRC detection (see recent reviews by Liu *et al*[49], Lam *et al*[50] and Rasmussen *et al*[51]). However, the reproducibility of these results was often problematic, and attempts to combine multiple methylated genes in panels were undertaken to increase assay reliability. It is remarkable that high CRC detection sensitivity and specificity values could be achieved by combining methylation testing for *BMP3* and *NDRG4*[49] or *VIM* and *SFRP2*[53] genes, but these results need to be corroborated. The Colosure™ test detecting methylated *VIM* in stool was the first methylation-based commercial test for CRC[141]. This diagnostic product was marketed in the USA but has recently been replaced by a more efficient multimarker Cologuard® test considered later in this sub-section.

Table 2 demonstrates that in the context of CRC diagnostics, DNA methylation markers detectable in blood attract at least as much attention as similar markers in stool. Although investigations of different groups often produce conflicting results, it is now apparent that *SEPT9* methylation detection is the best studied option amongst these blood tests[57]. This test has recently been commercialised and regulated for clinical application as Epi proColon® 2.0 CE[142], but its use appears to be limited to opportunistic CRC screening[57]. Moreover, DNA methylation analysis in biological samples is relatively laborious (especially for multimarker panels) and difficult to present in POC format. These factors limit diagnostic potential of this approach. In addition, Table 2 shows that samples of stool, blood, bowel lavage fluid and colorectal mucus were also tested for total and ALU-based DNA quantification, DNA integrity

assessment, examination of gene expression and long non-coding RNA expression. However, none of these assays could provide sufficiently high values for diagnostic sensitivity and specificity.

It is now becoming clear that tests involving DNA markers tend to perform better only when markers of different types are combined. Long-term research projects led by a United States company, Exact Sciences, allowed the design of a multitarget stool test that demonstrated high levels of sensitivity and specificity for CRC detection. An early version of this test that included *K-ras* mutation, methylation of *VIM, BMP3, NDRG4* and *TFPI2* genes, DNA measurement by *β-actin* assessment and the HemoQuant test for haemoglobin achieved diagnostic sensitivity between 78% and 85% at specificity between 85% and 90% in a case-control study[47]. It is remarkable that this test performed significantly better when directly compared with the test for methylated *SEPT9* in plasma (similar to Epi proColon)[143]. The multitarget test was then simplified, and its final version includes only determination of *K-ras* mutation, *BMP3* and *NDRG4* promoter methylation, DNA measurement by *β-actin* assessment and FIT. Screening application of this test in a large study produced CRC detection sensitivity of 92.3% at a specificity of 86.6%[48], which makes this assay the best among all available tests involving DNA markers. The test was approved by the United States Food and Drug Administration in 2014 and is now marketed as Cologuard®. However, this test, which can be regarded as an enhanced version of FIT, requires stool collection, remains technically complex, with a multistep analytical procedure required[144], and is very expensive at over $600.

MicroRNA markers

MicroRNAs (a sub-class of small non-coding RNA molecules) were discovered and characterised during the last decade of the XX century. Since that time, it was established that microRNAs are important regulators of gene expression intimately involved in the pathogenesis of many diseases including cancer[145]. As many of them are associated with the presence of colorectal tumours, it was suggested that microRNA determination in stool or blood samples may provide a new diagnostic modality for CRC early detection and screening[73]. MicroRNA variants investigated as potential CRC markers are listed in Table 3. Several published studies that used stool sample analysis highlight miR-21 as the best-studied marker of this type, but do not show outstanding sensitivity and specificity values[73]. MiR-451 and miR-223 detectable in stool produced high sensitivity and specificity values in a small study[75]; however, these markers looked less impressive in other studies, when combined with other microRNAs[73,76]. It is impossible to exclude that these discrepancies may be associated with either technical problems or different ethnic composition of the studied patient groups since clinical studies providing material for microRNA analyses were performed mostly in East Asia.

Table 3 also indicates that microRNA markers of CRC were intensely investigated in blood. Hitherto most of these studies produced modest or inconsistent results. Again, miR-21 was assessed by many groups, and conflicting results were published. Although very high diagnostic sensitivity (96.6%) and specificity (97.8%) values were reported by Ng *et al*[80] for miR-139-3p, which was shown to be downregulated in the serum of CRC patients, this finding remains to be confirmed. Combinations of microRNA markers detectable in plasma or serum were also tested as diagnostic panels. Among these panels (Table 3), combinations of downregulated miR-144-3p, miR-425-5p and miR-1260b[88] and upregulated miR-19a, miR-19b, miR-15b, miR-29a, miR-335 and miR-18a[90] demonstrated sensitivity and specificity levels exceeding 90%.

In addition, it should be noted that a recent small study has revealed that quantification of miR-21 in saliva samples resulted in CRC detection with 97% sensitivity and 91% specificity[93]. However, these highly intriguing results remain to be corroborated.

Although microRNAs constitute a group of promising CRC biomarkers, further research in this relatively new area is needed to establish clinically valid diagnostic techniques using these markers. The relative technical complexity of laboratory procedures used in microRNA analysis (RNA extraction, reverse transcription and qPCR analysis) and the necessity of careful assay optimisation and standardisation[146] should also be taken into account when the diagnostic potential of this interesting approach is considered.

Volatile organic compounds (VOC) and small metabolite biomarkers

Metabolomics is a new discipline that focuses on evaluating a wide variety of endogenous metabolites produced by the organism[17,18,28]. These metabolites can serve as late stage biomarkers of either normal physiological or pathophysiological events, and cancer metabolome is defined as the entire suite of low molecular weight (< 1500 Da) cancer-specific metabolites[17]. Interestingly, some of these metabolites are VOC-s

Table 3 Non-invasive microRNA biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s) and detection methods	Sensitivity (or its range)	Specificity (or its range)	Ref.
Case-control	Stool	MicroRNA	miR-18a, upregulated	61.00%	69.00%	[73]
Case-control	Stool	MicroRNA	miR-20a, upregulated	55.00%	82.00%	[73]
Case-control	Stool	MicroRNA	miR-21, upregulated	56.0%-86.0%	73.0%-81.1%	[73,74]
Case-control	Stool	MicroRNA	miR-92a, upregulated	72.00%	73.00%	[73]
Case-control	Stool	MicroRNA	miR-106a, upregulated	34.00%	97.00%	[73]
Case-control	Stool	MicroRNA	miR-135b, upregulated	78.00%	68.00%	[73]
Case-control	Stool	MicroRNA	miR-144*, upregulated	74.00%	87.00%	[73]
Case-control	Stool	MicroRNA	miR-221, upregulated	62.00%	74.00%	[73]
Case-control	Stool	MicroRNA	miR-223, upregulated	77.00%	96.00%	[75]
Case-control	Stool	MicroRNA	miR-451, upregulated	88.00%	100.00%	[75]
Case-control	Stool	MicroRNA panel	miR-223 and mir-92a, both upregulated	97.00%	75.00%	[73]
Case-control	Stool	MicroRNA panel	miR-17-93 cluster and miR-135b, all upregulated	74.00%	79.00%	[73]
Case-control	Stool	MicroRNA panel	miR-144-5p, miR-451a and miR-20b-5p, all upregulated	66.00%	95.00%	[76]
Case-control	Plasma	MicroRNA	miR-17-3p, upregulated	64.00%	70.00%	[73,77]
Case-control	Plasma	MicroRNA	miR-18a, upregulated	73.10%	79.10%	[77]
Case-control	Plasma	MicroRNA	miR-20a, upregulated	46.00%	73.40%	[73,77]
Case-control	Serum/plasma	MicroRNA	miR-21, upregulated	65.0%-91.4%	74.4%-95.0%	[73,77-79]
Case-control	Plasma	MicroRNA	miR-24, downregulated	78.40%	83.80%	[77]
Case-control	Plasma	MicroRNA	miR-29a, upregulated	69.00%	89.10%	[77]
Case-control	Serum/plasma	MicroRNA	miR-29b, downregulated	61.4%-77.0%	72.5%-75.0%	[77]
Case-control	Plasma	MicroRNA	miR-92, upregulated	89.00%	70.00%	[77]
Case-control	Serum/plasma	MicroRNA	miR-92a, upregulated	65.5%-84.0%	71.2%-82.5%	[73,77]
Case-control	Plasma	MicroRNA	miR-96, upregulated	65.40%	73.30%	[73,77]
Case-control	Plasma	MicroRNA	miR-106a, upregulated	74.00%	44.40%	[77]
Case-control	Serum	MicroRNA	miR-139-3p, downregulated	96.60%	97.80%	[80]
Case-control	Serum	MicroRNA	miR-139a-5p, upregulated	76.70%	88.00%	[81]
Case-control	Plasma	MicroRNA	miR-155, upregulated	58.20%	95.00%	[73]
Case-control	Plasma	MicroRNA	miR-182, upregulated	78.00%	91.00%	[82]
Case-control	Serum	MicroRNA	miR-194, downregulated	72.00%	80.00%	[77]
Case-control	Serum	MicroRNA	miR-196b, upregulated	63.00%	87.40%	[84]

Case-control	Plasma	MicroRNA	miR-200c, upregulated	64.10%	73.30%	[77]
Case-control	Serum	MicroRNA	miR-210, upregulated	74.6%-88.6%	73.5%-90.1%	[77,79]
Case-control	Plasma	MicroRNA	miR-221, upregulated	86.00%	41.00%	[73,77]
Case-control	Plasma	MicroRNA	miR-320a, downregulated	92.80%	73.10%	[77]
Case-control	Serum	MicroRNA	miR-338-5p, upregulated	76.30%	92.50%	[84]
Case-control	Serum	MicroRNA	miR-372, upregulated	81.90%	73.30%	[77]
Case-control	Serum	MicroRNA	miR-375, downregulated	76.90%	64.60%	[77]
Case-control	Plasma	MicroRNA	miR-423-5p, downregulated	91.90%	70.80%	[77]
Case-control	Plasma	MicroRNA	miR-506, upregulated	76.80%	60.70%	[85]
Case-control	Plasma	MicroRNA	miR-601, downregulated	69.20%	72.40%	[77]
Case-control	Plasma	MicroRNA	miR-760, downregulated	80.00%	72.40%	[77]
Case-control	Serum	MicroRNA	miR-1290, upregulated	70.10%	91.20%	[86]
Case-control	Plasma	MicroRNA	miR-4316, upregulated	76.80%	75.00%	[85]
Case-control	Plasma	MicroRNA panel	miR-19a and miR-19b, both upregulated	78.60%	77.40%	[77]
Case-control	Serum	MicroRNA panel	miR-21 and miR-92a, both upregulated	68.00%	91.20%	[73,77]
Case-control	Plasma	MicroRNA panel	miR-29a and miR-92a, both upregulated	83.00%	84.70%	[73,77]
Case-control	Plasma	MicroRNA panel	miR-200c and miR-18a, both upregulated	84.60%	75.60%	[36,77]
Case-control	Plasma	MicroRNA panel	miR-223 and miR-92a, both upregulated	76.00%	71.00%	[73]
Case-control	Plasma	MicroRNA panel	miR-320d, downregulated; miR-1290, upregulated	81.20%	90.70%	[87]
Case-control	Plasma	MicroRNA panel	miR-431 and miR-139-p3, both upregulated	91.00%	57.00%	[77]
Case-control	Plasma	MicroRNA panel	miR-601 and miR-760, both downregulated	83.30%	69.10%	[73,77]
Case-control	Plasma	MicroRNA panel	miR-19a, miR-19b and miR-15b, all upregulated	78.60%	79.20%	[77]
Case-control	Plasma	MicroRNA panel	miR-24, miR-320a and miR-423-5p, all downregulated	92.80%	70.80%	[36,77]
Case-control	Plasma	MicroRNA panel	miR-144-3p, miR-425-5p and miR-1260b, all downregulated	93.80%	91.30%	[88]
Case-control	Serum	MicroRNA panel	miR-145, downregulated; miR-106a and miR-17-3p, upregulated	78.50%	82.80%	[73,77]
Case-control	Plasma	MicroRNA panel	miR-409-3p, upregulated; miR-7 and miR-93, downregulated	82.00%	89.00%	[73,77]

Case-control	Plasma	MicroRNA panel	miR-18a, miR-21, miR-22 and miR-25, all upregulated	67.00%	90.00%	[89]
Case-control	Serum	MicroRNA panel	miR-23a-3p, miR-27a-3p, miR-142-5p and miR-376c-3p, all upregulated	89.00%	81%	[36]
Case-control	Plasma	MicroRNA panel	miR-29a, miR-92a, upregulated; miR-601, miR-760, downregulated	83.30%	93.10%	[77]
Case-control	Serum	MicroRNA panel	miR-21, miR-29, miR-92, miR-125, miR-223, all upregulated	84.70%	98.70%	[78]
Case-control	Plasma	MicroRNA panel	miR-19a, miR-19b, miR-15b, miR-29a, miR-335, miR-18a, all upregulated	91.00%	90.00%	[90]
Case-control	Plasma	MicroRNA panel	miR-21, let-7g, upregulated, mir-31, mir-92a, miR-181b, miR-203, downregulated	96.00%	81.00%	[73]
Case-control	Plasma	MicroRNA panel	miR-103a-3p, miR-127-3p, miR-151a-5p, miR-17-5p, miR-181a-3p, miR-18a-5p, miR-18b-5p, all upregulated	76.90%	86.70%	[91]
Case-control	Plasma	Exosomal MicroRNA panel	miR-27a, miR-130a, both upregulated	82.50%	75.00%	[92]
Case-control	Saliva	MicroRNA	miR-21, upregulated	97.00%	91.00%	[93]

that are present in the gas phase of various excreted biological materials and can potentially be used for detecting malignancies including CRC[99]. The outcomes of metabolomic studies on CRC detection are summarised in Table 4. Remarkably, very impressive results (with CRC detection sensitivity reaching 97% at 99% specificity) were achieved by Sonoda *et al*[97], when dog scent judgment was applied to faeces and exhaled breath samples for discriminating between CRC patients and controls. Unfortunately, it is not realistic to expect that this natural phenomenon could constitute a reliable diagnostic tool. Hence, advanced Electronic Nose technologies are being developed and tested for CRC detection (Table 4) alongside widely used combinations of gas chromatography (GC) and mass spectrometry (MS)[18,94,99]. The latter approach, albeit regarded as the technical gold standard, is complex, costly and unsuitable for population screening. This point is especially important because most of the numerous studies applying metabolomic approaches to detecting CRC-related metabolites (non-VOC-s) in biological substances use various versions of MS (Table 4). Although some of the studies listed in Table 4 produced sensitivity and specificity values above 90% for CRC detection[102,109,113,116,125], cost and complexity issues remain major obstacles to the introduction of these assays into routine clinical practice. In this context, the use of electronic noses sensing CRC-associated VOC-s appears to be more promising, especially in view of CRC detection sensitivity and specificity both reaching 95% in a recent study by Zonta *et al*[98].

Markers of CRC-associated changes in gut microbiome

The structure of the gastrointestinal tract engenders permanent interactions between its epithelial tissue and luminal microbiota, thus significant microbial impact in colorectal carcinogenesis appears to be likelier than in any other neoplasia. Steadily accumulating evidence indicates a pivotal role for the gut microbiome in influencing the development of CRC[19]. It is now believed that bacterial effects predisposing to CRC include impacts in gut surface barrier disruption, induction of colonic inflammation, direct genotoxic action against epithelial cells and dysbiosis leading to CRC-promoting shifts in gut microflora composition and the colonic microenvironment[19,147]. These advances prompted interest in evaluating gut microbiome shifts as possible diagnostic markers for CRC[148]. The results of several recent studies (presented in Table 5) show that alterations in gut microbiome composition can potentially serve as non-invasive diagnostic markers for this disease.

Table 4 Non-invasive volatile organic compounds and small metabolite biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s) and detection methods	Sensitivity (or its range)	Specificity (or its range)	Ref.
Case-control	Stool	VOCs	Hydrogen sulphide, Dimethylsulphide, Dimethyldisulphide, *mlz* 90 - detected by selected ion flow tube (SIFT) mass spectrometry (MS)	72.00%	78.00%	[94]
Case-control	Stool	VOCs	Propan-2-ol, 3-methylbutanoic acid - detected by gas chromatography (GC) and MS	87.90%	84.60%	[95]
Case-control	Stool	VOCs	Methyl mercaptan (increased) and hydrogen (decreased) – detected by GC	90.00%	57.70%	[96]
Case-control	Stool	VOCs	Pattern recognition technique - canine scent judgment	97.00%	99.00%	[97]
Case-control	Stool	VOCs	Pattern recognition technique (eNose Cyranose® 320)	85.00%	87.00%	[94]
Case-control	Stool	VOCs	Pattern recognition technique (SCENT A1)	95.00%	95.00%	[98]
Case-control	Urine	VOCs	Ion mobility spectroscopy technology (FAIMS)	88.00%	60.00%	[99]
Case-control	Urine	VOCs	Ion mobility spectroscopy technology (FAIMS)	63.00%	63.00%	[100]
Case-control	Urine	VOCs	Pattern recognition technique (eNose applied)	78.00%	79.00%	[99]
Case-control	Breath	VOCs	Pattern recognition technique - canine scent judgment	91.00%	99.00%	[97]
Case-control	Breath	VOCs	Acetone (increased), ethyl acetate (increased), ethanol (decreased) and 4-methyl octane (decreased) detected by GC-MS	85.00%	94.00%	[99]
Case-control	Breath	VOCs	Nonanal, decanal, 4-methyl-pentanone, 2-methylbutane, 4-methyloctane, 4-methylundecane, 2-methylpentane, methylcyclopentane, cycloxehane, methylcyclohexane, trimethyldecane-1,2-pentadiene, 1,3-dimethylbenzene, 1,4-dimethylbenzene – detected by GC-MS	86.00%	83.00%	[99]
Case-control	Stool	Magnetic resonance spectra	Magnetic resonance spectra patterns	85.20%	86.90%	[101]
Case-control	Stool	Small metabolites	Acetate – detected by proton magnetic resonance spectroscopy (PMRS)	94.70%	92.30%	[102]

Case-control	Stool	Small metabolites	Succinate – detected by PMRS	91.20%	93.50%	[102]
Case-control	Serum	Aromatic carboxylic acids	Benzoic acid – detected by CE-time of flight (TOF) MS	89.00%	82.00%	[103]
Case-control	Serum	Fatty acids	GTA-446 – detected by flow injection analysis MS	83.30%	84.80%	[104]
Case-control	Plasma	Amino acid metabolites	L-kynurenine – detected by high-performance liquid chromatography (HPLC)	85.20%	100.00%	[105]
Case-control	Plasma	Fatty acids	Decanoic acid – detected by CE-TOFMS	87.80%	80.00%	[106]
Case-control	Serum	Multiple metabolites	38 metabolites detected by GC-MS	85.00%	86.00%	[107]
Case-control	Serum	Phospholipids (sphingomyelins and phosphatidylcho-lines)	SM (34:1), PC (34:1), PC (34:2), PC (36:4), PC (36:2), PC (36:3) - detected by MS	♂77.3%; ♀80.8%	♂92.4%; ♀85.9%	[108]
Case-control	Serum	Unsaturated free fatty acids (panel)	C16:1, C18:3, C20:4, C22:6, all downregulated – detected by MS	93.80%	92.20%	[109]
Case-control	Serum	Amino acids (panel)	8 amino acids – detected by LC-MS/MS	65.00%	95.00%	[110]
Case-control	Serum	Amino acids, fatty acids, carbohydrates	13 metabolites – detected by LC-MS/MS	96.00%	80.00%	[111]
Case-control	Serum	Metabolite panel	2-hydroxy-butyrate, aspartic acid, kynurenine, cystamine – detected by GC-MS	83.10%	81.00%	[112]
Case-control	Serum	Lipid metabolites (panel)	Palmitic amide, oleamide, hexadecaneodioic acid, octadecanoic acid, eicosatrienoic acid, LPC(18:2), LPC(20:4), LPC(22:6), myristic acid, LPC(16:0) – detected by ion cyclotron resonance MS	98.10%	100.00%	[113]
Case-control	Serum	Panel of hydroxylated polyunsaturated ultra long-chain fatty acids	C28H46O4, C28H48O4 and C28H50O4, all downregulated – detected by LC-MS/MS and nuclear MR	75.00%	90.00%	[114]
Case-control	Serum	Multiple metabolites (panel)	11,14-eicosadienoic acid, 12a-hydroxy-3-oxocholadienic acid, 12-ketodeoxycholic acid, 12-keto-tetrahydro-leukotriene B4, 13-cis-retinoic acid, 1b-hydrocholic acid, 1-methylhistamine, 1-monopalmitin, 2,3-dihydroxybutanoic acid, 24-hydroxycalcitriol – detected by GC-TOFMS and UPLC-QTOFMS	83.70%	91.70%	[115]

Case-control	Plasma	Amino acids, fatty acids, carbohydrates	8 metabolites – detected by CT-TQMS	99.30%	93.80%	[116]
Case-control	Plasma	Choline-containing phospholipids (panel)	Total saturated lysophosphatidyl-cholines (LPCs), 18:2 LPC and sphingosylphosphorylcholine – detected by LC-MS/MS	88.30%	80.00%	[117]
Case-control	Plasma	Choline-containing phospholipids (panel)	Total saturated lysophosphatidyl-cholines (LPCs) and the difference between 18:2 LPC and 18:1 LPC – detected by LC-MS	82.00%	93.00%	[118]
Case-control	Dried blood	Amino acids and acylcarnitines (panel)	C16, Arg, C4/C8, C5/C3, Val, Phe/Tyr, Ala, C4/C3 – detected by direct infusion MS	81.20%	83.90%	[119]
Case-control	Urine	Polyamines	N1, N12-diacetylspermine – detected by ELISA	75.80%	96.00%	[120]
Case-control	Urine	Polyamines and amino acid metabolites	N1, N12-diacetylspermine and kynurenine – detected by LC-MS	80.00%	80.00%	[121]
Case-control	Urine	Amino acids and acetoacetate (panel)	Alanine, glutamine, aspartic acid and acetoacetate – detected by PMRS	87.50%	91.30%	[122]
Case-control	Urine	Nucleosides (panel)	5-hydroxymethyluracil and 8-oxo-7,8-dihydroguanine – detected by UPLC-MS/MS	78.60%	75.00%	[123]
Case-control	Urine	Nucleosides (panel)	Cytidine, 3-methylcitidine, 1-methyladenosine, 2-deoxyguanosine, adenosine, inosine – detected by HPLC-MS/MS	69.00%	98.00%	[124]
Case-control	Urine	Metabolite panel	Citrate, Hippurate, p-cresol, 2-aminobutyrate, myristate, putrescine and kynurenate - detected by UPLC-QTOFMS	97.50%	100%	[125]
Case-control	Urine	Nucleosides (panel)	Adenosine, N4-acetylcytidine, cytidine, guanosine, inosine, 1-methyladenosine, 1-methylguanosine, 1-methylinosine, 2-methylguanosine, 2,2-methylguanosine, N6-methyladenosine, uridine, 3-methyluridine+5-methyluridine, pseudouridine – detected by reverse phase HPLC	76.90%	90.40%	[126]

Case-control	Urine	Nucleosides (panel)	Adenosine, N4-acetylcytidine, cytidine, guanosine, inosine, 1-methyladenosine, 1-methylguanosine, 1-methylinosine, 2-methylguanosine, 2,2-methylguanosine, N6-methyladenosine, 5-methyluridine, pseudouridine, uridine – detected by column switching HPLC	71.00%	96.00%	[127]

One remarkable common feature of all the studies listed in Table 5 is the obligatory presence of *Fusobacterium nucleatum* (*F. nucleatum*) as one of the components of all tested panels. Indeed, *F. nucleatum*, an anaerobic oral commensal, is now identified as a pathogenetic factor contributing to multiple disorders comprising among others inflammatory bowel disease and CRC[19,148,149]. This interesting diagnostic approach is being actively investigated; however, further studies are necessary to firmly establish the value of the gut microbiome in non-invasive CRC detection.

NON-INVASIVE BIOMARKER TESTING USE IN CRC SCREENING TODAY AND FUTURE CHALLENGES

The existing plethora of potential non-invasive approaches to CRC detection briefly reviewed in this paper looks impressive in terms of numbers, but often disappointing in terms of outcome. Most of the published results clearly fail to transform into diagnostic or screening tests that would be highly sensitive and specific, simple to perform and not associated with excessive cost. As a matter of fact, the choice of available biomarker-based tests practically used for CRC screening remains strictly limited. Today FIT is by far the most popular option[2,9,31] owing to its relative simplicity and affordability. The recently introduced and widely advertised multitarget Cologuard® stool test or Epi proColon test targeting *SEPT9* methylation in plasma, albeit approved for clinical use, are technically complex and prohibitively expensive. Comparative studies addressing the health economics of CRC screening have demonstrated that the multitarget stool test, being more cost-effective that no screening, is significantly less cost-effective when compared to the FIT or invasive endoscopic testing[150-152]. Likewise, methylated SEPT9 detection in plasma samples[153] is clearly less cost-effective than the FIT. Considering a unit cost of $8 for the FIT (sampling kit and analysis only), Lansdorp-Vogelaar *et al*[154] concluded that a biomarker-based test that detects CRC with higher levels of sensitivity and specificity (up to 100%) should never be more expensive than $57 to be cost-effective. These estimates seem to indicate that in practical terms the FIT is currently the most cost-effective test for non-invasive CRC screening. Other authors argue that a highly specific non-invasive biomarker with an improved sensitivity for advanced adenomas (that progress to CRC) would probably be cost-effective at higher threshold costs[155], but the $600 price tag currently attached to Cologuard® is obviously excessive.

In any case, it is apparent that the FIT is not a perfect screening test. Its specificity reaching 95% is sufficiently high to be deemed satisfactory, but the sensitivity of this test remains relatively modest[31]. There is, however, an opinion that repeated FIT testing with one-year intervals may compensate for the lack of sensitivity[12]. Moreover, accurate identification of individuals with different levels of CRC risk could lead to creating objective approaches to risk stratification and personalised screening[12,155,156].

The effectiveness of a screening strategy is defined not only by screening test performance characteristics, but also by screening participant adherence[12]. One additional practical problem in CRC screening programmes employing faecal tests is insufficient screening uptake[157,158] that often results from participants' reluctance to collect stool samples[159,160]. The use of non-invasively collected colorectal mucus samples[24,138] in FIT-like tests can help solve this problem, but this new approach remains to be thoroughly evaluated, and this will require large comparative randomised trials that usually take several years to complete[155]. The existing

Table 5 Non-invasive faecal bacterial biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s)	Sensitivity (or its range)	Specificity (or its range)	Ref.
Case-control	Stool	Bacterial	*Fusobacterium nucleatum*	54.0%-92.8%	79.8%-91.0%	[128-131]
Case-control	Stool	Bacterial	*clbA*-positive bacteria	56.4%	81.5%	[131]
Case-control	Stool	Bacterial panel	*Fusobacterium nucleatum*, *Bacteroides clarus*, *Roseburia intestinalis* and *Clostridium hathewayi*	92.8%	79.8%	[130]
Case-control	Stool	Bacterial panel	*clbA*-positive bacteria and *Fusobacterium nucleatum*	84.6%	63.1%	[131]
Case-control	Stool	Bacterial panel	Ratio of *Fusobacterium nucleatum* to *Bifidobacterium*	84.6%	92.3%	[132]
Case-control	Stool	Bacterial panel	Combination of ratios of *Fusobacterium nucleatum* to *Bifidobacterium* and *Fusobacterium nucleatum* to *Faecalibacterium prausnitzii*	90.0%	90.2%	[132]
Case-control (CRC and adenomatous polyps in the case group)	Stool	Bacterial panel	*Fusobacterium nucleatum*, *Enterococcus faecalis*, *Streptococcus bovis*, *Enterotoxigenic Bacteroides fragilis*, and *Porphyromonas* spp	91.4%	93.5%	[133]

CRC: Colorectal cancer.

combination of the FIT and confirmatory colonoscopy is the strategy of choice today, and its further optimisation is currently regarded as the main factor in improving CRC screening effectiveness.

The present strong position of the FIT as the test of choice for non-invasive CRC screening will certainly be temporary as this test has one intrinsic deficiency that is impossible to eliminate. The FIT detects blood, which is shed but not produced by tumours, and bleeding may not occur in some CRC patients. For this reason, FIT sensitivity will never approach 100%, and it is likely that this target will become achievable only when a screening test employing CRC-specific biomarker(s) is developed. As no single biomarker detectable in all colorectal tumours has been identified so far, multitarget strategies combining either multiple markers of the same type or different assays (such as Cologuard®) emerge as CRC screening options advocated by some experts. However, these complex assays usually require sophisticated laboratory equipment and are laborious and expensive. Although future technological advances can help in eliminating these deficiencies, the search for more reliable and easily detectable single CRC biomarkers should continue.

It can be expected that rapid progress in cancer biomarker research accompanied by accelerated development of new non-invasive tests promises forthcoming breakthroughs in CRC screening and prevention of this disease.

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2020 February 15; 12(2): 149-172

DOI: 10.4251/wjgo.v12.i2.149

ISSN 1948-5204 (online)

REVIEW

Caffeine and its main targets of colorectal cancer

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Author contributions: Sang LX designed the study; Cui WQ wrote the original draft; Cui WQ, Wang ST, Pan D, Chang B and Sang LX reviewed and edited; All authors read, revised and approved the final manuscript.

Supported by the innovative talents support program of institution of higher learning of Liao Ning province, No. 2018-478; The innovative talents of science and technology support program of young and middle-aged people of Shenyang, No. RC170446.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Manuscript source: Unsolicited manuscript

Received: September 12, 2019

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Abstract

Caffeine is a purine alkaloid and is widely consumed in coffee, soda, tea, chocolate and energy drinks. To date, a growing number of studies have indicated that caffeine is associated with many diseases including colorectal cancer. Caffeine exerts its biological activity through binding to adenosine receptors, inhibiting phosphodiesterases, sensitizing calcium channels, antagonizing gamma-aminobutyric acid receptors and stimulating adrenal hormones. Some studies have indicated that caffeine can interact with signaling pathways such as transforming growth factor β, phosphoinositide-3-kinase/AKT/mammalian target of rapamycin and mitogen-activated protein kinase pathways through which caffeine can play an important role in colorectal cancer pathogenesis, metastasis and prognosis. Moreover, caffeine can act as a general antioxidant that protects cells from oxidative stress and also as a regulatory factor of the cell cycle that modulates the DNA repair system. Additionally, as for intestinal homeostasis, through the interaction with receptors and cytokines, caffeine can modulate the immune system mediating its effects on T lymphocytes, B lymphocytes, natural killer cells and macrophages. Furthermore, caffeine can not only directly inhibit species in the gut microbiome, such as *Escherichia coli* and *Candida albicans* but also can indirectly exert inhibition by increasing the effects of other antimicrobial drugs. This review summarizes the association between colorectal cancer and caffeine that is being currently studied.

Peer-review started: September 12, 2019
First decision: October 14, 2019
Revised: October 25, 2019
Accepted: November 13, 2019
Article in press: November 13, 2019
Published online: February 15, 2020

P-Reviewer: Fiori E, Huang HC, Schuller HM
S-Editor: Zhang L
L-Editor: Filipodia
E-Editor: Qi LL

Key words: Caffeine; Colorectal cancer; Epidemiology; Signaling pathway; Immune response; Cell cycle

Core tip: Increasing evidence indicates that caffeine has wide-ranging effects on pathogenesis, metastasis and prognosis of colorectal cancer. This study systematically reviewed the literature on the targets and effects of caffeine on colorectal cancer. The effects were categorized into five groups: (1) communicating with cell signaling; (2) modulating immune response; (3) influencing gut bacteria; (4) regulating cell cycle; and (5) redox homeostasis.

Citation: Cui WQ, Wang ST, Pan D, Chang B, Sang LX. Caffeine and its main targets of colorectal cancer. *World J Gastrointest Oncol* 2020; 12(2): 149-172
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/149.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.149

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related deaths[1]. It has been reported that there will be 1456000 new cases of CRC in 2019, with an estimated 51020 people dying of this disease in the United States[2]. According to statistics, the incidence and mortality rates of CRC have stabilized or declined in a number of the high human-development-index countries such as the United States, Australia, New Zealand and several Western European countries[3]. However, in Asia the incidence continues to increase at an alarming rate without any sign of abating[4]. Age and gender are both risk factors for CRC. People older than 50 years of age are more predisposed to be affected by CRC, and incidence in males is greater than in females[5]. Additionally, CRC is often accompanied by metastasis; statistics show that one in five patients with CRC suffer from simultaneous metastatic disease[6]. Due to venous drainage of the large bowel being achieved *via* the portal system, the first site of hematogenous dissemination for CRC is usually the liver, followed by the lungs, bone and brain[7].

CRC is a multifactorial disease involving genetic changes, the host immune response, gut microbiota and other environmental and lifestyle risk factors, which result in a series of pathologic changes that finally transform normal colonic epithelium into invasive carcinoma[1,8]. CRC involves many genetic changes and certain signaling pathways are clearly singled out as key factors in tumor formation. For example, the activation of the Wnt/β-catenin signaling pathway, which is associated with mutations of adenomatous polyposis coli, is regarded as the initiating event in CRC. The second step is the inactivation of the p53 pathway. Then, the mutational inactivation of the transforming growth factor β (TGF-β) signaling pathway is viewed as the third step in the progression to CRC[9]. Furthermore, aberrational activation of phosphoinositide-3-kinase (PI3K) and the induction of AKT activity can mediate the metastasis of CRC[10]. KRAS expression is also required for CRC, and the loss of its expression can cause the apoptosis of primary and metastatic colon adenocarcinomas[11].

As for the host immune response, it is well known that chronic inflammation induces dysplasia in intestinal epithelial cells, which can contribute to the initiation or progression of CRC[12]. Some pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) can contribute to inflammation-related tissue damage and are associated with tumor initiation[13]. In the tumor microenvironment, pre-existing T lymphocyte cells play an important role in CRC regression by attacking cancer cells throughby recognizing abnormally expressed neoantigens[14]. Both $CD4^+$ and $CD8^+$ effector T lymphocytes have anti-tumor properties and independently correlate with improved outcomes of CRC[15]. Additionally, low activity of natural killer (NK) cells is correlated with an increased risk of CRC compared with patients with high NK cell activity[16]. In tumor cases, tumor-derived factors attract circulating monocytes into the tumor tissue where they differentiate into macrophages called tumor-associated macrophages[17]. Tumor-associated macrophages are enriched in tumors compared with normal tissue and confer a poorer prognosis[18]. During the development of CRC, tumor-associated macrophages potentiate the angiogenic capacity of the tumor

microenvironment in an oxidative stress-dependent manner and promote CRC cell metastasis[19,20]. Oxidative stress, defined as an imbalance between pro- and antioxidants, has been implicated in the initiation, promotion and progression of carcinogenesis[21].

CRC has increased levels of different markers of oxidative stress, such as increased levels of reactive oxidative species (ROS) and nitric oxide, suggesting that oxidative stress may be one possible pathway to affect CRC[22]. An imbalance of gut bacteria can also lead to abnormal immune activation, chronic inflammation or hyperproliferation, which finally contributes to the development of CRC through specific mechanisms such as enhancing toxic bacterial products, decreasing beneficial bacterial metabolites, disrupting tissue barriers and translocation[8]. For example, *H. pylori* infection can be a risk factor for CRC and adenomatous polyps[23]. Moreover, *E. coli* may contribute to microbiome-driven CRC through damaging DNA, inducing senescence and leading to immune activation[24].

CAFFEINE

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid that belongs to the methylxanthine group. As one of major components in coffee, it was first isolated in 1820, and it is also present in tea leaves, cocoa beverages, soft drinks and chocolate products[25,26]. Caffeine is even available in a number of over-the-counter remedies, including some pain killers[26]. In both animals and humans, after its oral ingestion, caffeine is rapidly and completely absorbed into the gastrointestinal tract. Then, it enters into the water compartment of tissues, crosses all biological membranes, and eventually distributes in all body fluids. Once it is filtered by the glomeruli, 98% of caffeine is reabsorbed from the renal tubules, and only 0.5%–2% of unmetabolized caffeine is excreted in urine[27].

The metabolism of caffeine is a complex process that occurs in the liver, which involves successive N-demethylations and a C-8 oxidation by CYP1A2, N-acetyltransferase or xanthine oxidase to form metabolites with variable pharmacological actions[27,28]. The N-demethylation pathways of caffeine mediated by CYP1A2[29] involve the formation of paraxanthine (PX) through N3-demethylation[28]. PX is then metabolized by CYP1A2 in the human liver to an unknown unstable intermediate with an open ring structure. This intermediate is acetylated by N-acetyltransferase to form 5-acetyl-amino-6-formylamino-3-methyluracil, or the ring is closed to form 1-methylxanthine[30]. 1-methyluric acid is also the product of following 7-demethylation of PX. All of the above accounts for 67% of PX clearance. Furthermore, the renal excretion of unchanged PX, 1,7-dimethyluric acid and 7-methylxanthine comprise 9%, 8% and 6% of PX clearance, respectively[31].

Through *N*1-demethylation, caffeine gives rise to theobromine. In this case, theobromine can be further metabolized to form 7-methylxanthine, 7-methyluric acid, 3-methylxanthine, 6-amino-5[N-methylformylamino]-1-methyluracil and a small amount of 3,7-dimethyluric acid[32]. Theophylline (TP) formed from *N*7-demethylation is degraded to both 3-methylxanthine (major product) and 1-methylxanthine (minor product), and they are further demethylated to form xanthine[33]. The N3, N1 and N7 demethylations account for 84%, 12% and 4%, respectively, of caffeine metabolism in humans[27,28]. C8-hydroxylation only takes up 1% of metabolism; it can cause the formation of trimethyluric acid, which further gets degraded to 3,6,8-trimethylallantoin[29,34]. All of the above metabolites are then further metabolized in the liver by additional demethylations and oxidation to urates[35] (Figure 1). Caffeine exerts its functions by regulating important target molecules as described below.

Modulator of Ca^{2+} release channels

Intracellular Ca^{2+} signaling is a universal, evolutionarily conserved and versatile regulator of cell biochemistry, which is involved in angiogenic progression, cell proliferation, differentiation, migration and apoptosis[36,37]. Many of the molecules involved in Ca^{2+} remodeling are expressed differentially in multiple tumor cells and may significantly contribute to cancer hallmarks in a series of cancers including CRC[38]. In electrically inexcitable cells, most Ca^{2+} signals are initiated by receptors that stimulate phospholipase C and thereby induce the transformation from phosphatidylinositol-4,5-bisphosphate (PIP2) to diacylglycerols and inositol 1,4,5-trisphosphate (IP3). IP3 then binds to IP3 receptors to stimulate Ca^{2+} release from the endoplasmic reticulum[39]. The depletion of endoplasmic reticulum Ca^{2+} stores triggers the process called store-operated Ca^{2+} entry, which activates the endoplasmic reticulum-resident Ca^{2+} sensor protein stromal interaction molecule to gate and open the ORAI Ca^{2+} channels in the plasma membrane[37,40]. Caffeine is known to influence

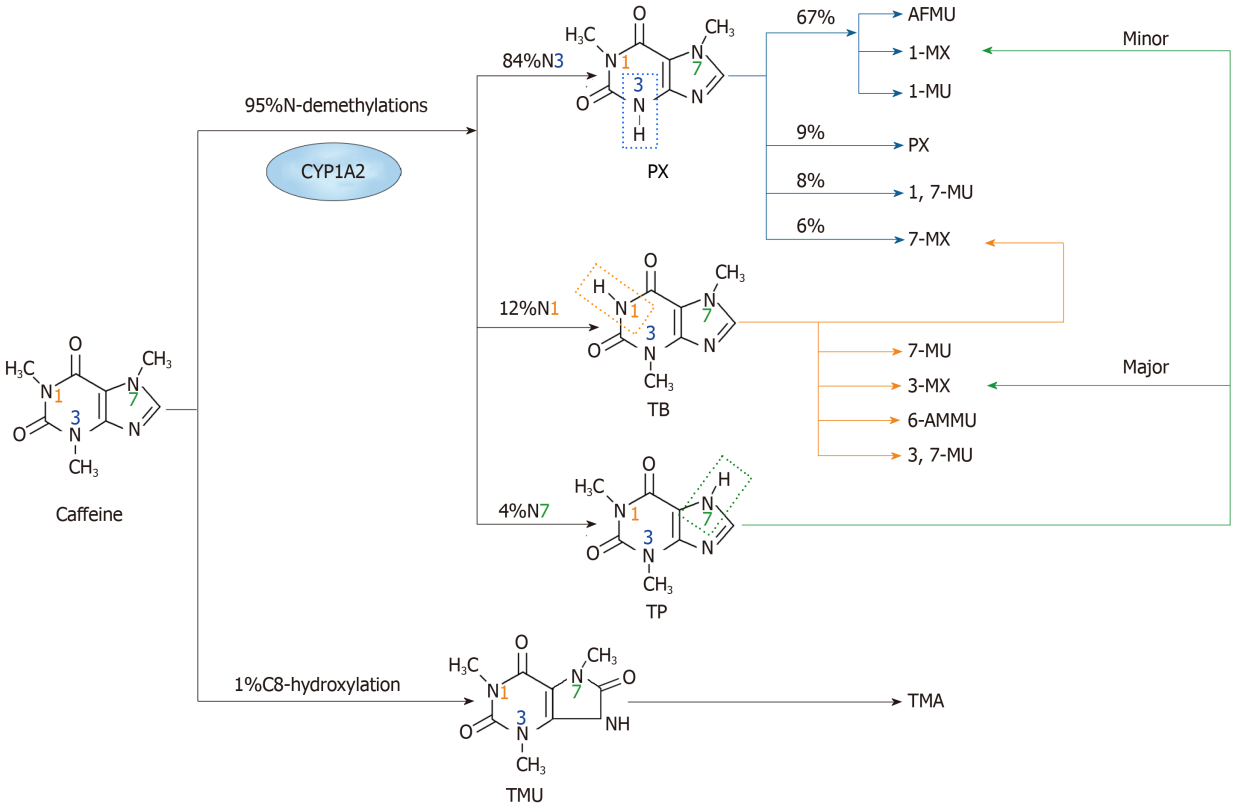

Figure 1 The major metabolism pathways of caffeine. The primary metabolic action of caffeine involves two main pathways. One is N-demethylation, which includes N1, N3 and N7-demethylations. Through N3-demethylation, caffeine can be degraded to form paraxanthine, which may be further degraded to 5-acetyl-amino-6-formylamino-3-methyluracil, 1-methylxanthine, 1-methyluric acid, 1,7-methyluric acid and 7-methylxanthine. A small amount of paraxanthine can be cleared in its unchanged form. Theobromine can be formed through N1-demethylation of caffeine, and it can be metabolized to form 7-methylxanthine, 7-methyluric acid, 3-methylxanthine, 6-amino-5[N-methylformylamino]-1-methyluracil and 3,7-methyluric acid. Moreover, caffeine can be metabolized to theophylline through N7-demethylation, and then theophylline can be further degraded to 1-methylxanthine and 3-methylxanthine. The other pathway is C8-hydroxylation in which caffeine is metabolized to trimethyluric acid and then 3,6,8-trimethylallantoin. PX: Paraxanthine; AFMU: 5-acetyl-amino-6-formylamino-3-methyluracil; 1-MX: 1-methylxanthine; 1-MU: 1-methyluric acid; 7-MU: 7-methyluric acid; 1,7-MU: 1,7-methyluric acid; 7-MX: 7-methylxanthine; TB: Theobromine; 3-MX: 3-methylxanthine; 6-AMMU: 6-amino-5[N-methylformylamino]-1-methyluracil; 3,7-MU: 3,7-methyluric acid; TP: Theophylline; TMU: Trimethyluric acid; TMA: 3,6,8-trimethylallantoin.

intracellular Ca^{2+} homeostasis in two different ways: one is by inhibiting the activation of the IP3 receptors through which the release of Ca^{2+} from intracellular stores and its following activation of store-operated Ca^{2+} entry can be suppressed. Another way is by activating ryanodine receptor (RyR) mediated Ca^{2+} release[25,41]. For the reason that both IP3 and RyR are present in the colonic epithelium, Ca^{2+} remodeling may be one possible mechanism by which caffeine acts on CRC[42] (Figure 2).

Antagonist of adenosine receptor

The tumor microenvironment exhibits high concentrations of adenosine due to the contribution of immune and stromal cells, tissue disruption and inflammation[43]. Adenosine, a purine nucleoside derived from a decrease in cellular adenosine triphosphate, is released into the extracellular space and may have significant influences on the vasculature, resistance to immune attacks, modulation of inflammation and growth of tumor masses by binding to specific G-protein-coupled A1, A2A, A2B, and A3 cell surface receptors[44]. A1R and A3R belong to the group of Gi-coupled proteins that inhibit adenylate cyclase-mediated production of cyclic adenosine 3′,5′-monophosphate (cAMP). In contrast, A2AR and A2BR are Go/Gs-coupled receptors that raise intracellular levels of cAMP[45]. Methylxanthines are inhibitors of adenosine action, most notably caffeine and TP, except those actions that are mediated by A3 receptors, as these methylxanthines are almost 100 times less potent at that receptor than on the other three[46,47]. Caffeine and some of its metabolites are all antagonists of adenosine receptor. However, caffeine is a less potent inhibitor of adenosine at its receptors than its two metabolites TP and PX[46].

Antagonist of phosphodiesterase

Compared with cells of the related benign mucosa, CRC cells bind to a significantly increased level of intracellular cAMP and decreased levels of cyclic guanosine 3′-5′

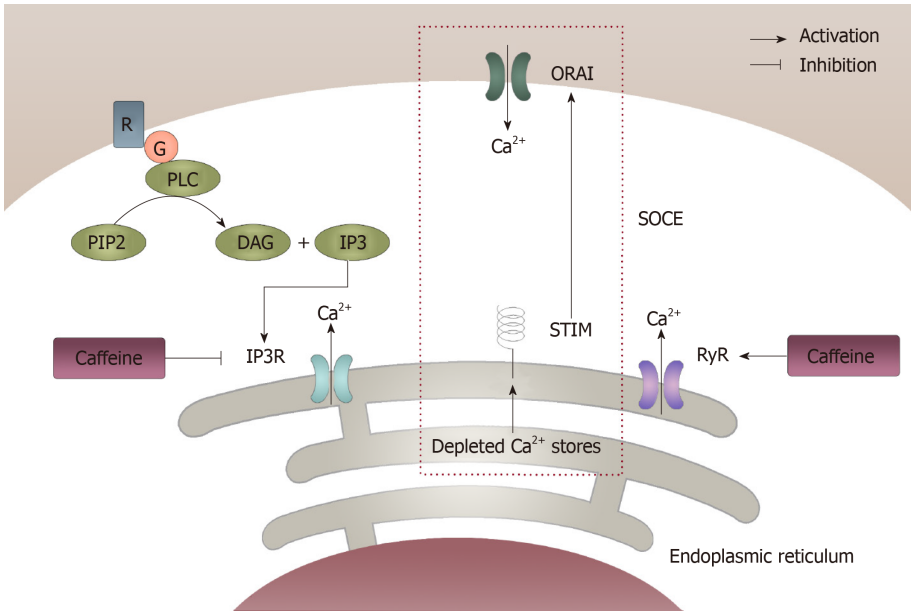

Figure 2 Modulation of intracellular calcium homeostasis by caffeine. Caffeine can modulate intracellular calcium homeostasis of inexcitable cells in two different ways. One way is by inhibiting the inositol 1,4,5-trisphosphate receptor through which Ca^{2+} released from the endoplasmic reticulum can be decreased, which results in a decrease in intracellular calcium levels. However, caffeine can increase the intracellular calcium levels by activating the ryanodine receptor, which causes an increase in Ca^{2+} being released from the endoplasmic reticulum. In this case, the store-operated Ca^{2+} entry, which was initiated from the depletion of Ca^{2+} from the endoplasmic reticulum can be activated and then result in an increase in extracellular Ca^{2+} getting into the intracellular compartment. IP3: 1,4,5-trisphosphate; IP3R: 1,4,5-trisphosphate receptor; ER: Endoplasmic reticulum; RyR: Ryanodine receptor; SOCE: Store-operated Ca^{2}+ entry.

monophosphate (cGMP)[48,49]. cAMP and cGMP levels are normally regulated by the balance between the activities of two types of enzymes, the generating enzymes (adenylate cyclases/guanylyl cyclase) and the degrading enzymes (PDEs)[48]. PDE enzymes are encoded by 21 genes in humans and classified into 11 different families (PDE1–PDE11), several of which contain isoform subfamilies[50]. They are a large superfamily of enzymes that can cause cleavage of the phosphodiester bond in the second messengers like cAMP and cGMP and then degrade them into 5′-GMP and 5′-AMP[51]. Phosphodiesterases 1, 2, 3, 10 and 11 are dual substrate-degrading isozymes, whereas phosphodiesterases 5, 6 and 9 are selective for cGMP and phosphodiesterases 4, 7 and 8 are cAMP selective[52].

PDEs show different expression levels in CRC. For example, the expression of PDE5 and PDE10 is higher in colon adenomas and adenocarcinomas compared with in the normal colonic mucosa[50,53], and the inhibition of PDE5 and PDE10 can suppress colon tumor cell growth[54]. Thus, caffeine, as a non-specific PDE antagonist, can lead to an increase in intracellular cAMP and cGMP concentrations, which exert a variety of cell responses such as the inhibition of CRC proliferation[41]. Moreover, its metabolite, theobromine has an approximately equipotent ability to caffeine to inhibit PDE, while TP is more potent, and PX is less potent[44].

cAMP, accumulated by the inhibition of PDE, influences the initiation of CRC. For example, the β-catenin pathway, an initial step for CRC development, can be activated through positive action of cAMP on protein kinase A[9,55]. Also, cAMP can suppress AKT/mammalian target of rapamycin (mTOR) signaling, which is a critical regulator of CRC development[56]. Meanwhile, the apoptosis of CRC cells can be suppressed by cAMP through the extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) pathways[57]. Additionally, cAMP promotes vascular endothelial growth factor expression, resulting in neoplastic vascularization[58]. As for cGMP, when it acts on its effector, protein kinase G, it can exert its functions of opposing intestinal epithelial cell proliferation by upregulating the nuclear transcription of cell cycle inhibitors (p21 and p27) and by suppressing pro-proliferative transcription mediated by the β-catenin/T cell factor[59]. Meanwhile, increasing cGMP in the colon epithelium activates forkhead box class O 3a, which plays important roles in coordinating environmental stressors through the regulation of cell growth and tissue homeostasis, and it can upregulate antioxidant gene expression to protect against redox stress and barrier dysfunction[60]. Also, cGMP

signaling promotes the DNA damage repair process and opposes chromosomal instability in healthy tissue. Also, it can inhibit epithelial–mesenchymal transition following tumorigenesis, regulating intestinal inflammation and altering the microbiome composition[59]. Therefore, the dysregulation of cGMP and cAMP signaling can contribute to CRC[55,59] (Figure 3).

Stimulation of adrenergic signaling

Stress, as one of the environmental factors, is reported to enhance CRC cell growth *in vivo* and *in vitro* and is linked to the occurrence and progression of CRC[61]. Catecholamines, including norepinephrine and epinephrine, are the primary neurotransmitters involved in stress response and originate from the sympathetic nerves of the autonomic system[62]. In people suffering from acute or chronic stress, both epinephrine and norepinephrine are elevated[61]. Caffeine ingestion is widely associated with stimulation of the sympathetic nervous system and with subsequent elevations in the plasma concentrations of the catecholamines epinephrine and norepinephrine[63,64]. Catecholamines can stimulate beta-adrenergic receptors by the beta-adrenoceptor-adenylylcyclase-protein kinase A cascade.

Beta-adrenergic receptors belong to the family of G-protein coupled receptors, and stimulation of the cascade can cause an accumulation of the second messenger cAMP resulting in modulation of varied pathways[65]. They can influence a lot in CRC because beta-2 adrenergic receptors have a high expression level in the neoplastic cells from colorectal adenocarcinoma. Moreover, it has significant association with tumor grading[66]. Meanwhile, it also has effects on tumor growth including promoting tumorigenesis, tumor cell proliferation, antiapoptotic mechanisms and promoting metastasis by stimulating the expression of angiogenic growth factors such as vascular endothelial growth factor and interleukin (IL)-6 and inducing epithelial–mesenchymal transition, motility and invasion[67]. In addition, it can also cause the modulation of the immune system. For example, it can induce a Th1/Th2 imbalance in the mouse immune system, which is considered critical during colon cancer progression[68]. Moreover, use of blockers of beta-adrenergic receptors has been proven to be associated with longer survival in patients with stage IV CRC[69].

Antagonist of GABAA receptors

An increasing amount of evidence suggests that the increased migration of tumor cells is not only a consequence of genetic alterations but is also due to chemokines, neuropeptides and neurotransmitters such as γ-aminobutyric acid (GABA)[70] (Figure 4). Caffeine acts as an antagonist of GABAA receptors (GABAAR) at the benzodiazepine-positive modulatory site[25].

EPIDEMIOLOGY

Coffee is one of the major sources of caffeine and is among the most widely consumed beverages in the world. It contains many substances that affect the human body, the majority of which include caffeine, caffeic acid, trigonelline, chlorogenic acid and diterpenes[71]. Evidence shows a protective effect of coffee consumption on CRC in the United States[72]. Moreover, the results of a large United States prospective cohort study consistent with the former study showed that caffeinated coffee drinkers had a significantly lower risk of CRC. The results also indicated the protective effect can be different at specific anatomic subsites that decrease the risk more for the proximal than for the distal colon[73]. A similar result was found in a prospective cohort study in Japanese men. However, it mentioned that the level of coffee consumption, which decreased the risk for recurrence in the proximal colon, can significantly increase the risk in the distal colon[74]. Furthermore, it has been shown that the association between CRC and coffee consumption can be influenced by the concentration of coffee. A large population-based case-control study demonstrated that modest coffee consumption (≥ 1 and < 2 servings/d) is associated with a 90% reduction in the odds of developing CRC and that the highest category of consumption (> 2.5 servings/d) is associated with a 54% reduction in the odds of developing CRC[75]. Additionally, findings within two large prospective cohort studies suggested that a higher intake of coffee was associated with a lower risk of CRC-specific mortality and all-cause mortality with stage I to III disease (an association that was stronger in stage III disease)[76]. However, some studies have also shown that drinking coffee is not associated with the colon cancer risk[77,78].

The reason why the results of epidemiological studies exploring the correlation between coffee consumption and CRC risk have been varied may be due to the following reasons. Firstly, the result may vary with ethnicity, which may present with cultural, dietary and genetic variants[75]. Secondly, associations appear to be very

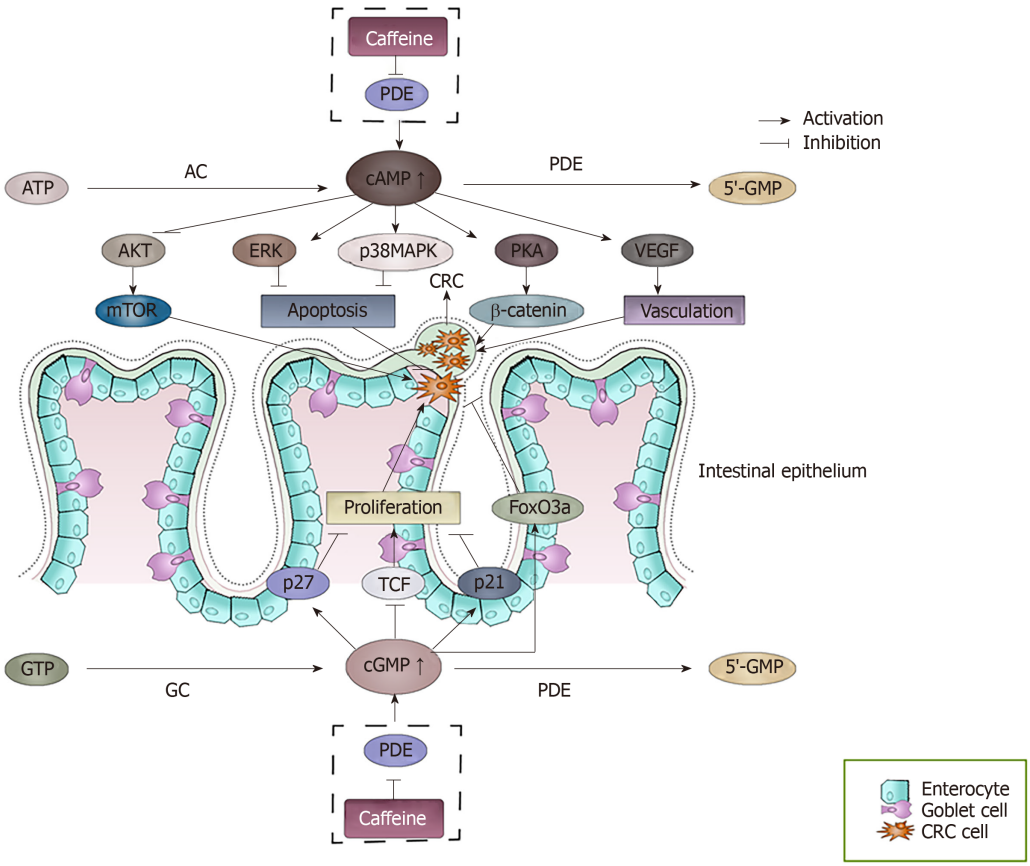

Figure 3 The effect of accumulated cAMP and cGMP caused by the inhibition of phosphodiesterase by caffeine. cAMP and cGMP levels are regulated by the balance between the activities of two types of enzymes, the generating enzymes (adenylate cyclase/guanylyl cyclase) and the degrading enzymes (phosphodiesterase). Caffeine can inhibit the degrading enzymes, which causes the accumulation of cAMP and cGMP. The accumulated cAMP and cGMP can interact with many factors and finally may result in a series of events of colorectal cancer cells. cAMP: Cyclic adenosine 3',5'-monophosphate; cGMP: Cyclic guanosine 3'-5' monophosphate; AC: Adenylate cyclases; GC: Guanylyl cyclase; PDEs: Phosphodiesterase; PKA: Protein kinase A; VEGF: Vascular endothelial growth factor; TCF: T cell factor.

complex and are influenced by other factors such as cigarette smoking status. Studies have shown that among non-smoking populations, coffee increases the risk of CRC, while in populations that smoke cigarettes, the opposite has been observed[79]. Finally, many studies did not specify whether the coffee blend was caffeinated or decaffeinated, filtered or unfiltered, processed at a certain roasting level, or made with a certain brewing method. Also, studies did not distinguish between coffee brewed from *Coffea arabica* and *Coffea canephora* (*robusta*) beans[71].

EFFECT ON SIGNALING PATHWAYS

Many signaling pathways have been reported to be associated with CRC such as the TGF-β, phosphatase and tensin homolog (PTEN)[80], PI3K/AKT/mTOR, β-catenin[10], MAPK[81] and NF-KB[82] pathways (Figure 5).

TGF-β pathway

Caffeine was reported to have the ability to block the elevation of TGF-β in a concentration-dependent manner[83,84]. The TGF-β signaling pathway is one of the most commonly inactivated signaling pathways in CRC[80]. It is well accepted that the TGF-β family members are key regulatory polypeptides that participate in many aspects of cellular function such as proliferation, differentiation and apoptosis[85]. TGF-β functions as a ligand by binding to the type II receptor, which recruits and phosphorylates the type I receptor (TGFBR1)[85]. TGF-β type I receptor then phosphorylates the receptor-associated SMAD2 and SMAD3, and then the activated SMAD2 and SMAD3 bind to the common mediator SMAD4, with the consequent relocation of this molecular complex at the level of the nucleus, resulting in the final regulation of target genes related to a wide range of cellular processes, including

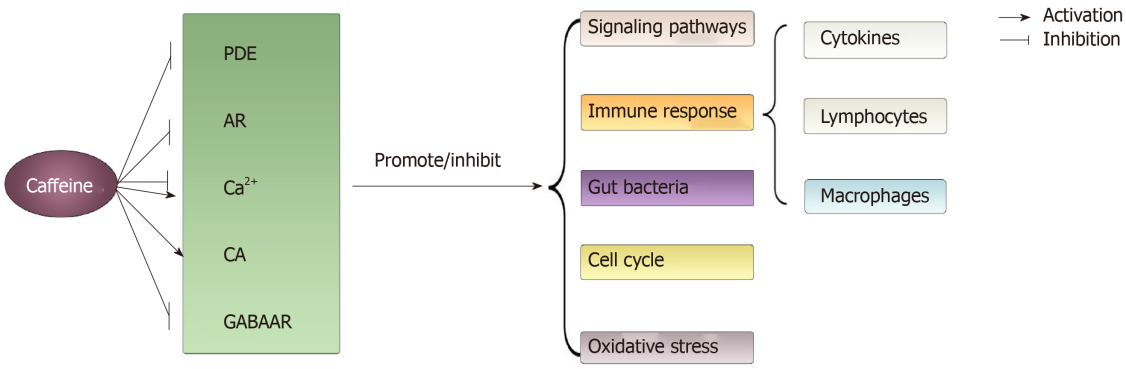

Figure 4 The main acting sites and physiological processes modulated by caffeine on colorectal cancer involved in this article. Through modulating acting sites such as phosphodiesterase, adenosine, Ca^{2+}, catecholamines and γ-aminobutyric acid receptor, caffeine can exert varied effects (promote or inhibit) on physiological processes such as signal pathways, immune response, gut bacteria, cell cycle and oxidative stress. PDE: Phosphodiesterase; AR: Adenosine receptor; CA: Catecholamines; GABAAR: γ-aminobutyric acid receptor.

cancer initiation and progression, proliferation, differentiation, apoptosis and migration[86].

Multiple studies have demonstrated that TGF-β can exert a dual function in the process of developing human cancers: In normal cells and early carcinomas, it acts as a tumor suppressor[87,88], while in aggressive and invasive tumors, it acts as a promoter of tumor metastasis[87]. A possible pathway for the switching of the dual function of TGF-β is that MAPK is activated by TGF-β *via* a SMAD4-dependent mechanism in CRC cells, leading to the upregulation of expression of cyclin-dependent kinase 2 inhibitor p21[89], which act as a tumor suppressor and contributes to cell cycle arrest in response to various stimuli[90]. The upregulation of p21 induced by TGF-β decreases over time, which can lead to different cell cycle functions[89]. TGF-β can regulate downstream factors, CCN-family protein 2 (CCN2) and transgelin, in a TGF-β/SMAD3 dependent fashion[91]. Mediated by the induction of its transcriptional target CCN2, TGF-β plays crucial pro-metastatic, lymphangiogenesis, stromal infiltration and activation roles[92]. CCN2, also known as connective tissue growth factor, is markedly activated in human CRC tissues compared with the corresponding normal colon tissues during both tumorigenesis and metastasis[93].

Caffeine and its metabolites can suppress both TGF-β-dependent and independent CCN2 expression *via* a mechanism that involves reduction of the steady state concentration of total SMAD2 protein and the decline of phosphorylation of SMAD3[91]. Transgelin, an actin-binding protein of the calponin family, has the potential to alter cell motility through direct interaction with the actin cytoskeleton, which plays an important role in promoting invasion, survival and resistance to the anoikis of CRC[94]. However, caffeine is able to inhibit transgelin promoter activity in a dose dependent manner[91] (Figure 6).

PI3K/AKT/mTOR pathway and PTEN pathway

Caffeine inactivates PI3K and AKT and activates PTEN, resulting in the apoptosis of tumor cells[95,96]. Apart from the TGF-β pathway, the PI3K/AKT pathway is another commonly dysregulated pathway in CRC[80], and mutational activation of PI3K and induction of AKT activity can mediate the metastasis of CRC[10]. PI3K is a family of intracellular lipid kinases, including phosphatidylinositol, phosphatidylinositol-4-phosphate (PIP) and PIP2 whose substrate is the phosphatidylinositol lipid matrix. Once activated, PI3K can catalyze the phosphorylation of PIP2 to phosphorylate 3,5,5-triphosphate (PIP3)[97]. PI3K can be divided into three classes: class I, class II and class III[98]. Only class IA PI3Ks play a role in human cancer[99], and caffeine can directly inhibit both class I and class II PI3Ks[100]. Epidermal growth factor receptor activates survival signaling pathways including the RAS/RAF/ERK, p38 MAPK, JNK and PI3K/AKT/mTOR signaling pathways, leading to cell growth and proliferation[101]. When PI3K is activated, PIP3 is generated, and increased PIP3 recruits AKT to the membrane where it is activated by other kinases that are also dependent on PIP3[102].

Caffeine exerts pharmacological activity by inhibiting AKT activation *via* modulating the cAMP level *in vitro*[103]. Maximal activation of AKT is dependent on the phosphorylation of two residues: Thr308, which is dependent on the activity of the enzyme PI3K-dependent kinase 1, and Ser473. Caffeine has been reported to reduce the phosphorylation of AKT at both Thr308 and Ser473 residues[104]. Activated AKT promotes cell growth and survival by inhibiting the pro-apoptotic proteins of the Bcl-

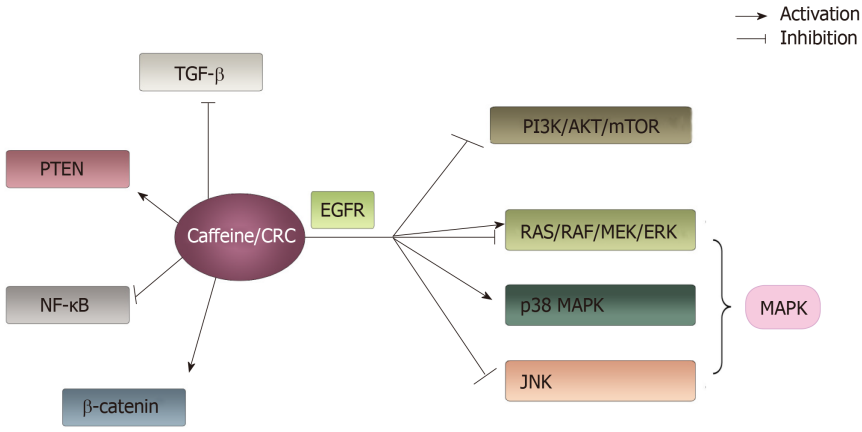

Figure 5 The main signaling pathways associated with colorectal cancer that can be influenced by caffeine involved in this article. Caffeine can interact with a number of signaling pathways. It shows an active effect on phosphatase and tensin homolog, β-catenin and p38 mitogen-activated protein kinase pathways. Additionally, it has negative effects on transforming growth factor β, NF-KB, phosphoinositide-3-kinase/AKT/mammalian target of rapamycin and c-Jun N-terminal kinase pathways. Moreover, caffeine showed a dual function on RAS/RAF/MEK/ERK pathways depending on concentration of caffeine. PTEN: Phosphatase and tensin homolog; MAPK: Mitogen-activated protein kinase; TGF-β: Transforming growth factor β; PI3K: Phosphoinositide-3-kinase; mTOR: Mammalian target of rapamycin; JNK: c-Jun N-terminal kinase.

2 family, enhancing the degradation of p53 and stimulating the mTOR group of proteins[105]. mTOR is a serine/threonine protein kinase that regulates protein synthesis and degradation, cell survival, proliferation and longevity[103]. Activation of the mTOR pathway has been noted in squamous cancers including CRC, and caffeine was found to be an inhibitor of mTOR[106]. PTEN acts as an important negative regulator of the PI3K/AKT signaling pathway[96,107], and its inactivation is a common cause of increased PI3K activity in cancers[81]. PTEN is a lipid phosphatase specific for PIP3, which opposes the effects of PI3K on cellular PIP3 levels and consequently regulates cell proliferation and survival through various signaling molecules. Caffeine can lead to the activation of PTEN through the elevation of intracellular cAMP[96].

MAPK pathway

MAPKs are serine-threonine kinases that are located downstream of many growth-factor receptors, including epidermal growth factor receptor[81,108]. They mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, differentiation, survival, death and transformation[109]. There are three major subfamilies of MAPK: The extracellular-signal-regulated kinases (MAPK/ERK, RAS/RAF/mitogen-activated ERK-regulating kinase (MEK)/ERK; the c-Jun N-terminal or stress-activated protein kinases (JNK); and p38 MAPKs[81].

RAS/RAF/MEK/ERK PATHWAY

There is growing evidence that activation of the RAS/RAF/MEK/ERK pathway is involved in the pathogenesis, progression and oncogenic behavior of human CRC[81]. It was reported that, mediated by activation of the MEK/ERK signaling pathway, 20 μM of caffeine can prevent paclitaxel-induced apoptosis in Colo205 CRC cells *in vitro*[26].

KRAS belongs to the RAS family of genes that encode GTP-binding proteins[81]. KRAS expression is required for tumor maintenance, even in situations where KRAS activation is not an initiating event. The loss of KRAS expression has been shown to cause the apoptosis of primary and metastatic colon adenocarcinomas[111]. Coffee and its component caffeine reduced KRAS expression in Caco-2 human colon carcinoma cells by activating two miRNAs, miR-30c and miR-96, which are known to target the KRAS gene[110]. In KRAS wild-type tumor cells, the binding of EGF to its receptor, epidermal growth factor receptor, causes GTP-loading of KRAS, which activates RAF to phosphorylate MEK, which phosphorylates ERK[111]. ERK is constitutively active in CRC cells, suggesting that MEK is activated in primary colorectal tumors[112]. A high concentration of caffeine was shown to activate the ERK1/2 pathway and induce autophagy, while a moderate concentration of caffeine is thought to have an inhibitory effect on the ERK pathway[95].

p38 MAPK pathway

p38 MAPK is a MAPK, and its canonical activation is mediated by the module in

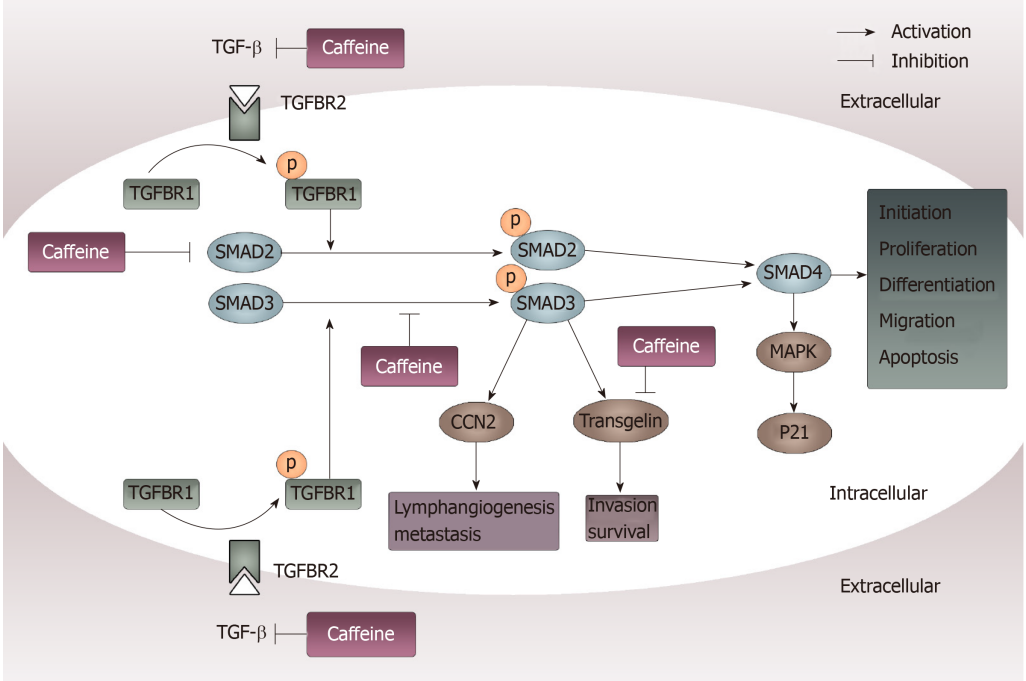

Figure 6 Inhibition of transforming growth factor β pathways caused by caffeine. When transforming growth factor (TGF-β) acts on its type II receptor, the type I receptor can be recruited and phosphorylated. Then, mediated by the activated TGF-β type I receptor, SMAD2 and SMAD3 are phosphorylated and exert their functions by modulating downstream factors such as CCN-family protein 2 and transgelin or by binding to SMAD4. Caffeine may influence this pathway not only through inhibiting TGF-β at the beginning of the pathway but also by reducing the steady state concentration of total SMAD2 and decreasing phosphorylation of SMAD3. Furthermore, caffeine can directly inhibit transgelin, which plays an important part in invasion and survival. TGF-β: Transforming growth factor β; CCN2: CCN-family protein 2; TGFBR2: Transforming growth factor binding to the type II receptor; TGFBR1: Transforming growth factor binding to the type I receptor; MAPK: Mitogen-activated protein kinase.

which MAPK kinase kinases phosphorylate and activate MAPK kinases, which activates p38 MAPK through dual phosphorylation on threonine and tyrosine residues[113]. p38 MAPK has four family members: p38α (MAPK14), p38β (MAPK11), p38δ (MAPK13) and p38γ (MAPK12)[113]. p38α MAPK signaling has an important protective function in colorectal tumorigenesis. On one hand, p38 MAPK protects intestinal epithelial cells against colitis-associated CRC by regulating the intestinal epithelial barrier function. On the other hand, it suppresses tumor initiation in epithelial cells[114]. Caffeine was reported to trigger the phosphorylation of p38 MAPK through an increase in the intracellular Ca^{2+} concentration and ROS generation in U937 cells[115] (Figure 7).

Other pathways

Compared to normal colorectal epithelial cells, CRC cells exhibit aberrant constitutive NF-KB activation[82]. Caffeine inhibits NF-KB, which plays an important role in multiple signaling cascades related to carcinogenesis, including the survival, invasion and migration of cancer cells[95]. In addition, ultraviolet radiation irradiation stress-induced activation of the apoptosis signal-regulating kinase-1/SEK1/JNK signaling pathway can interact with caffeine[116], and the activation of JNK signaling pathways was implied to be involved in piperlongumine-mediated apoptosis in human colorectal cancer HCT116 cells[117]. Furthermore, abnormal activation of the Wnt/β-catenin pathway is responsible for the initiation of more than 90% of colon cancers. β-catenin accumulates in the nucleus, binds to T cell factor or lymphoid enhancer factor transcription factors and induces the expression of Wnt target genes that have key roles in tumor progression[10]. Caffeine was shown to increase the frequency of β-catenin mutations, and the colon tumors almost exclusively harbor β-catenin mutants with direct substitutions of glycine 34[118].

EFFECT ON INTESTINAL HOMEOSTASIS

Intestinal homeostasis is maintained through interactions involving the immune response and the microbial content in the gut[119].

Effect on the immune system

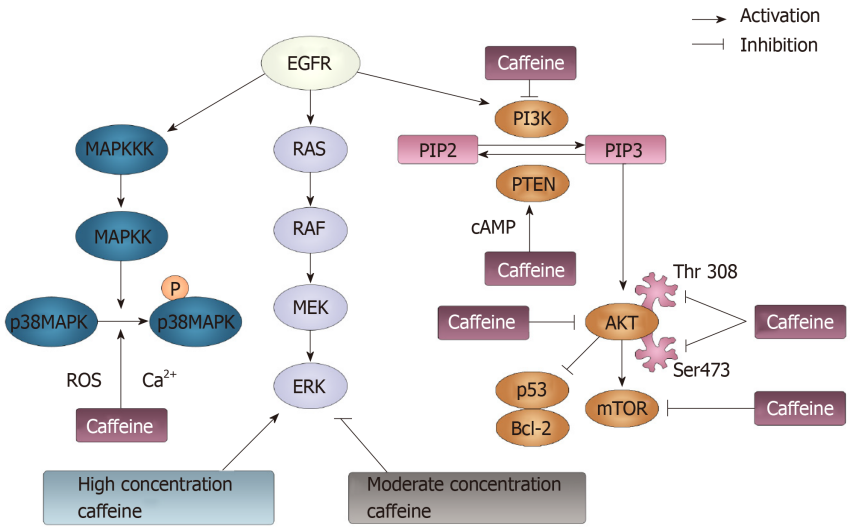

Figure 7 Phosphoinositide-3-kinase, phosphatase and tensin homolog, p38 mitogen-activated protein kinase and RAS pathways modulated by caffeine. Three signaling pathways can be initiated by epidermal growth factor receptor: p38 mitogen-activated protein kinase (MAPK), RAS/RAF/MEK/ERK and PI3K/AKT/ mTOR pathways. p38 MAPK can be phosphorylated following the activation of MAPK kinase kinases and MAPK kinases. Caffeine can inhibit the activation of p38 MAPK though modulating the Ca^{2+} concentration and reactive oxygen species generation. Additionally, caffeine can exert a dual function on ERK, the downstream factor of the RAS/RAF/MEK pathway. At a high concentration, caffeine activates the ERK pathway. At a moderate concentration, caffeine shows an inhibitory effect on the ERK pathway. PI3K can convert phosphatidylinositol-4-phosphate (PIP) 2 to PIP3, and PTEN can reverse this conversion. Caffeine can activate PTEN and inhibit PI3K, in which case PIP3 can be reduced and PIP2 can be increased. Then, PIP3 can activate AKT, through which p53 and Bcl-2 can be activated, and the mTOR pathway can be inhibited. Caffeine can inhibit the phosphorylation of AKT by suppressing its residues Thr308 and Ser473. Moreover, caffeine can directly inhibit mTOR pathways. EGFR: Epidermal growth factor receptor; ERK: Extracellular-signal regulated kinases; mTOR: Mammalian target of rapamycin; MAPK: Mitogen-activated protein kinase; MAPKK: MAPK kinases; MAPKKKs: MAPK kinase kinases; ROS: Reactive oxygen species; PI3K: Phosphoinositide-3-kinase; PIP: Phosphatidylinositol-4-phosphate; PTEN: Phosphatase and tensin homolog.

The immune system plays a crucial role in cancerogenesis, and it can prevent tumor development or the progression of existing neoplasms[120].

General effects of caffeine on cytokines: The development, growth, activation and functions of innate and adaptive immune cells are controlled largely by cytokines, and their effects on tumor-associated immune cells are extremely influential[121]. Caffeine shows different effects on cytokines at different concentrations. Once caffeine reaches therapeutic levels, preferential blockade of A1R increases cAMP accumulation, thereby decreasing cytokine production. However, because caffeine is a non-specific AR antagonist, a higher concentration also blocks A2Rs, thereby decreasing cAMP and increasing pro-inflammatory cytokine transcription. Thus, it can reverse the anti-inflammatory effect that is observed at a lower concentration[122].

TNF-α is a well-known pro-inflammatory cytokine that plays important roles in various cellular events, such as cell proliferation, differentiation, cell death, inflammation and carcinogenesis[121]. Increased levels of TNF-α are associated with metastatic disease in several cancer types including CRC[123]. In an *in vitro* study, caffeine at a concentration of 50 μM in culture was shown to attenuate TNF-α secretion by blocking A1R on lipopolysaccharide-activated human cord blood monocytes[124], which is mediated by its effect on the cAMP/protein kinase A pathway[125].

Moreover, caffeine produces transcriptional downregulation of IL-10, which may result from lipopolysaccharide-induced upregulation of A2R expression by caffeine[122]. IL-10 is a potent anti-inflammatory cytokine secreted from T helper cells, monocytes, macrophages, dendritic cells and a myriad of immune effector cell types including B cells, cytotoxic T cells, NK cells, mast cells and granulocytes like neutrophils and eosinophils[126], which have significantly elevated expression in metastatic colon adenocarcinoma compared with primary colon adenocarcinoma tumors[127]. In CRC cells, the secretion of IL-10 was shown to suppress anti-tumor inflammatory effects by inhibiting T cell–mediated systemic immunity[128]. Besides, IL-10 can cause down-regulation of pro-inflammatory cell signaling pathways like the NF-KB pathway, suppressing Th1 cell activation[129].

Furthermore, caffeine has also been reported to suppress lymphocyte function by reducing the production of IL-2 and IL-4, which is mediated by RyR[130]. The expression of IL-4 and the IL-4 receptor is involved in the process of local metastasis in CRC[131]. IL-2 is a cytokine that is essential for T-cell proliferation[132] and has emerged as a key cytokine in regulating the survival, proliferation and differentiation of activated T cells and NK cells through activating the key transcription factor signal transducer and activator of transcription 5[133]. The production of IL-2 is mainly regulated at the transcriptional level through multiple transcription factors, and the nuclear factor of activated T cells has been reported to bind to several motifs within the IL-2 promoter[134]. In HL-60 cells, caffeine is also reported to downregulate IL-2 receptor expression. In which case, decreased IL-2 and decreased membrane-bound IL-2 receptor can decline the enhancement effect of monocyte production of superoxide and hydrogen peroxide[135]. Furthermore, caffeine can almost completely inhibit the concanavalin A-stimulated increase of the expression of IL-2 and interferon (IFN)-γ in cells[136]. Endogenous IFN-γ acts as a rate-limiting factor in the development of adenomatous polyposis coli-mediated CRC[137] (Figure 8).

General effects of caffeine on lymph cells: Caffeine interacts with lymph cells mainly by sensitizing calcium channels, inhibiting phosphodiesterases, stimulating release of adrenal hormones and binding to adenosine receptors. Calcium signaling plays important roles in various cell types including lymphocytes, where it has been shown to be essential for both the activation and effector phases[138]. In T lymphocytes, the intracellular Ca^{2+} concentration increases within seconds of T-cell antigen-receptor stimulation and initiates the synthesis and secretion of IL-2[132]. Furthermore, in B lymphocytes, pro-inflammatory transcriptional regulators, like NF-KB and JNK, were found to be selectively activated by a large, transient intracellular calcium increase, while the regulation of nuclear factor of activated T cells was activated by a low and sustained intracellular calcium level. Caffeine alters intracellular calcium signaling in naive and primary lymphocytes *via* the RyR-mediated pathway (RyR-3 in T lymphocytes and RyR-1 in B lymphocytes) and IP3-induced Ca^{2+} release[136,138]. Additionally, pre-treatment with caffeine can also reduce the concanavalin A-induced rise in cytosolic calcium in lymphocytes[136].

In T cells, cAMP is known to be a potent negative regulator, which inhibits T-cell antigen-receptor signaling and T-cell activation[139]. In addition, regulatory T-cells mediate their suppressive action by acting directly on conventional T-cells or dendritic cells. One possible mechanism of regulatory T-cell suppression is by increasing the cAMP levels in target cells[140]. Moreover, prolonged elevation of the intracellular cAMP concentration leads to the inhibition of proinflammatory cytokine production and NK cell-mediated cytotoxicity[141]. T and NK cells express both AR (T cells, A2A, A2B and A3; NK cells, A1, A2A, and A2B) and β2-adrenoreceptors, with the density of these receptors increasing following activation[63]. Caffeine might modify the intracellular levels of cAMP in a number of ways including AR antagonism, catecholamine stimulation and PDE inhibition[63,64].

A2AR modulates intracellular cAMP accumulation *via* coupling to a heterotrimeric Gs-protein, which stimulates AC and causes an increase in cAMP[142]. ARs also seem to be involved in the regulation of T-cell receptor-triggered activation-related events, such as antibody production, cell proliferation, IL-2 production, upregulation of the IL-2 receptor α-chain and lymphocyte-mediated cytolysis[143]. Cytokine production can be affected *via* AR. Upregulated A2BARs are functional and elicit a significant reduction in IL-2 production[143]. Additionally, activation of both Th1 and Th2 cells during the early and late stages of lymphocyte activation can be inhibited strongly by activated A2AR. These inhibitory effects can also be extended to other inflammation-inducing Th subsets such as Th17 cells[144,145]. Moreover, differentiation from CD4+ T cell towards regulatory T-cells can be promoted by activated A2AR, probably due to an increase in TGF-β and a decrease in the IL-6 level following A2AR activation[144]. Furthermore, NK cells can also be modulated by caffeine in an AR-dependent manner and exert dual functions. For one thing, the activation of NK cells can be increased *via* A2AR antagonism. For another, its activation can be decreased *via* A1R antagonism and/or increased epinephrine stimulation[146].

Activation of β2-adrenoceptor was also reported as a possible pathway through which caffeine might modify intracellular levels of cAMP[63]. The sympathetic nervous system is able to modulate immune functions *via* adrenoceptor such as β2-adrenoceptor[147]. Norepinephrine released during stress responses is one of the primary catecholamines of the sympathetic nervous system that can be stimulated by caffeine[63,148]. Moreover, mediated by β2-adrenoceptor, norepinephrine induces inflammatory cytokine production while simultaneously reducing the production of growth-related cytokines, leading to reduced activation-induced expansion of memory CD8+ T cells[148]. Furthermore, β2-adrenoceptor signaling plays the greatest

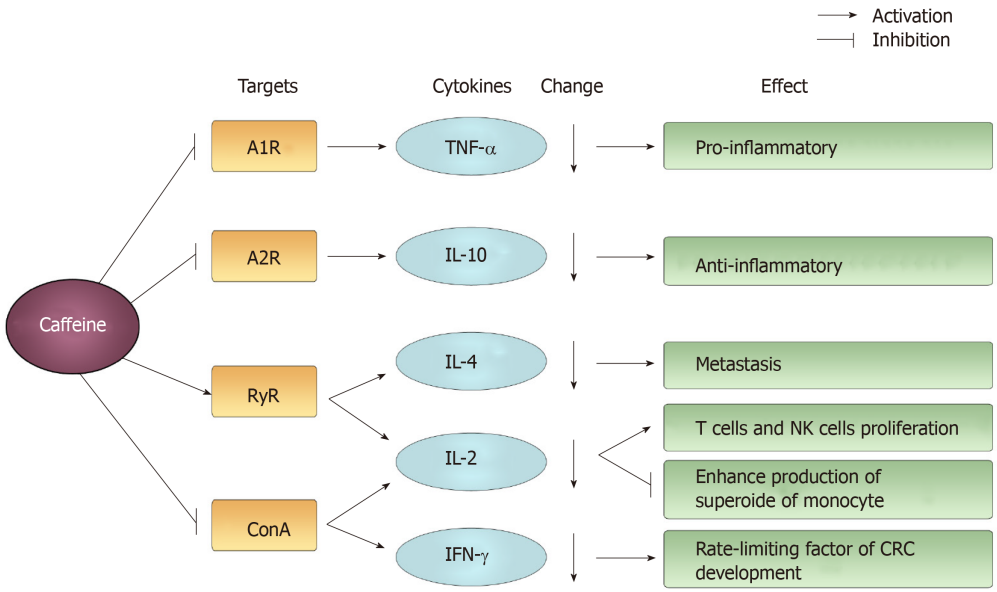

Figure 8 The main targets and changes induced by caffeine acting on cytokines and their effects. Examples of mediated targets are A1R, A2R, ryanodine receptor and concanavalin A. Caffeine induces a decline in cytokines, such as tumor necrosis factor-alpha, interleukin (IL)-10, IL-4, IL-2, interferon-γ and exerts a differential effect. RyR: Ryanodine receptor; ConA: Concanavalin A; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; IFN: Interferon.

role in the mobilization of many subtypes of CD8+ T cells (*e.g.*, central memory, effector memory and the terminally differentiated cells) and NK cells to the bloodstream[149].

GENERAL EFFECTS OF CAFFEINE ON NON-SPECIFIC IMMUNE RESPONSE

Cases of CRC have high macrophage infiltration compared with adenomatous colon polyps. Macrophage infiltration significantly increases in parallel with clinical stage and lymph node metastasis however not with the histologic tumor grade[150]. Macrophages are key cellular components of the innate immunity, acting as the main player in the first-line defense against the pathogens and modulating homeostatic and inflammatory responses[151]. One of the types of receptors on macrophages is the toll-like receptors (TLRs), which recognize pathogen-specific associated patterns and play a crucial role in initiating the innate inflammatory signaling cascade[152]. Adult monocytes express TLR1, TLR2 and TLR4. When exposed to caffeine, lipopolysaccharide-activated cord blood monocytes inhibit TLR1 and TLR2 and the induction of TLR4 expression[124]. *Via* influencing TLR, caffeine can exert dual functions on the non-specific immune response. On the one hand, it may inhibit TLR-mediated inflammatory cascades in macrophages by suppressing calcium mobilization. On the other hand, it may also trigger inflammation by preventing the AR-mediated antagonism of TLRs and perhaps by changing their expression[124].

Macrophages exist in two distinct polarized states: The classically activated state is activated by Th1 cytokines and possesses anti-tumor activity; the alternatively activated state is activated by Th2 cytokines and promotes tumor invasion and metastasis[150]. When treated with caffeine, the conditioned medium of mesenchymal stem cells can potentiate the transformation of macrophages toward an anti-inflammatory phenotype by preserving the activity of macrophages, increasing phagocytosis, reducing the production of potentially harmful ROS and NO by macrophages and decreasing inflammatory cytokine IL-12[153]. At a concentration of 1 mM, caffeine interferes with the activity state and viability of macrophages[153]. At low concentrations (< 5 nM), caffeine prevents the apoptosis of macrophages, whereas at moderate concentrations (5–20 nM), caffeine induces apoptosis in macrophages[154] (Figure 9).

Effect on the microbial content in the gut

The human intestine serves as a host for the densest population of microorganisms in the body with over 10^{11} microbes/mL by intestinal volume[59]. Many studies have recognized caffeine as having an antimicrobial effect. Different mechanisms have been

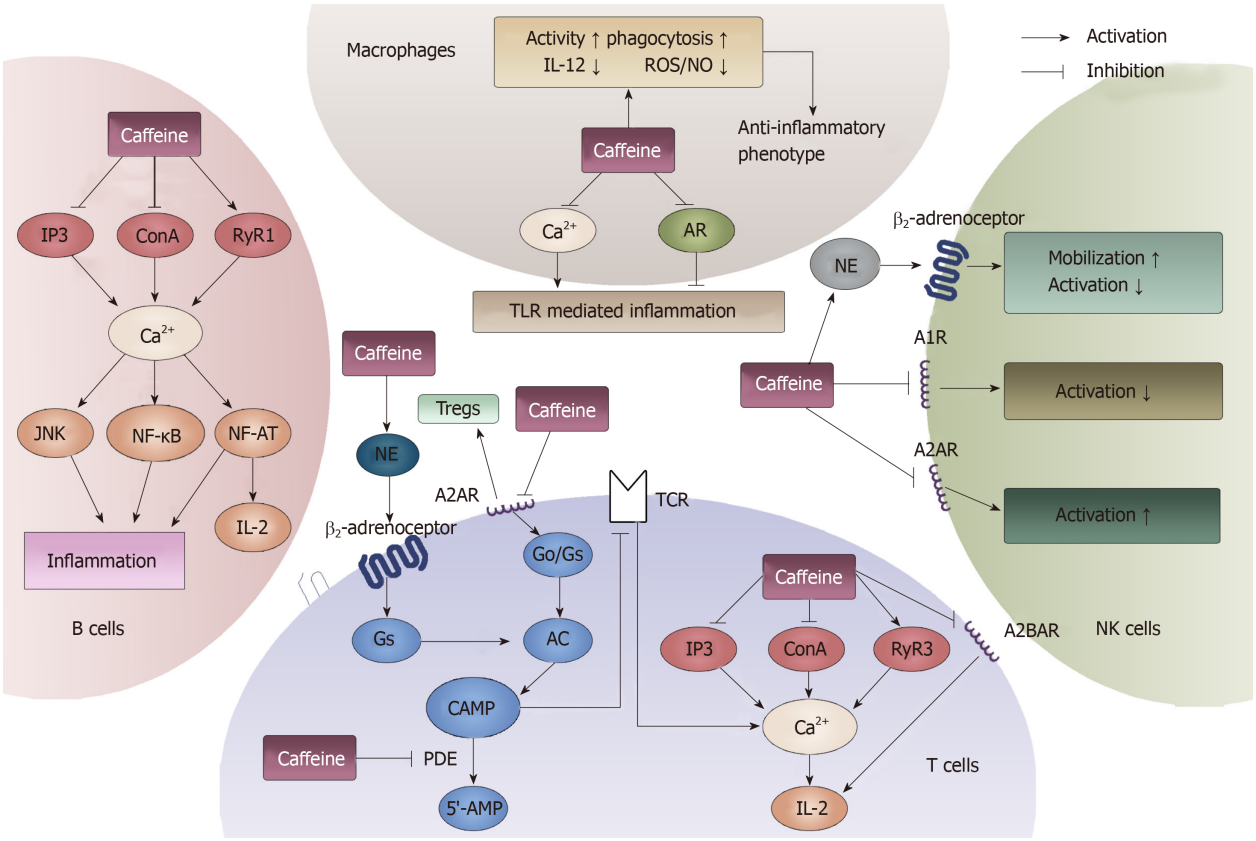

Figure 9 General effects of caffeine on lymphocytes and macrophages. Four types of immune cells can be influenced by caffeine. In T cells, cAMP and Ca^{2+} can be modulated by caffeine through the binding of adenosine receptors, modulating norepinephrine and inhibiting phosphodiesterase. In B cells, caffeine can influence the immune response through interacting with Ca^{2+}. In macrophages, caffeine can both inhibit and activate toll-like receptor-mediated inflammation by modulating Ca^{2+} and binding to adenosine. Furthermore, in natural killer cells, caffeine exerts a dual function on its activation. When it acts on A1R, it inhibits. When it acts on A2R, it induces. Moreover, by stimulating norepinephrine , caffeine can promote the mobilization of natural killer cells. AR: Adenosine receptors; NE: Norepinephrine; PDE: Phosphodiesterase; TLR: Toll-like receptor; NK: Natural killer; IL: Interleukin.

mentioned for the antibacterial activity of caffeine such as inhibiting the incorporation of adenine and thymidine in the synthesis of DNA *via* inhibiting thymidine kinase and DNA synthesis and increasing the sensitivity of bacterial and human cells to different antibiotics[155,156].

Under normal conditions, *E. coli*, as part of the intestinal flora, coexists harmoniously with its host, which promotes normal intestinal homeostasis and rarely causes disease. However, some pathogenic strains have acquired the ability to induce chronic inflammation and/or produce toxins, such as cyclomodulin, which can participate in the carcinogenesis process[157]. There is a statistically significant relationship between high levels of mucosa-associated *E. coli* and poor CRC TNM stages[157]. In an *in vitro* study, caffeine was reported to inhibit the activity of *E. coli* K12 strains likely through its interaction with UmuC, a gene that is regulated by the bacterial DNA repair pathway, and the inhibition of translesion synthesis[158]. Moreover, caffeine induced-replication errors of *E. coli* can result in frameshift mutations[159]. In addition, an *in vivo* study indicated that the percentages of *Blautia*, *Coprococcus* and *Prevotella*, which have been implicated in inflammation, changed significantly in Tsumura Suzuki obese diabetes mice treated with caffeine or coffee[160]. Furthermore, caffeine can also influence the communication between bacteria as a potential quorum sensing inhibitor. Quorum sensing is a form of cell-cell communication system for bacteria. It enables bacteria to control gene expression in response to the cell density. It regulates a variety of bacterial physiological functions such as biofilm formation, bioluminescence, virulence factors and swarming, which have been shown to contribute to bacterial pathogenesis[161].

Additionally, caffeine has shown antibacterial properties along with potent antifungal activity against *Candida albicans*[162]. Secreted aspartic proteases are considered key virulence factors of *Candida albicans*, and the level of *SAP7* expression correlates with the importance of this gene for the early stage of Caco-2 CRC intestinal tissue invasion[163]. Apart from its direct effect on the microbiome, caffeine also indirectly acts on the microbiome in combination with cell-wall-targeting antibiotics

such as penicillin and cephalosporin, and the combination can yield synergistic effects that might be due to the antibiotics facilitating the diffusion of caffeine into microorganisms and therefore allowing better interaction with DNA[155]. Moreover, by increasing the antimicrobial actions of carbenicillin, ceftizoxime, and gentamicin, caffeine can enhance their inhibitory effects on *Staphylococcus aureus* and *P. aeruginosa*[162]. However, caffeine may have an inverse effect by significantly reducing defensins, which induce a decrease in antimicrobial peptides. Antimicrobial peptides in the intestine are produced mainly by intestinal epithelial cells to protect against pathogens and maintain microbiota–host homeostasis[119].

EFFECT ON REGULATING THE CELL CYCLE

The intestinal epithelium is continuously exposed to DNA damaging agents including both exogenous agents, such as radiation and microorganisms, and endogenous agents, such as ROS generated by metabolically active crypt cells[59]. In response to these DNA damaging agents, checkpoint pathways are activated, which can result in stoppage of the cell cycle, allowing DNA repair systems to correct replication errors. If the DNA errors can be repaired successfully, checkpoint signals will be attenuated, and the cell cycle will be restarted. If the DNA damage cannot be repaired properly, the cells' fate may be permanent senescence or apoptosis, or cells will continue to divide with aberrant DNA[164], which causes accumulated genomic instability and may lead to the development of cancer[165]. The cell cycle consists of four distinct phases: the G1 phase, S phase, G2 phase and the mitosis phase. Thus, tightly controlled checkpoints include the G1/S, G2/M, intra-S phase and mitotic checkpoints[166]. The control of mammalian cell cycle division is subject to numerous cyclin-dependent kinase (Cdk)–cyclin complexes. In the early G1 phase of the cell cycle, Cdk4/6–Cyclin D complexes are activated. Subsequently, entrance into and progression through the S phase are regulated by Cdk2–Cyclin E and Cdk2–Cyclin A, respectively, while the onset of mitosis is governed by Cdk1–Cyclin B[166]. It is well understood that caffeine has an effect on cell cycle function by inducing programmed cell death or apoptosis and perturbing key cell cycle regulatory proteins[167]. Additionally, the effects of caffeine on cell growth inhibition and apoptosis appear to be sustained even after caffeine withdrawal for 0–16 h[168].

Tumor suppressor protein p53, a key regulator of the G1/S checkpoint, is regarded as the best-characterized guardian of genomic integrity in the DNA repair process[166,167]. When DNA damage occurs, p53 is phosphorylated by ataxia-telangiectasia mutated and ataxia telangiectasia and Rad3-related (ATR), which results in *p53* stabilization and accumulation[167]. Caffeine has been shown to inhibit the activation of ataxia-telangiectasia mutated and ATR proteins, which results in a decrease in phosphorylated p53 and dysfunction of its target genes[165]. p53 regulates its target genes *p21* and *Bax* to modulate cellular G1 arrest and apoptosis, and then protect them from mutations and genomic aberrations[167]. Bax protein, a Bcl-2 family member, controls cell death through its participation in mitochondria disruption, and subsequently cytochrome c is released[168]. The relative mRNA expression level of *Bax* was found to be higher in CRC cells than in adjacent colon tissue[169]. The translocation of Bax from the cytosol to the mitochondria is a novel step in apoptosis[170], and this event can be promoted by caffeine[171].

In addition, a low concentration of caffeine can induce p53-dependent apoptosis through the Bax and caspase 3 pathways[172]. During p53-dependent apoptosis, when Bax protein enters the cytosol, cytochrome c induces the oligomerization of APAF-1, which recruits procaspase-9. Then, cytochrome c activates procaspase-9 to caspase-9, and caspase-9 converts procaspase-3 to cleaved caspase-3[170,172], which is a primary mechanism of apoptosis[169]. However, this apoptosis pathway can be suppressed by Bcl-2[173]. Therefore, the ratio of Bax/Bcl-2 is an essential index that illustrates the apoptosis progression of tumor cells. Research has demonstrated that caffeine reduces the expression level of Bcl-2, while it does not elevate the expression of Bax, leading to augmentation of the ratio of Bax/Bcl-2[174], which promotes apoptosis.

Additionally, another target, p21 (also known as $p21^{cip1/waf1}$), is a cyclin-dependent kinase inhibitor controlling cell cycle arrest *via* cdk1 and cdk2 inhibition and is a master regulator of multiple tumor suppressor pathways *via* both p53-dependent and independent mechanisms. Moreover, it is a known target gene of TGF-β in CRC[175]. The loss of both the expression and topological regulation of p21 is commonly detected in CRC[176]. Furthermore, researchers have found that caffeine exerts its functions not only through p53-dependent pathways but also through p53-independent pathways[167]. The ATR–Chk1–Cdc25C pathway, which is a p53-independent pathway, has been proven to induce G2/M cell cycle arrest in human

CRC cells[177] (Figure 10).

EFFECT ON REDOX HOMEOSTASIS

Normally, the cellular level of ROS is in balance with the body's natural antioxidant defense system to maintain redox homeostasis. When ROS overproduction occurs or antioxidant function is deficient, a pathological condition called oxidative stress can occur, which ultimately leads to disease development through the oxidation of lipids, proteins and DNA[22,178]. ROS have dual functions depending on their concentration level. A moderate level of ROS leads to cell damage, DNA mutation and inflammation, which promotes the initiation and development of cancer. On the contrary, an excessively high level of ROS induces cancer cell death, showing an anti-cancer role[179]. The activation of oxidative stress-related cell signaling pathways, such as MAPKs and NF-KB, is also involved in the initiation and development of inflammatory bowel disease, which may result in CRC[179].

There is no antioxidant activity present in caffeine at micromolar concentrations[180], while at millimolar concentrations caffeine has significant antioxidant activity, protecting membranes from oxidative damage induced by three of the major reactive oxygen species, namely the hydroxyl radical, peroxyl radical and singlet oxygen[181]. The mechanism of caffeine against ROS is mediated by its reaction with the hydroxyl radical as the most reductant substrate, leading to the formation of a caffeine-derived, oxygen-centered radical, involving carbonyl oxygen at C-6[159]. Also, caffeine can influence other sources of ROS such as the immune cells infiltrated in CRC[121] and the altered gut microbiota composition[178]. Meanwhile, the main metabolites of caffeine, 1-methylxanthine and 1-methyluric acid, are also highly effective antioxidants at physiologically relevant concentrations (40 mmol/L)[180].

OTHERS

Caffeine was reported to induce the inhibition of prostaglandin biosynthesis in rat microglia[182]. Prostaglandin E2 is generated from arachidonic acid by the sequential actions of the cyclooxygenases and terminal synthases. An increased level of COX-2, with a concomitant elevation of prostaglandin E2, is often found in CRC[183]. The suppression of prostaglandin E2 was reported to protect against CRC due to its function in controlling immunoregulatory cell expansion within the colon-draining mesenteric lymph nodes[183]. Evidence is accumulating that folate deficiency is implicated in carcinogenesis, particularly in rapidly proliferative tissues such as the colorectal mucosa. Folate deficiency causes cytogenetic damage in mice, and caffeine acts synergistically with inadequate folate status to augment this damage[184].

CONCLUSIONS

In summary, there is substantial evidence from laboratory, animal, and epidemiological studies suggesting that caffeine can influence the pathogenesis and prognosis of CRC through many aspects. Through antagonizing ARs and GABAARs, inhibiting PDE, sensitizing calcium channels, stimulating adrenal hormones and communicating with signaling pathways such as the TGF-β, PI3K/AKT/mTOR and MAPK pathways, caffeine exerts a broad range of effects, such as modulating the cell cycle, intestinal homeostasis and redox homeostasis. When acting on the cell cycle, caffeine can inhibit ataxia-telangiectasia mutated and ATR, resulting in a decrease in phosphorylated p53 and dysfunction of its target genes p21 and Bax. As for antioxidants, at high concentrations caffeine induces the formation of the caffeine-derived oxygen-centered radical, which results in a decrease in ROS and protection from cell damage, DNA mutation and inflammation. Moreover, it can not only affect immune cells like T and B lymphocytes, NK cells and macrophages, but it can also affect cytokines, such as TNF-α and IL-2, which have a variety of functions. Furthermore, caffeine can also directly and indirectly act on the gut microbiome, which plays an important part in CRC formation.

Overall, the majority of studies have consistently expressed the opinion that caffeine has a protective effect on CRC. However, because caffeine-containing drinks are difficult to standardize (filtered or unfiltered, coffee bean roasting level, brewing method and species of Coffea beans), epidemiological studies in humans cannot draw consistent conclusions, which indicates the need for additional high-quality studies, preferably prospective, interventional and randomized, in order to further investigate

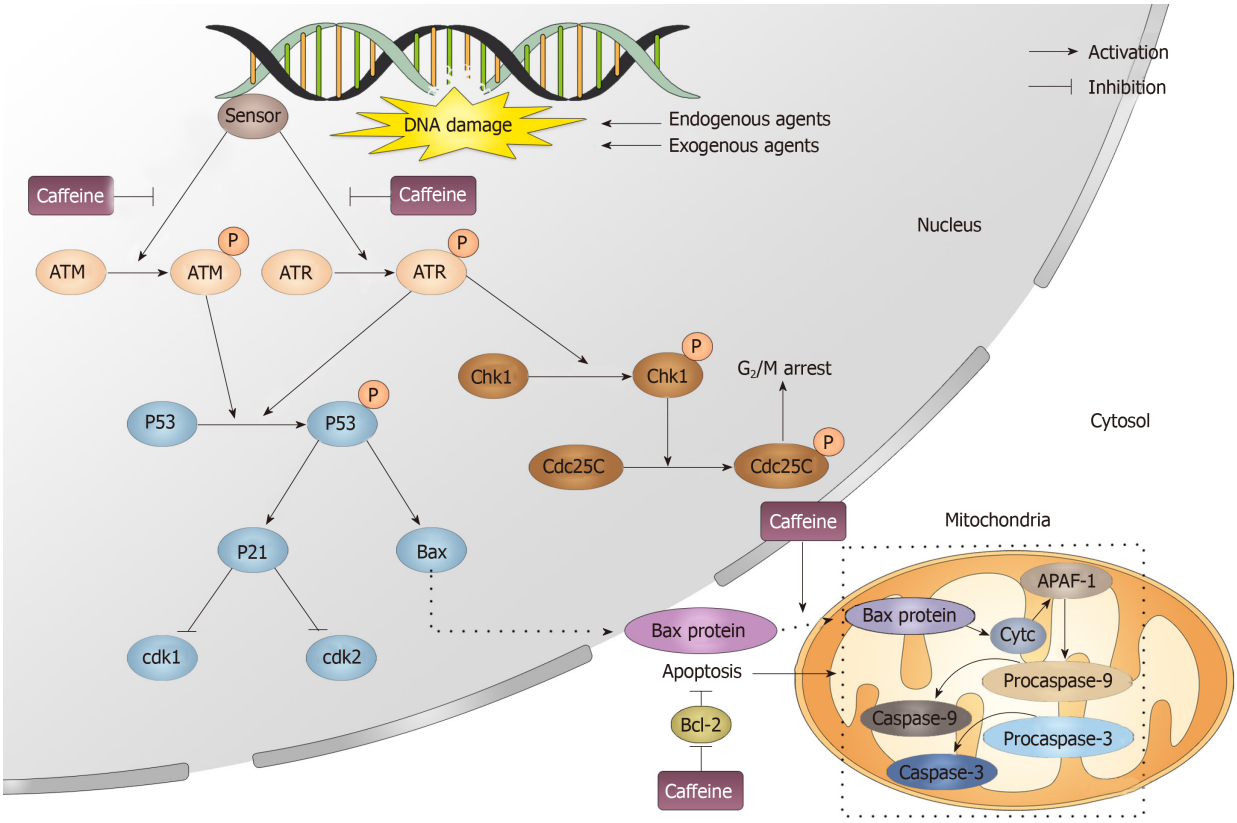

Figure 10 Caffeine influences the DNA repair process. When exogenous and endogenous agents attack DNA, DNA can be damaged, which immediately activates the DNA repair process. In this process, a sensor detects the damage and then causes the phosphorylation of ataxia-telangiectasia mutated and ataxia telangiectasia and Rad3-related. Caffeine can inhibit the activation of both ataxia-telangiectasia mutated and ataxia telangiectasia and Rad3-related. Phosphorylated ataxia telangiectasia and Rad3-related and ataxia-telangiectasia mutated can activate cyclin-dependent kinase 1, which induces G2/M arrest and p53. p53 then modulates its downstream target p21, which can influence the cell cycle by inhibiting Cdk1 and Cdk2. Moreover, Bax is also downstream of p53, and when it enters the cytosol, it can initiate the apoptosis process. Caffeine can promote apoptosis by inhibiting the translocation of Bax from the nucleus to the mitochondria and also by promoting the apoptosis inhibitor Bcl-2. ATM: Ataxia-telangiectasia mutated; ATR: Ataxia telangiectasia and Rad3-related; Chk: Cyclin-dependent kinase.

the relationship and exact mechanism of caffeine's effects on CRC.

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2020 February 15; 12(2): 173-181

DOI: 10.4251/wjgo.v12.i2.173

ISSN 1948-5204 (online)

MINIREVIEWS

Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvironment and immunotherapy

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Author contributions: Sarantis P, Koustas E and Papadimitropoulou A have equal contribution; Sarantis P, Koustas E and Papadimitropoulou A made substantial contributions to acquisition, analysis and interpretation of data; Sarantis P, Koustas E, Papadimitropoulou A, Papavassiliou AG and Karamouzis MV made substantial contributions in the conception, design and interpretation of the data; Papavassiliou AG and Karamouzis MV made substantial contributions in drafting the manuscript and revising it critically for important intellectual content.

Conflict-of-interest statement: No potential conflicts of interest.

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal diseases, with an average 5-year survival rate of less than 10%. Unfortunately, the majority of patients have unresectable, locally advanced, or metastatic disease at the time of diagnosis. Moreover, traditional treatments such as chemotherapy, surgery, and radiation have not been shown to significantly improve survival. Recently, there has been a swift increase in cancer treatments that incorporate immunotherapy-based strategies to target all the stepwise events required for tumor initiation and progression. The results in melanoma, non-small-cell lung cancer and renal cell carcinoma are very encouraging. Unfortunately, the application of checkpoint inhibitors, including anti-CTLA4, anti-PD-1, and anti-PD-L1 antibodies, in pancreatic cancer has been disappointing. Many studies have revealed that the PDAC microenvironment supports tumor growth, promotes metastasis and consists of a physical barrier to drug delivery. Combination therapies hold great promise for enhancing immune responses to achieve a better therapeutic effect. In this review, we provide an outline of why pancreatic cancer is so lethal and of the treatment hurdles that exist. Particular emphasis is given to the role of the tumor microenvironment, and some of the latest and most promising studies on immunotherapy in PDAC are also presented.

Key words: Pancreatic ductal adenocarcinoma; Tumor microenvironment; Immunotherapy; Gemcitabine; Treatment; Cancer stem cells

Manuscript source: Invited manuscript

Received: March 29, 2019
Peer-review started: April 3, 2019
First decision: November 11, 2019
Revised: November 28, 2019
Accepted: December 13, 2019
Article in press: December 13, 2019
Published online: February 15, 2020

P-Reviewer: Lin JM, Mohamed SY
S-Editor: Dou Y
L-Editor: A
E-Editor: Qi LL

Core tip: Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal malignancies. Treatments such as surgery, radiation, and chemotherapy have limited efficacy due to the extensive heterogeneity of genetic mutations and the dense stromal environment, among other causes. In recent years, immunotherapy has been successfully applied in the treatment of various types of cancers, and immunotherapy combined with the above treatments could create more favorable conditions for the fight against PDAC.

Citation: Sarantis P, Koustas E, Papadimitropoulou A, Papavassiliou AG, Karamouzis MV. Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvironment and immunotherapy. *World J Gastrointest Oncol* 2020; 12(2): 173-181
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/173.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.173

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive lethal malignancy due to the lack of early diagnosis and limited response to treatments. It is the most prevalent type of pancreatic neoplasm, and it is developed in the exocrine compartment and accounts for more than 90% of pancreatic cancer cases. Despite scientific progress on the elucidation of PDAC tumor biology and the development of novel therapeutic regimes, it has an average 5-year survival rate of less than 10%[1] and is anticipated to become the second leading cause of cancer–related mortality by 2020. Almost 60%-70% of PDAC cases arise from the head of the pancreas, and these cases are usually diagnosed earlier than tumors arising from the body and tail, as the head of the pancreas contains the common bile duct[2]. Tumors of the body and tail are associated with a worse prognosis[3]. Weight loss, abdominal pain, and jaundice[4] are the most common symptoms observed in patients with PDAC, while less common symptoms include new-onset type 2 diabetes[5] and thromboembolic disease[6]. Classical treatments such as chemotherapy, surgery and radiation have been widely used, but they have not exhibited any significant improvements in clinical outcomes[7,8]. The overall survival for metastatic pancreatic cancer remains poor, and less than 20% of patients survive past the end of the first year[9]. Surgical resection and chemotherapy (gemcitabine and FOLFIRINOX, a combination of oxaliplatin, irinotecan, fluorouracil, and leucovorin) have managed to improve survival of patients with early-stage pancreatic cancer, but these treatments are not sufficient for patients with late stages of the disease[10]. Novel immunotherapies have provided promising results in various solid tumors, such as melanoma or renal cell carcinoma, in a number of cases surpassing chemotherapy as a first-line therapeutic selection[11]. Although immunotherapy began a new era in the field of cancer treatment, it is challenging in the context of PDAC as this type of cancer has a nonimmunogenic, immune-suppressive and therapy-resistant microenvironment.

TREATMENT HURDLES

PDAC development is associated with a poor prognosis due to its complicated and multifactorial nature. There is a lack of simple, early detection methods and is typically diagnosed at a late stage because symptoms do not appear until the disease has progressed and metastasized to distinct sites[12]. As mentioned above, surgical resection with chemotherapy provides the best treatment option for PDAC and is beneficial in patients whose cancer cells have not spread to critical abdominal vessels and adjacent organs[13].

The major difficulties in treating pancreatic cancer lie at both the genetic and cellular levels. The extent of mutational changes in pancreatic tumors generates gene instability that appears to play an essential role in PDAC tumor growth and resistance to treatments. PDAC is characterized by considerable genetic heterogeneity not only among patients but also within a single primary tumor. Targeted treatments are effective in cancers that have a relatively high percentage of patients with the same cancer-causing mutation, such as *EGFR* in lung cancer[14] or *BRAF* in melanoma[15].

Pancreatic cancer, on the contrary, presents a variety of mutations that lead to cancer, and each mutation is present in a small percentage of patients[16].

The presence of multiple signaling pathway alterations could partially explain the presence of multiple resistance mechanisms. Although the underlying biology of PDAC has not been fully elucidated, key mutations of specific genes such as *Kras*, *CDKN2A/p16*, *TP53* and *SMAD4* and the concomitant activation of downstream signaling pathways appear to play an essential role in the resistance to treatments[17]. Additionally, the existence of cancer stem cells (CSCs) contributes to the acquisition of a more resistant tumor state. Pancreatic CSCs account for 0.5%-1.0% of all pancreatic cancer cells[18]; CSCs have an increased capacity for self-renewal and exhibit unique metabolic, autophagic and chemoresistance properties that allow them to escape any therapeutic interventions. CSCs are considered tumor-initiating cells that are able to promote tumor development and therapy resistance, leading to disease progression and relapse. One more reason why current treatment fails to exhibit considerable efficacy and beneficial clinical outcomes is that they do not adequately target CSCs[19].

Furthermore, the metastatic potential of PDAC is also responsible for the poor outcome and the lack of effective treatment modules. Recently, genomic and proteomic analyses in the primary PDAC tumor have revealed subclones with different metastatic potentials[20] and probably different responses to specific therapeutic regimes. Additionally, PDAC metastasizes microscopically early in the disease course, limiting the effectiveness of local therapies such as surgery and radiation[21].

Finally, multiple studies have demonstrated that components within the PDAC microenvironment are responsible for poor prognosis and the difficulty in establishing efficacious therapeutic strategies[22-24]. The tumor microenvironment (TME) is characterized by dense desmoplasia and extensive immunosuppression. Extensive desmoplasia results in decreased stromal vascularization, altered immune cell infiltration and hypoxia, inducing tumor growth and hindering drug activity[25].

TUMOR MICROENVIRONMENT

As mentioned above, the PDAC microenvironment is characterized by increased desmoplasia and the presence of several noncellular components, such as hyaluronic acid, and various cell types, such as cancer-associated fibroblasts (CAFs), pancreatic stellate cells (PSCs), muscle fibroblasts and immune cells. Cellular components account for 10%-30%, but the stroma generates most of the tumor mass[26]. The PSC and CAF components are the dominant cells of pancreatic cancers that produce the extracellular matrix in the TME[27]. These components are responsible for the generation of a rigid barrier that results in elevated tumor pressure, diminished vascularization and attenuated drug delivery. Conventional drugs, such as gemcitabine, cannot penetrate the rich and thick layer of the stoma in PDAC and result in drug resistance[28]. Targeting stroma has demonstrated contradictory results among preclinical studies. A study by Olive *et al*[29] in mouse models showed that inhibition of Sonic Hedgehog-dependent desmoplasia increased gemcitabine delivery and overall survival, while other studies exhibited results contradictory to those of conditional Shh ablation; however, Shh inhibition diminished stroma formation, induced a more aggressive phenotype and decreased survival[30,31]. Additionally, the limited availability of oxygen in the PDAC microenvironment and the minimal vascularization detected were identified as promising targets for therapy. However, clinical trials focused on VEGF-A inhibition combined with chemotherapy did not have the anticipated results. The dense ECM provoked elevated intratumoral pressure that negatively regulated vasculature and diffusion. This phenomenon was reversed with the use of hyaluronidase, but it had a limited beneficial effect because of the increased risk for thrombus[32]. In addition, the extensive immune suppression observed in PDAC comes as a result of the coordinated action of regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs) and macrophages, which block $CD8^+$ T cell duties in tumor recognition and clearance.

In recent years, the impact of the TME on chemotherapy has become the target of many studies. Chemotherapy can induce immunogenic cell death in certain tumors, which could activate the immune system. Gemcitabine can affect the TME through the inhibition of the expansion of MDSCs and the induction of T2H cells, which leads to the polarization of M2 polarized TAMs[33]. Furthermore, other chemotherapeutic drugs, such as cisplatin or carboplatin, have been identified as inducers of IL-6 and prostaglandin E2 and IL-10-producing M2 polarized TAMs[34].

A highly heterogeneous subpopulation of cells is a characteristic of pancreatic cancer. This complex structure of cancer cells and stromal and immunosuppressive

cells consequently alters the effect of immunotherapy. The predominant cell types in the PDAC TME are MDSCs, Tregs and macrophages[35]. Furthermore, several other cell types have also been identified in the PDAC TME, such as fibroblasts, ECM, and PSCs; there is also a high ratio of Treg/Teffs. The accumulated population of T cells in the TME leads T cells to exhaustion during an immune response.

Moreover, approximately 50% of PDAC tumors are characterized by the invasion of MDSCs and the upregulation of PD-L1 through IFN-γ[36]. Thus, PDAC tumors establish an immunosuppressive environment[37,38]. In more advanced tumors, several studies have identified that Tregs and Teffs inhibit the normal function of T cells and enhance the immunosuppressive environment of the TME[39].

Several studies also underline the lack of recognition by T cells of cancer antigens through the degradation of downregulation of major histocompatibility complex I in cancer cells. Furthermore, a mutation in the IFN-receptor 1 or 2 gene increases immune suppression in TME and helps cancer cells escape the T cell antitumor response[40]. Moreover, two phenotypes, often called "cold" and "hot" tumors, are categorized based on the degree of immune infiltration of T-lymphocytes[41]. Hot tumors are characterized by a variation in CD8+ and Tregs in response to immunotherapeutic drugs, and cold tumors, in the early stage of tumorigenesis, show a 20%-40% response to immune checkpoint inhibitors[37,42].

IMMUNOTHERAPY

PDAC is a disease with increased heterogeneity and exhibits unique immunologic hallmarks. The principal basis of cancer immunotherapy is to activate a patient's T cells so that they can kill their tumors. The key steps are briefly described as follows: (1) Decrease of tumor-specific antigen presentation; (2) Activation of T cells; (3) Infiltration of T cells into tumors; (4) Recognition of cancer cells by T cells; and (5) Elimination of cancer cells[43]. There are several types of cancer immunotherapies, such as monoclonal antibodies, adoptive cell transfer[44-46], cancer vaccines[47,48], immune checkpoint inhibitors[49], and immune modulators, all currently tested in clinical trials for the determination of their efficacy. Promising results have been demonstrated after the administration of inhibitors against two major T cell response checkpoints, ipilimumab (anti-CTLA-4 IgG1 humanized antibody) and Nivolumab/Pembrolizumab (anti-PD-1), in various immunogenic cancers, such as melanoma and non-small-cell lung cancer[50-52]. CTL-4 binds to its ligands on antigen-presenting cells (APCs) and exerts its immunosuppressive role by reducing T effector cell activation while increasing Treg activity[53].

Similarly, binding of PD-1, which is predominantly expressed on T cells, with its ligands PDL-1/PDL-2, which are found on tumor cells and tumor-infiltrating lymphocytes, results in diminished T cell proliferation and antitumor cytokine release. Despite the encouraging evidence from the aforementioned cancer studies, these treatments exhibited poor efficacy to pancreatic cancer when administered alone. In a phase II study, ipilimumab was not able to induce tumor response in patients with advanced pancreatic cancer, and the anti-PD-L1 monoclonal antibody BMS-93655 had no efficacy in a phase I study[54-56]. The incompetence of these compounds to elicit pancreatic tumor growth inhibition was probably due to the immune quiescence, excessive desmoplasia and the lack of consensus expression of PD-L1 in this type of cancer[57]. Therefore, the incorporation of additional therapies for administration of combinatorial strategies appears to be the ideal approach to achieving the most efficient response. A broad spectrum of clinical trials in pancreatic cancer have been completed or are currently ongoing using immune checkpoint monotherapies, dual checkpoint combination therapies and checkpoint inhibitors combined with vaccines, cytotoxic chemotherapy and other inhibitory agents. Below, there are some examples of the therapeutic strategies followed in these clinical trials: (1) Monotherapies include the administration of various inhibitors against CTL-4 (ipilimumab, tremelimumab) and PD-1 (pembrolizumab, MPDL3280A, MEDI4736), and dual checkpoint inhibition including the combinations of these agents with each other or with other agents such as mogamulizumab (anti-CCR-5); (2) Immune checkpoint inhibitors in combination with chemotherapeutic agents consist of combinations of CTL4 and/or PD-1 inhibitors with conventional chemotherapeutic agents such as gemcitabine, Nab-paclitaxel, FOLFOX, and carboplatin[58-60]. A phase I clinical study investigating the efficacy of gemcitabine and tremelimumab in metastatic pancreatic cancer showed a partial response in some patients, and the disease remained stable for more than ten weeks[61]. In another study of unresectable pancreatic cancer, ipilimumab and gemcitabine combinatorial treatment had similar results[58]. Two clinical pilot studies based on the combination of chemotherapy and

anti-PD-1 antibodies (pembrolizumab and FOLFOX for advanced GI cancer and pidilizumab and gemcitabine for resected pancreatic cancer) were initiated after increased tumor infiltration of $CD8^+$ T cells and complete responses were observed in treated mice[59]; (3) Vaccine immunotherapy is based on the delivery of tumor antigens to APCs and the subsequent induction of an orchestrated immune response. Cancer-specific DNA alterations create neo-antigens, which results in a unique peptide sequence. Vaccine immunotherapies include whole-cell vaccines, DC vaccines, DNA and peptide vaccines, but despite the improved immune profiles, they have shown a poor clinical outcome[48]. The most widely studied vaccine in pancreatic cancer is GVAX, an allogenic irradiated whole-cell tumor vaccine genetically engineered to secrete granulocyte macrophage-colony stimulating factor (GM-CSF) and stimulate cytolytic activity against tumors[62]. In a phase I clinical study, GVAX administration in resectable pancreatic cancer before and after radiotherapy exhibited extended DFS[63], and in phase II clinical studies, GVAX in combination with cyclophosphamide or 5-FU-based chemoradiation demonstrated similar results regarding DFS and MS[64,65]. When combined with the aforementioned immune checkpoint inhibitor ipilimumab in a phase I trial in patients with advanced refractory pancreatic cancer, GVAX resulted in improved survival compared to ipilimumab alone, a fact that was associated with the extensive presence of T cells[66]. Other vaccines targeting KRAS, MUC1, VEGF-R, or survivin alone or in combination with GVAX are also under clinical investigation for the determination of their efficacy[60]; (4) Adoptive T cell immunotherapy is based on the modification of autologous T cells, engineered to express a chimeric antigen receptor (CAR) and stimulate the immune response against the tumor. Despite the impressive results gained by a clinical study utilizing CAR-T technology to target leukemia[67,68], the majority of patients receiving CAR-T cells targeting mesothelin, a membrane antigen overexpressed in pancreatic cancer, showed satisfying tolerance but failed to exhibit a good response[60]. In addition to mesothelin, other cancer-associated antigens are being tested in ongoing clinical trials as potential targets of CAR-T-based therapeutic regimes (anti-CEA, anti-CD-133, anti-ROR1, anti-WT1) alone or in combination with chemotherapy[60]; and (5) Immune modulating agents targeting the dense pancreatic microenvironment could also exert substantial antitumor activity. Promising data have been derived from the use of anti-CD40 agonistic antibodies along with gemcitabine in PDAC patients, where tumor regression was attributed to stromal alterations provoked by the effect of the anti-CD40 antibody[69,70]. Another molecule currently being tested in clinical trials against PDAC is CCR2, a chemokine receptor that mediates the chemotaxis of immune cells. In a phase 1 clinical trial, half of PDAC patients treated with PF-04136309, an inhibitor of CCR2, in combination with FOLFIRINOX, exhibited partial response and stable disease[71].

CONCLUSION

Pancreatic cancer remains a devastating disease with poor prognosis. This is due to factors such as the lack of early diagnostic markers, delayed detection, diverse genetics and rapid metastasis. The extensive TME that grows around the tumor plays crucial roles in this disease. Due to the dense and immunosuppressive TME, the penetrance of therapeutic regimes for the elimination of cancer cells is hindered. The interaction between the microenvironment and cancer cells remains to be further elucidated. However, in recent years, immunotherapy has been successfully applied in the treatment of various types of cancers. Combination therapies have been developed to optimize the clinical outcome and prolong the survival of patients with pancreatic cancer (Figure 1).

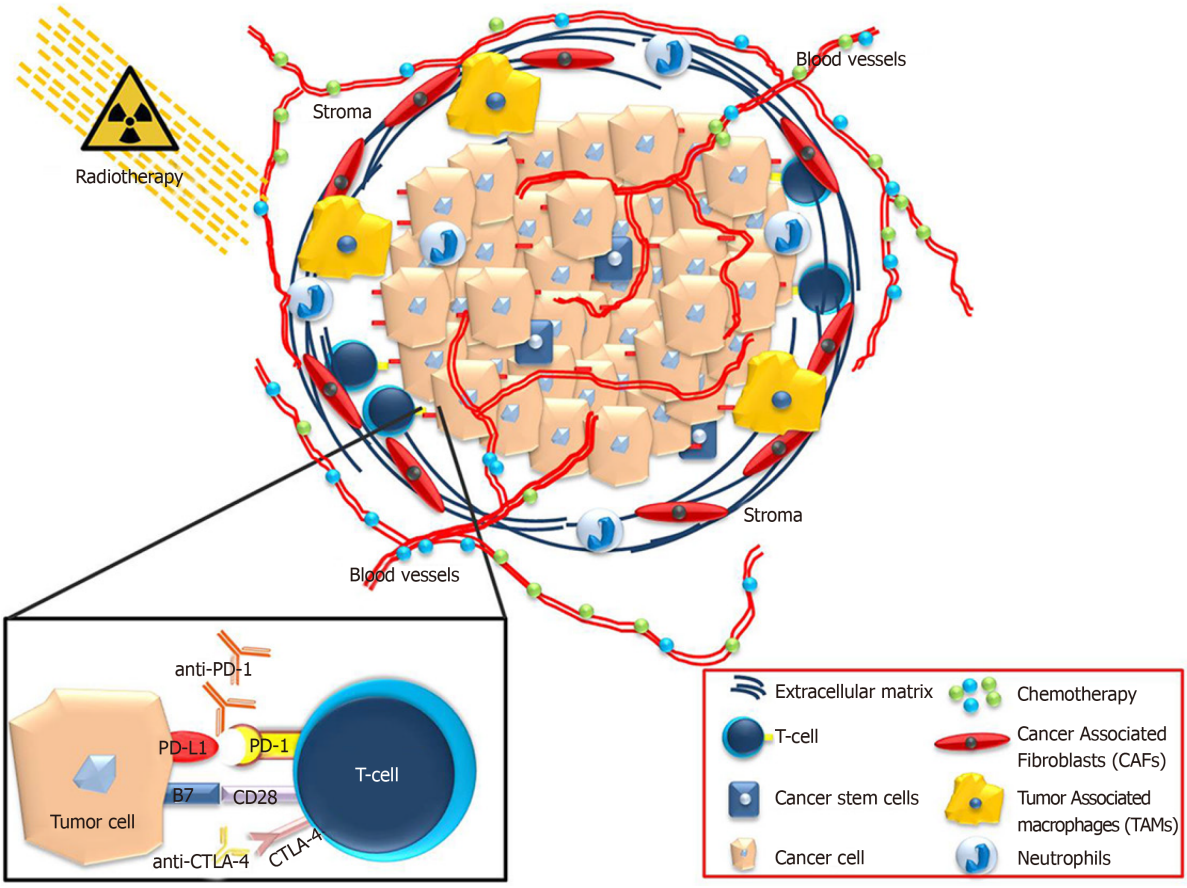

Figure 1 The pancreatic ductal adenocarcinoma microenvironment consists of a significant hurdle for the efficient application of chemotherapy drugs or immunotherapeutic compounds. Combination treatments of chemotherapy, immunotherapy and radiation might render pancreatic ductal adenocarcinoma microenvironment more vulnerable to inhibition and promote effective treatment strategies.

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2020 February 15; 12(2): 182-194

DOI: 10.4251/wjgo.v12.i2.182

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

FOLFIRINOX *vs* gemcitabine/nab-paclitaxel for treatment of metastatic pancreatic cancer: Single-center cohort study

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Author contributions: Cho IR analyzed and interpreted data and wrote the paper; Kang H, Jo JH, Lee HS, Chung MJ performed the data analysis; Park JY interpreted data and performed statistical analysis; Park SW, Song SY interpreted data and supervised study; An C, Park MS analyzed and interpreted radiologic findings; Bang S interpreted data and designed and supervised study.

Institutional review board statement: The study was reviewed and approved for publication by our Institutional Reviewer.

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

Conflict-of-interest statement: All the Authors have no conflict of interest related to the manuscript.

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Abstract

BACKGROUND

FOLFIRINOX and gemcitabine plus nab-paclitaxel (Gem + nabPTX) were recently introduced for metastatic pancreatic cancer treatment. However, studies that compared these two regimens and studies in Asian populations are lacking.

AIM

To compare the treatment outcomes of FOLFIRINOX and Gem + nabPTX regimen for metastatic pancreatic cancer treatment in Korean population.

METHODS

Patients with metastatic or recurrent pancreatic cancer treated with FOLFIRINOX (n = 86) or Gem + nabPTX (n = 81) as the first-line since January 2015 were identified using the Severance Hospital Pancreatic Cancer Cohort Registry. Treatment efficacy, treatment-related adverse events and economic aspects were compared.

RESULTS

Patients in the FOLFIRINOX group were significantly younger (54 *vs* 65 years; P < 0.001) and had better performance statuses at diagnosis. The median overall survival (10.7 *vs* 12.1 mo; P = 0.157), progression-free survival (8.0 *vs* 8.4 mo; P = 0.134), and objective response rates (33.7% *vs* 46.9%; P = 0.067) were not

STROBE statement: The authors have read the STROBE Statement–checklist of items, and the manuscript was prepared and revised according to the STROBE Statement–checklist of items.

Manuscript source: Unsolicited manuscript

Received: October 11, 2019
Peer-review started: October 11, 2019
First decision: November 11, 2019
Revised: December 19, 2019
Accepted: December 30, 2019
Article in press: December 30, 2019
Published online: February 15, 2020

P-Reviewer: Aosasa S, Tang Y
S-Editor: Zhang L
L-Editor: A
E-Editor: Qi LL

significantly different when compared with Gem + nabPTX group. Grade ≥ 3 neutropenia and gastrointestinal adverse events were more common in the FOLFIRINOX group. The drug costs of both regimens were similar.

CONCLUSION

Treatment efficacy and economic burdens were comparable between the two regimens. But, the details of adverse event were different. Gem + nabPTX regimen might be considered preferentially in certain conditions.

Key words: Pancreatic cancer; Chemotherapy; FOLFIRINOX; Gemcitabine; Nab-paclitaxel; Survival

Core tip: Both FOLFIRINOX and gemcitabine plus nab-paclitaxel combination therapy are widely used as a treatment of choice in patients with metastatic pancreatic cancer. However, the treatment choice and sequence are not firmly established. In addition, researches on Asian populations in this regard are scarce. In the present study, we compared the treatment efficacy, safety, and economic aspects of FOLFIRINOX and gemcitabine plus nab-paclitaxel combination therapy. We believe that this study can help physicians and patients to select appropriate regimens while avoiding and preventing unnecessary complications.

Citation: Cho IR, Kang H, Jo JH, Lee HS, Chung MJ, Park JY, Park SW, Song SY, An C, Park MS, Bang S. FOLFIRINOX *vs* gemcitabine/nab-paclitaxel for treatment of metastatic pancreatic cancer: Single-center cohort study. *World J Gastrointest Oncol* 2020; 12(2): 182-194
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/182.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.182

INTRODUCTION

Pancreatic cancer demonstrates a very poor prognosis and is one of the main causes of cancer-related death worldwide[1,2]. While the treatment outcomes of other cancers have gradually improved, progress in the treatment outcomes of metastatic pancreatic cancer has remained stagnant[3]. Since the late 1990s, several efforts have been made to treat metastatic pancreatic cancer[4-6]. Recently, two effective regimens were introduced through large-scale clinical trials.

The FOLFIRINOX regimen, which consists of 5-fluorouracil (5-FU), leucovorin, oxaliplatin, and irinotecan, was introduced by the PRODIGE4/ACCORD11 trial[7]. In this clinical trial, FOLFIRINOX yielded superior survival rates when compared to gemcitabine monotherapy. Another randomized phase III trial, MPACT, showed that a combination of gemcitabine and nab-paclitaxel (Gem + nabPTX) yielded a statistically significant survival benefit and response rate when compared with gemcitabine monotherapy[8]. As a result, these two regimens are recommended as the first-line therapy for metastatic pancreatic cancer[9,10].

However, there are two possible impediments when treating patients in a clinical setting. The first is the treatment choice and sequence between the two standard regimens. There is a lack of data regarding a direct comparison of the two regimens in terms of the treatment outcome. In addition, reliable guidelines that help to select the appropriate regimen according to each patient are lacking. The second impediment concerns ethnic differences between western and east-Asian populations. Even though we reported the efficacy and adverse events of Gem + nabPTX in Korean population were similar to the western population, there are still lack of evidences for supporting the results of MPACT trial in Asian countries[11]. In terms of pharmacoethnicity, an understanding of the differences in treatment response and adverse events according to ethnicity helps to improve chemotherapeutic tolerability and effectiveness[12].

Therefore, the purpose of this study was to compare the efficacy, safety, and economic aspects of FOLFIRINOX and Gem + nabPTX in the treatment of metastatic pancreatic cancer in Korean population.

MATERIALS AND METHODS

Study population

Patients with metastatic or recurrent pancreatic cancer who were treated with FOLFIRINOX or Gem + nabPTX since January 2015 were identified using the Severance Hospital Pancreatic Cancer Cohort Registry, which is a prospectively collected database of pancreatic cancer patients who received anticancer therapy at Severance Hospital since 2015. During the study period, a total of 924 patients were registered in the cohort registry.

The inclusion criteria were as follows: (1) ≥ 18 years of age; (2) Pathologically confirmed metastatic or recurred pancreatic adenocarcinoma; (3) At least one measurable or evaluable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1[13]; (4) Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2; (5) No prior anti-tumor treatment for metastatic or recurred pancreatic adenocarcinoma; and (6) Adequate organ function (absolute neutrophil count ≥ 1.5 × 10^9/L, serum creatinine < 1.5 mg/dL, or calculated creatinine clearance ≥ 60 mL/min per the Cockcroft and Gault formula) before chemotherapy.

Finally, 167 patients who met the enrolment criteria were identified as eligible patients. This study was approved by the Yonsei University Health System Institutional Review Board (Approval number: 4-2015-1058) and conducted in accordance with the principles set forth in the Declaration of Helsinki. Written informed consent was obtained from all patients.

Treatment schedule and response evaluation

In patients who received the FOLFIRINOX regimen, oxaliplatin (85 mg/m^2), leucovorin (400 mg/m^2), and irinotecan (180 mg/m^2) were delivered *via* intravenous infusion, which was followed by 400 mg/m^2 (bolus) and 2400 mg/m^2 (continuous intravenous infusion over a 46-h period) of 5-FU administered every 2 wk. Patients treated with Gem + nabPTX received a slow (over 30–40 min) intravenous infusion of nab-paclitaxel (125 mg/m^2) and gemcitabine (1000 mg/m^2) on days 1, 8, and 15 of a 28-d cycle (every 4 wk). The dose of the chemotherapeutic agent was reduced and/or administration was delayed if serious treatment-related adverse events (AEs) occurred that made treatment intolerable. Chemotherapy was discontinued when life-threatening AEs or disease progression was identified.

At the beginning of treatment, the following tumor-related factors were examined and recorded: Patient demographics, patient body mass index (BMI), date of diagnosis, tumor size and location, location and number of metastases, and laboratory data including levels of carbohydrate antigen 19-9. To evaluate treatment efficacy, computed tomography, magnetic resonance imaging, or 18F-fluorodeoxyglucose-positron emission tomography was performed every 8 wk. All imaging studies were conducted and reviewed according to institutional standard protocols. Treatment responses according to the RECIST criteria were reported by designated radiologists and final disease assessments were independently performed by the responsible physicians.

Assessment of treatment-related adverse events and drug costs

To monitor for treatment-related AEs, the presence of an AE was carefully examined by physicians and registered nurses at each visit during chemotherapy. The category and severity grade of the AEs were precisely recorded in the patients' medical records. Treatment-related AEs were assessed and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0[14].

The anticancer drug cost that was actually paid by the patient was calculated based on the median body surface area (1.61 m^2). The total cost for 4 wk of treatment administration were compared between the two regimens, since each regimen had a different administration protocol per cycle. Then, 4 d of hospital costs per cycle were added to the cost of FOLFIRINOX because these patients were required to be hospitalized during the chemotherapy. When calculating the hospital cost, the cheapest room covered by the Korean National Health Insurance Service (NHIS) was used.

Study endpoints and statistical analysis

The primary endpoints were overall survival (OS) and progression-free survival (PFS). The secondary endpoints were the rate and severity of treatment-related AEs. OS was calculated as the date of diagnosis until the date of the most recent follow-up or death. PFS was computed from the date of diagnosis to disease progression (or the most recent follow-up or death). Object response was defined as complete response or partial response and disease control was defined as complete response + partial response and stable disease according to the RECIST criteria.

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, United States), SAS version 9.4 (SAS Institute, Cary, NC, United States), and R version 3.3.0 (The R Foundation for Statistical Computing, Vienna, Austria). Baseline patient characteristics, laboratory data, and the grade and frequency of AEs were used to calculate descriptive statistics. Student's *t*-tests were used to compare continuous variables and chi-square or Fisher's exact tests were used to compare categorical variables. Survival times and rates were estimated using the Kaplan-Meier method (with log-rank test). Estimated medians with 95% confidence intervals (CI) are reported. A Cox proportional-hazards model was used for the subgroup analysis to estimate the hazard ratios for OS and PFS.

RESULTS

Patient characteristics

The baseline characteristics of all patients are presented in Table 1. The patients who received FOLFIRINOX were significantly younger (54 *vs* 65 years; $P < 0.001$) and had better performance status scores at baseline (proportion of ECOG-PS score 0: 83.7% *vs* 70.4%; $P = 0.040$) than those who received Gem + nabPTX. The most common metastatic sites were the liver and peritoneum. Liver metastasis was more common in the FOLFIRINOX group (66.3% *vs* 49.4%; $P = 0.027$) and peritoneal carcinomatosis was more common in the Gem + nabPTX group (51.9% *vs* 40.7%; $P = 0.148$). There was no difference in the number of metastasis sites between the two groups. In terms of baseline laboratory data, a significantly higher neutrophil count was observed in the FOLFIRINOX group. Other laboratory data, including carbohydrate antigen 19-9, showed no differences between the two groups.

Treatment data and efficacy

The median follow-up period for all patients was 7.9 (range, 1.5–23.4) mo; during this period, 78 (46.7%) patients died and 101 (60.5%) patients experienced disease progression. The treatment data and efficacy of the two groups are presented in Table 2. The median number of chemotherapy cycles received by each patient in the FOLFIRINOX and Gem + nabPTX groups was 8 and 5, respectively. There was no statistically significant difference in the median duration of chemotherapy (FOLFIRINOX, 138 d *vs* Gem + nabPTX, 154 d; $P = 0.249$). The median relative dose intensities of gemcitabine and nab-paclitaxel were 93.3% and 86.2%, respectively. In the FOLFIRINOX group, 80% of the planned dose of 5-FU and 75% of oxaliplatin and irinotecan were administered to patients.

In aspect of efficacy, there was no statistically significant difference in the objective response rate between the two groups ($P = 0.082$). However, the Gem + nabPTX group showed a significantly higher disease control rate than the FOLFIRINOX group (84.0% *vs* 69.8%; $P = 0.030$). The median overall survival was 12.1 mo (95%CI, 10.7- not estimable) in the Gem + nabPTX group and 10.7 mo (95%CI, 9.1–12.3) in the FOLFIRINOX group ($P = 0.157$, Figure 1A). The median progression-free survival was 8.4 mo (95%CI, 5.0–11.8) in the Gem + nabPTX group and 8.0 mo (95%CI, 6.5–9.5) in the FOLFIRINOX group ($P = 0.134$, Figure 1B).

Subgroup analysis

The treatment efficacy was consistently similar in both groups across the majority of subgroups (Figure 2). In patients who had pancreatic body/tail cancer and a BMI > 23, the risk of death significantly reduced with the Gem + nabPTX regimen. Similar trends were observed for PFS according to subgroup. In addition to primary cancer site and BMI, the presence of liver metastasis and carcinomatosis at diagnosis were associated with the hazard ratio of disease progression.

Treatment-related AEs

The treatment-related AEs observed in this study population are shown in Table 3. Notable AEs occurred in both groups. In terms of haematologic AEs, the incidence of severe (grade 3 or more) anemia and thrombocytopenia were similar between the two groups. Both groups demonstrated a notable incidence of severe neutropenia, but the FOLFIRINOX group showed a statistically significantly higher rate of severe neutropenia (74.4% *vs* 46.9%; $P < 0.001$). The granulocyte-colony stimulating factor (G-CSF) administration rate was also significantly higher in the FOLFIRINOX group.

In the Gem + nabPTX group, more than half of the patients (46, 56.8%) showed peripheral neuropathy and 15 (18.5%) developed severe peripheral neuropathy after chemotherapy. On the other hand, the rate of neurologic AEs in the FOLFIRINOX group was significantly lower. The median time to onset of peripheral neuropathy was shorter in the Gem + nabPTX group, but not statistically significant (73.5 *vs* 120 d;

Table 1 Baseline characteristics of all patients, *n* (%)

Characteristics	FOLFIRINOX (*n* = 86)	Gem + Nab-paclitaxel (*n* = 81)	*P* value
Age (yr)	54 (30-78)	65 (42-79)	< 0.001
Male sex	49 (57.0)	37 (45.7)	0.144
ECOG-PS			0.040
0	72 (83.7)	57 (70.4)	
1	14 (16.3)	24 (29.6)	
Body mass index	22.13 (16.49-31.63)	21.97 (16.11-29.59)	0.432
Tumor location[1]			0.398
Head and neck	40 (46.5)	32 (39.5)	
Body and tail	46 (53.5)	48 (49.3)	
Metastasis site			
Liver	57 (66.3)	40 (49.4)	0.027
Lung	9 (10.5)	12 (14.8)	0.397
Bone	4 (4.7)	6 (7.4)	0.526[2]
Peritoneum (carcinomatosis)	35 (40.7)	42 (51.9)	0.148
Distant LN	33 (38.4)	28 (34.6)	0.610
Othersite (*e.g.* adrenal gland)	14 (16.3)	20 (24.7)	0.177
No. of metastasis site			0.726
1 site	39 (45.3)	38 (46.9)	
2 sites	30 (34.9)	24 (29.6)	
3 or more	17 (19.8)	19 (23.5)	
Laboratory data (at diagnosis)			
WBC count (cells/μL)	6765 (2830-21880)	6240 (2580-12240)	0.068
Neutrophil count (cells/μL)	4660 (1610-18930)	4045 (1410-8540)	0.035
Prothrombin time (INR)	1.01 (0.80-1.28)	1.00 (0.83-1.16)	0.176
Total bilirubin (mg/dL)	0.7 (0.2-13.5)	0.6 (0.2-23.4)	0.200
AST (IU/L)	24 (9-204)	22 (9-765)	0.286
ALT (IU/L)	27 (5-192)	17 (5-717)	0.117
Alkaline phosphatase (IU/L)	116 (43-957)	92 (37-2080)	0.798
CA 19-9 at diagnosis (U/mL)	585.3 (3.4-20000)	305.2 (0.6-20000)	0.678

[1]Except for one case that originated from an ectopic pancreas;
[2]Fisher's exact test. Gem + nabPTX: Gemcitabine plus nab-paclitaxel; ECOG-PS: Eastern Cooperative Oncology Group performance status; LN: Lymph node; WBC: White blood cell; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; CA: Carbohydrate antigen.

P = 0.051). In terms of other non-hematologic AEs, the incidences of nausea/vomiting and severe gastrointestinal AEs were higher in the FOLFIRINOX group while the incidence of dermatologic AEs was higher in the Gem + nabPTX group.

Compared to those observed in previous phase-III trials (PRODIGE4/ACCORD11 and MPACT population), the proportions of patients who experienced severe neutropenia and febrile neutropenia were higher in the present study, regardless of the treatment regimen administered. In addition, a larger number of patients in the FOLFIRINOX group showed severe anemia and nausea/vomiting compared to that observed in previous trials, and the incidence of severe fatigue was more than 10% higher in the Gem + nabPTX group when compared with the MPACT population. (Supplemental Table 1).

Dose reduction, delay of administration and cessation of administration

Dose modification, treatment delay and cessation are shown in Table 4. Proportion of patients who experienced dose reduction of chemotherapeutic agent was significantly higher in FOLFIRINOX group patients than Gem + nabPTX group (88.4 % *vs* 60.5%; *P* < 0.001). In the FOLFIRINOX group, most patients experienced dose reduction prior to the 1st response evaluation (68 of 76 patients), whereas in the Gem + nabPTX group, many patients experienced dose reduction after the 1st response evaluation (30 of 49 patients).

Among the FOLFIRINOX group, 47 (54.7%) patients experienced delayed treatment and 12 (14.0%) patients discontinued chemotherapy due to adverse events. In the

Table 2 Treatment data and efficacy of FOLFIRINOX and gemcitabine plus nab-paclitaxel, *n* (%)

	FOLFIRINOX (*n* = 86)	Gem + nabPTX (*n* = 81)	*P* value
Duration of chemotherapy			
Cycles	8 (2-24)	5 (2-16)	
Duration, days	138 (19-551)	154 (32-554)	0.249
Accumulation dose, mg/m^2			
Gemcitabine		14000 (4000-40000)	
Nab-paclitaxel		1562.5 (375-4875)	
5-Fluorouracil	16800 (5600-53200)		
Oxaliplatin	510 (170-1445)		
Irinotecan	1080 (360-3060)		
Relative dose intensity, %			
Gemcitabine		93.3 (54.3-100)	
Nab-paclitaxel		86.2 (22.7-100)	
5-Fluorouracil	80.0 (52.5-100.0)		
Oxaliplatin	75.0 (52.5-100.0)		
Irinotecan	75.0 (52.5-100.0)		
Best response of chemotherapy			0.067
Complete response	0 (0)	0 (0)	
Partial response	29 (33.7)	38 (46.9)	
Stable disease	31 (36.0)	30 (37.0)	
Progression of disease	26 (30.2)	13 (16.0)	
Response rates			
Objective response rate	29 (33.7)	38 (46.9)	0.082
Disease control rate	60 (69.8)	68 (84.0)	0.03

Gem + nabPTX: Gemcitabine plus nab-paclitaxel.

Gem + nabPTX group, 51 (63.0%) and 17 (21.0%) patients experienced treatment delay and discontinuation respectively. The proportion of treatment delay and cessation were not statistically different between both groups. The most common cause of delayed treatment was hematologic AE, and general weakness was the most common cause of early-termination of chemotherapy in both groups.

Drug costs

The anticancer drug cost was similar between the two groups. In patients treated with the FOLFIRINOX regimen, the cost of 1 cycle, which lasted 2 wk, was determined to be 52190 KRW. After adding the room charges, the total cost for 4 wk treatment of the FOLFIRINOX regimen was 138724 KRW. The cost for 4 wk of the Gem + nabPTX regimen was 168838 KRW. Drug costs of two regimens were not very different - only 30000 KRW (about 30 USD) per month.

DISCUSSION

In the present study, the oncologic outcomes of the FOLFIRINOX and Gem + nabPTX regimens were found to be similar. Although the disease control rate was higher in the Gem + nabPTX group, there was no significant difference in objective response rate, OS, or PFS. In the subgroup analysis, Gem + nabPTX regimen showed survival advantages in relation to the patients' baseline factors such as body/tail cancer, high BMI, presence of liver metastasis and peritoneal carcinomatosis. When comparing the two regimens in terms of safety, patients who received FOLFIRINOX were at higher risk for the development of high-grade neutropenia, while those who received Gem + nabPTX were at higher risk for neuropathy and fatigue.

Compared to previous clinical trial data, the treatment efficacy observed in this study population was favourable. Patients who received Gem + nabPTX showed improved OS (12.1 *vs* 8.5 mo) and PFS (8.4 *vs* 5.5 mo) than those in the MPACT. The FOLFIRINOX group patients also showed improved PFS (8.0 *vs* 6.4 mo) when compared to those in the PRODIGE 4/ACCORD 11 trial, with similar OS rates (10.8 *vs*

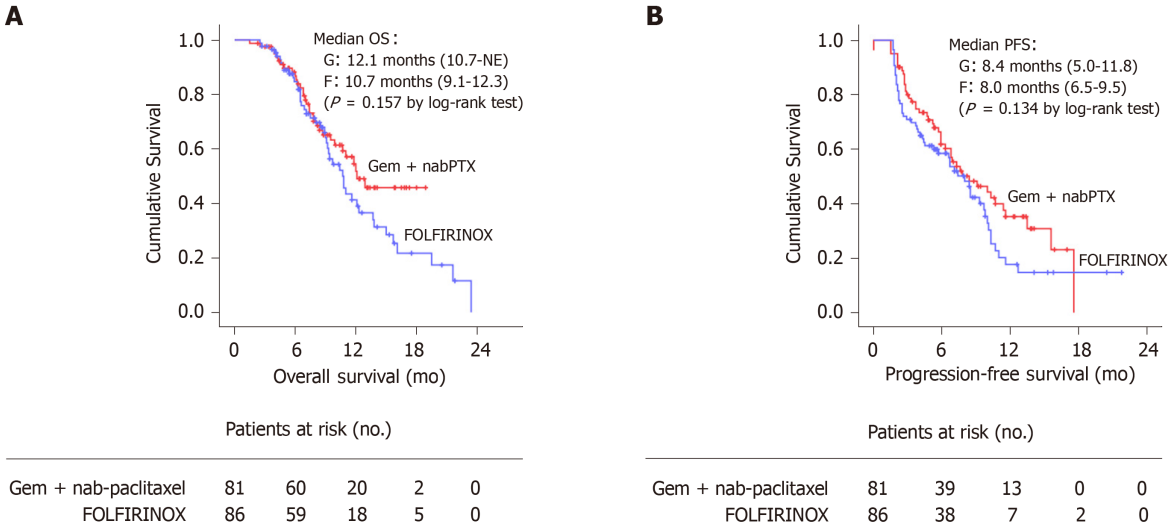


Figure 1 Overall survival and progression-free survival. A: Kaplan-Meier estimates of overall survival; B: Progression-free survival.

11.1 mo). On the basis of these data, we can consider that both the FOLFIRINOX and Gem + nabPTX regimens are very effective in Korean patients with metastatic pancreatic cancer.

However, treatment-related AEs were more common in this study population than in the previous clinical trials, especially hematologic AEs. Compared to the PRODIGE 4/ACCORD 11 trial[7] the FOLFIRINOX group patients in this study were younger (median age, 54 *vs* 61 years) and had better performance status scores at baseline (higher proportion of ECOG-PS score 0: 83.7% *vs* 37.4%). The median relative dose intensity of each agent was similar (5-FU, 80% *vs* 82%; oxaliplatin, 75% *vs* 78%; irinotecan, 75% *vs* 81%). However, the rate of hematologic AEs was remarkably high in this study population and similar to the proportion of hematologic AEs reported in a previous Japanese phase-II study[15]. Considering that the PRODIGE 4/ACCORD 11 trial was conducted at 48 French medical centers and that the rates of AEs in the Korean and Japanese population are similar, it can be assumed that there was an ethnic difference in the incidence of treatment-related AEs using the FOLFIRINOX regimen.

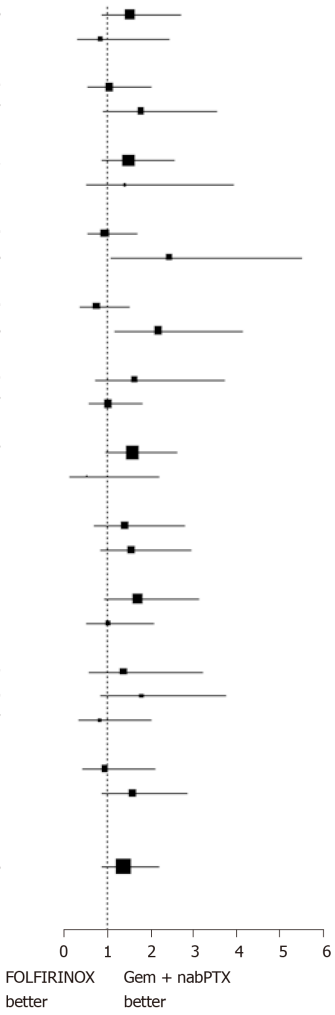
Ethnic differences in terms of drug efficacy or AEs are affected by local environment, dietary habits, genetic mutations, and genetic polymorphism[12]. Ethnic variations in polymorphisms can be an explanation for the racial differences in AEs. For example, the UGT1A1 polymorphism, which is related with the glucuronidation of SN-38 (an active metabolite of irinotecan) is associated with irinotecan-mediated diarrhea and neutropenia[16]. UGT1A1 No. 6 mutations, which are found predominantly in Asian populations, have been implicated in irinotecan toxicity[16-19]. Goetz *et al*[20] recommended UGT1A1 genotype-guided dosing of CAPIRINOX (capecitabine, oxaliplatin, and irinotecan) in their phase I study because the toxicity profile differed according to the presence of UGT1A1 polymorphisms. Defective cytochrome P450 3A4 (CYP3A4) variants are another example known to be related with paclitaxel-induced neuropathy[21,22]. Although direct associations with different pharmacokinetics according to ethnicity has not yet been established, ethnic differences in the frequency of polymorphisms of CYP3A4 have been reported[23].

Both regimens carry an unfavourable AE profile; therefore, dose modification strategies have been made. In terms of the FOLFIRINOX regimen, several studies used modified (reduced) doses through various methods to increase the patients' tolerance. For example, Mahaseth *et al*[24] replaced the 5-FU bolus injection with haematopoietic growth factors. Stein *et al*[25] used a modified dose with a 25% reduction in both irinotecan and the 5-FU bolus. Li *et al*[26] used modified FOLFIRINOX (no 5-FU bolus, 85% oxaliplatin, and 75% irinotecan) in Chinese patients with metastatic pancreatic cancer. In their studies, the incidence of severe neutropenia, fatigue, and vomiting were reduced, without any compromise in treatment efficacy. Ahn *et al*[27] presented a modified biweekly Gem + nabPTX regimen that could reduce the incidence of severe neutropenia and neurotoxicity when compared with that reported in the MPACT data[27].

In our study population, 43 patients (50% of FOLFIRINOX group) started receiving the FOLFIRINOX regimen as a modified (reduced) dose. There was no significant difference in treatment duration and efficacy. Moreover, no significant difference was

A

Subgroup	Gem + nabPTX	FOLFIRINOX	Hazard ratio for overall survival	(95%CI)	*P* value
	no. of events/no. of patients				
Age					
≤ 65	18/43	40/66		1.53 (0.87-2.69)	0.136
> 65	14/38	6/20		0.86 (0.31-2.42)	0.781
Sex					
Male	18/37	23/49		1.06 (0.56-2.00)	0.854
Female	14/44	23/37		1.79 (0.91-3.53)	0.093
ECOG					
0	22/57	40/72		1.51 (0.89-2.56)	0.127
1	10/24	6/14		1.42 (0.51-3.93)	0.502
BMI					
≤ 23	23/46	28/50		0.95 (0.54-1.68)	0.866
> 23	9/35	18/36		2.45 (1.09-5.51)	0.030
Primary site					
Head&Neck	17/32	17/40		0.76 (0.38-1.51)	0.433
Body&Tail	15/48	29/46		2.19 (1.17-4.13)	0.015
Liver metastasis					
No	12/41	13/29		1.63 (0.72-3.71)	0.241
Yes	20/40	33/57		1.03 (0.59-1.81)	0.909
Lung metastasis					
No	26/69	42/77		1.60 (0.98-2.62)	0.063
Yes	6/12	4/9		0.54 (0.13-2.19)	0.389
Carcinomatosis					
No	13/39	25/51		1.41 (0.71-2.79)	0.326
Yes	19/42	21/35		1.57 (0.84-2.95)	0.160
Distant LN metastasis					
No	18/53	27/53		1.71 (0.93-3.13)	0.085
Yes	14/28	19/33		1.03 (0.51-2.07)	0.939
Metastasis number					
1	10/38	14/39		1.38 (0.59-3.21)	0.455
2	11/24	22/30		1.80 (0.86-3.74)	0.117
3 or more	11/19	10/17		0.84 (0.35-2.00)	0.688
CA 19-9					
≤ 400	14/43	13/35		0.95 (0.43-2.09)	0.890
> 400	18/38	33/51		1.59 (0.89-2.85)	0.115
Total					
	32/81	46/86		1.39 (0.88-2.00)	0.161



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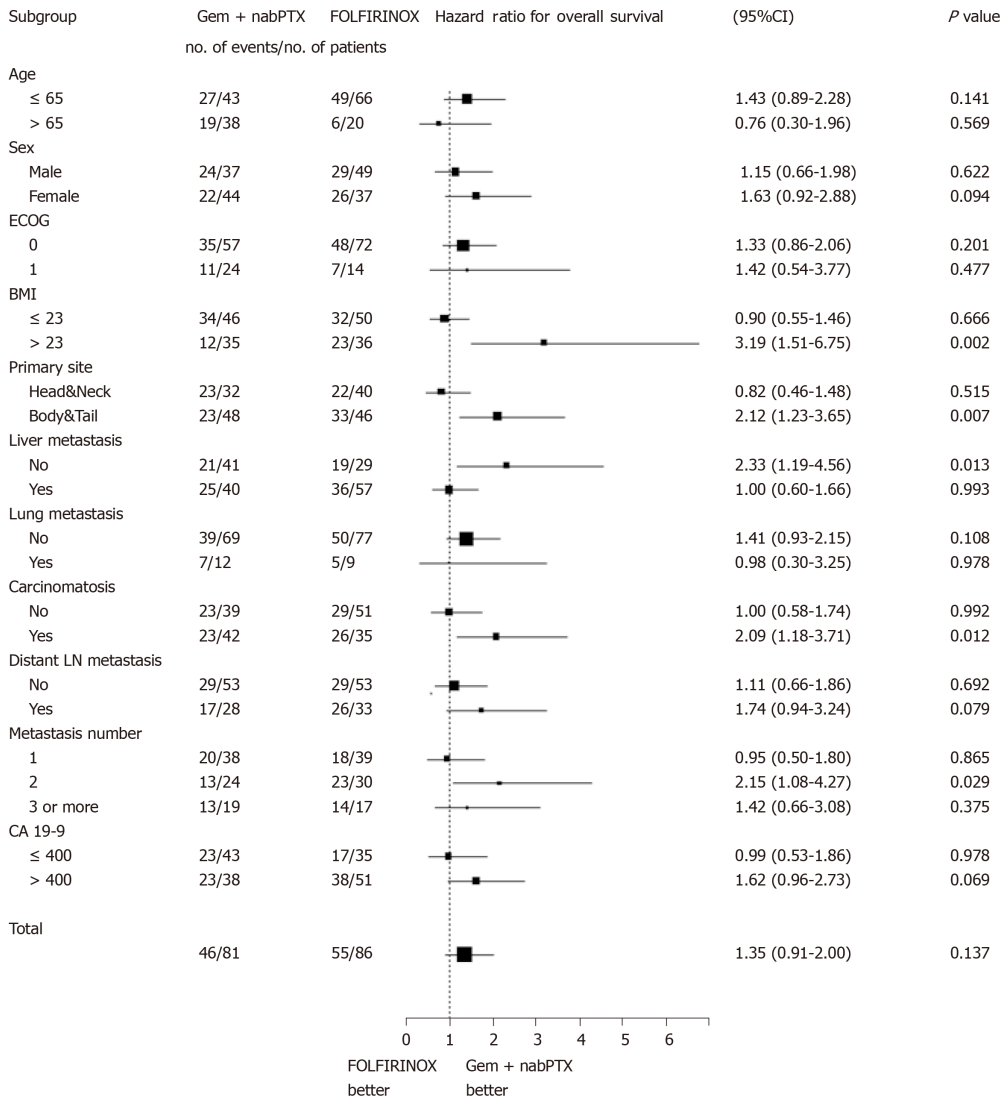

Figure 2 The treatment efficacy was consistently similar in both groups across the majority of subgroups. A: Forest plots of hazard ratio for overall survival; B: Progression-free survival according to subgroups.

noted in the incidence of non-hematologic AEs. However, the incidences of severe (grade ≥ 3) neutropenia and febrile neutropenia were lower in patients who received the modified dose than in those who received the full dose (severe neutropenia, 62.8% *vs* 86.0%; $P = 0.013$, febrile neutropenia, 18.6% *vs* 32.6%; $P = 0.138$). It may be helpful to use a modified dose when initiating chemotherapy for toxicity-susceptible patients identified *via* early screening tests.

Second-line chemotherapy could be considered after first-line therapy failure if patients continue to demonstrate good performance status. Even after a failure of first-line treatment, effective second-line chemotherapy can prolong patients' post-progression survival (PPS, overall survival from the notification of disease progression) and OS[28,29]. In this study population, 20 (24.7%) patients in the Gem + nabPTX group and 38 (44.2%) in the FOLFIRINOX group received second-line chemotherapy. XELOX (capecitabine plus oxaliplatin) was the most commonly prescribed second-line regimen in the Gem + nabPTX group and gemcitabine plus erlotinib was the most common second-line treatment administered in the FOLFIRINOX group. No difference in PPS was noted between the two groups (Gem + nabPTX group, 136 d [95%CI, 78.384–193.616]; FOLFIRINOX group, 148 d [95%CI, 120.576–175.424]; $P = 0.762$). Overall, patients who received second-line chemotherapy showed a significantly longer PPS than patients who did not (138 *vs* 39 d; $P < 0.001$) In particular, second-line chemotherapy was found to be more effective in patients who showed early progression within 3 mo after the first-line treatment (median PPS, 153 d). Recently, several studies have been conducted to assess the efficacy of second-line chemotherapy. Portal *et al*[30] showed that second-line Gem + nabPTX was effective

Table 3 Treatment-related adverse events due to the chemotherapy regimen, *n* (%)

	FOLFIRINOX (*n* = 86)	Gem + nabPTX (*n* = 81)	*P* value
Hematologic adverse event			
Grade ≥ 3 Anemia	17 (19.8)	12 (14.8)	0.398
Grade ≥ 3 Thrombocytopenia	7 (8.1)	5 (6.2)	0.623
Grade ≥ 3 Neutropenia	64 (74.4)	38 (46.9)	< 0.001
Febrile neutropenia	22 (25.6)	13 (16.0)	0.130
Administration of G-CSF	66 (76.7)	15 (18.5)	< 0.001
Neurologic adverse event			
Peripheral neuropathy	16 (18.6)	46 (56.8)	< 0.001
Grade ≥ 3 neuropathy	3 (3.5)	15 (18.5)	0.002
Median time to onset-days (range)	120 (15-278)	73.5 (17-284)	0.051
Gastrointestinal adverse event			
Nausea/Vomiting	43 (50.0)	17 (21.0)	< 0.001
Diarrhea	15 (17.4)	12 (14.8)	0.645
Grade ≥ 3 adverse events	39 (45.3)	16 (19.8)	< 0.001
General weakness	30 (34.9)	40 (49.4)	0.058
Dermatologic adverse event	12 (14.0)	34 (42.0)	< 0.001

G-CSF, granulocyte-colony stimulating factor; Gem + nabPTX: Gemcitabine plus nab-paclitaxel.

after the failure of first-line FOLFIRINOX[30]. The NAPOLI-1 trial revealed that nanoliposomal irinotecan (nan-IRI) plus 5-FU was effective in patients previously treated with gemcitabine-based chemotherapy[31]. Another clinical trial showed that oxaliplatin, folinic acid, and fluorouracil (OFF regimen) was effective in gemcitabine-refractory pancreatic cancer patients[32]. Furthermore, there are ongoing studies testing FOLFIRINOX after Gem + nabPTX failure[33].

As more options for second-line chemotherapy are being introduced, questions surrounding treatment choice and sequence have arisen. The advantage of using FOLFIRINOX as a first-line regimen is that Gem + nabPTX, which has similar efficacy, can be used as the secondary drug. On the other hand, patients who receive Gem + nabPTX as the first-line drug can choose diverse 5-FU based regimens (*i.e*. OFF, nal-IRI + 5-FU, or FOLFIRINOX) as second-line treatment depending on their performance status. Although more favourable sequences need to be studied, the active use of FOLFIRINOX or Gem + nabPTX as a second-line regimen or appropriate use of new agents such as nal-IRI may help to improve the prognosis of patients who show early progression.

When we consider the economic aspects of anticancer treatment, we have to consider both anticancer drug costs and general management costs such as hospitalization or medication fees for AE control. Gemcitabine, nab-paclitaxel, oxaliplatin, and irinotecan are expensive drugs. Fortunately, in Korea, the NHIS provides economic benefits for cancer patients-the NHIS provides 95% of the drug cost. Therefore, when NHIS coverage for cancer patients is reflected, the costs of the two regimens (per month) are similar. However, in terms of potential cost burden, the FOLFIRINOX regimen seems to have some disadvantages. Patients must be hospitalized to receive the FOLFIRINOX regimen. During hospitalization, additional costs that are not covered by the NHIS, such as private rooms, can occur. In addition, due to the higher hematologic AE rates, the cost for prolonged hospitalization, G-CSF administration, and infection control (related to febrile neutropenia) are more likely to occur in patients receiving FOLFIRINOX. However, for a more accurate comparison, additional quantitative and comparative analysis is also needed in terms of the decreased labour productivity or quality of life due to admission or severe AEs such as peripheral neuropathy, fatigue, or alopecia[34,35].

This study had several limitations. First, it was a retrospective cohort study conducted only in a single center. A prospective randomized controlled trial is needed to confirm and validate the results of this study. And, to determine the ethnic differences in efficacy and safety more clearly, a larger scale nation-wide study will be helpful. Second, this study did not quantify the change in the quality of life. There was a lack of medical records and questionnaires that could help to more precisely analyse quality of life. Finally, we could not perform more advanced genetic analyses. If new technologies (*e.g*., next-generation sequencing) are actively used in clinical

Table 4 Dose modification, treatment delay and cessation, *n* (%)

Variables	FOLFIRINOX (*n* = 86)	Gem + nabPTX (*n* = 81)	*P* value
Dose reduction	76 (88.4)	49 (60.5)	< 0.001
At beginning	43 (50)	7 (8.6)	
Before 1st RE	25 (29.1)	12 (14.8)	
1st RE-2nd RE	5 (5.8)	15 (18.5)	
After 2nd RE	3 (3.5)	15 (18.5)	
Delay of administration due to AE	47 (54.7)	51 (63.0)	0.346
Neurologic AE	2 (2.4)	14 (17.3)	
Hematologic AE	30 (34.9)	22 (27.2)	
Gastrointestinal AE	3 (3.5)	4 (4.9)	
General weakness	8 (9.3)	20 (24.7)	
Others	6 (7.0)	2 (2.5)	
Cessation of administration due to AE	12 (14.0)	17 (21.0)	0.307
Neurologic AE	2 (2.4)	3 (3.7)	
Hematologic AE	1 (1.2)	0 (0)	
Gastrointestinal AE	1 (1.2)	2 (2.5)	
General weakness	8 (9.3)	11 (13.6)	
Death	0 (0)	1 (1.2)	

RE: Response evaluation; AE: Adverse event; Gem + nabPTX: Gemcitabine plus nab-paclitaxel.

fields, it will be possible to collect and analyse genetic data more economically and easily.

The results of the present study suggest that the FOLFIRINOX and Gem + nabPTX regimens are similar in efficacy, but the type and rates of the AE are somewhat different: neurologic AEs were more common in the Gem + nabPTX group and hematologic AEs were more common in the FOLFIRINOX group. Given the subgroup analysis of this study, Gem + nabPTX regimen could be considered as a priority in patients with specific baseline conditions.

ARTICLE HIGHLIGHTS

Research background

FOLFIRINOX regimen and combination of gemcitabine and nab-paclitaxel (Gem + nabPTX) are recommended as the first-line therapy for metastatic pancreatic cancer. However, there is a lack of data regarding a direct comparison of the two regimens in efficacy and safety.

Research motivation

When treating metastatic pancreatic cancer patients, physicians would like to select appropriate chemotherapeutic regimens while avoiding and preventing unnecessary complications and economic burdens. By comparing the efficacy and safety of two regimens, this study can help physicians' decision of treatment choice and sequence.

Research objectives

The purpose of this study is to compare the efficacy, safety, and economic aspects of FOLFIRINOX and Gem + nabPTX in the treatment of metastatic pancreatic cancer in Korean population.

Research methods

Patients with metastatic or recurrent pancreatic cancer treated with FOLFIRINOX (n = 86) or Gem + nabPTX (n = 81) as the first-line since January 2015 were identified using the Severance Hospital Pancreatic Cancer Cohort Registry. Treatment efficacy, treatment-related adverse events and economic aspects were compared.

Research results

The median overall survival (FOLFIRINOX 10.7 *vs* Gem + nabPTX 12.1 mo; P = 0.157), progression-free survival (FOLFIRINOX 8.0 *vs* Gem + nabPTX 8.4 mo; P = 0.134), and objective response rates (FOLFIRINOX 33.7% *vs* Gem + nabPTX 46.9%; P = 0.067) were not significantly different between two regimens. Neurologic adverse events were more common in the Gem + nabPTX group and Grade ≥ 3 neutropenia and gastrointestinal adverse events were more common in the FOLFIRINOX group. The drug costs of both regimens were similar.

Research conclusions

Treatment efficacy and economic burdens were comparable between the two regimens. But, the type and rates of the adverse events were somewhat different. Given the subgroup analysis of this study, Gem + nabPTX regimen might be considered preferentially in patients with specific baseline conditions.

Research perspectives

This study will help clinicians choose an appropriate chemotherapeutic regimen for metastatic pancreatic cancer. To confirm and validate the results of this study, a larger scale prospective study would be helpful.

ACKNOWLEDGEMENTS

The authors thanks to Jun Tae Kim for his dedication in handling, managing and auditing of cohort data.

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2020 February 15; 12(2): 195-204

DOI: 10.4251/wjgo.v12.i2.195

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Study

Prognostic scoring system for synchronous brain metastasis at diagnosis of colorectal cancer: A population-based study

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Author contributions: Quan JC and Guan X wrote the manuscript and contributed equally to this work; Jiang Z and Wang XS conceived and designed the study and are the co-corresponding authors; Quan JC, Guan X and Ma CX collected the data; Ma CX, Liu Z, Yang M, Zhao ZX, Zhuang M, Sun P and Wang S analyzed the data; all authors made critical revisions for the manuscript and approved the final version.

Supported by National Key Research and Development Program of the Ministry of Science and Technology of China, No. 2016YFC0905303, 2016YFC0905300; and Beijing Science and Technology Program, No. D171100002617004.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China.

Informed consent statement: Informed consent was not required

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Abstract

BACKGROUND

Brain metastasis (BM) from colorectal cancer (CRC) is rarely encountered clinically, and its prognosis has not been fully evaluated.

AIM

To construct a scoring system and accurately predict the survival of patients with synchronous BM at diagnosis of CRC.

METHODS

A retrospective study of 371 patients with synchronous BM from CRC was performed, using the data from 2010 to 2014 from the Surveillance, Epidemiology, and End Results database. Survival time and prognostic factors were statistically analyzed by the Kaplan-Meier method and Cox proportional hazards models, respectively. A scoring system was developed using the independent prognostic factors, and was used to measure the survival difference among different patients.

RESULTS

For the 371 patients, the median overall survival was 5 mo, survival rates were 27% at 1 year and 11.2% at 2 years. Prognostic analysis showed that age, carcinoembryonic antigen level and extracranial metastasis to the liver, lung or bone were independent prognostic factors. A scoring system based on these three prognostic factors classified the patients into three prognostic subgroups (scores of 0-1, 2-3, and 4). The median survival of patients with scores of 0-1, 2-3 and 4

as the study is based on a publicly available database.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Received: September 8, 2019
Peer-review started: September 8, 2019
First decision: November 11, 2019
Revised: November 14, 2019
Accepted: November 28, 2019
Article in press: November 28, 2019
Published online: February 15, 2020

P-Reviewer: Kmietowicz Z, Tsikas D
S-Editor: Wang JL
L-Editor: Webster JR
E-Editor: Qi LL

was 14, 5 and 2 mo, respectively ($P < 0.001$). Subgroup analysis showed that there were significant differences in prognosis among the groups. Score 2-3 *vs* 0-1: hazard ratio (HR) = 2.050, 95%CI: 1.363-3.083; $P = 0.001$; score 4 *vs* 0-1: HR = 3.721, 95%CI: 2.225-6.225; $P < 0.001$; score 2-3 *vs* 4: HR = 0.551, 95%CI: 0.374-0.812; $P = 0.003$.

CONCLUSION
The scoring system effectively distinguishes long-term and short-term survivors with synchronous BM from CRC. These results are helpful in providing a reference for guiding therapy.

Key words: Colorectal cancer; Brain metastasis; Survival; Prognosis factors; Scoring system; Synchronous

Core tip: There is no prognostic scoring system specifically for synchronous brain metastasis (BM) from colorectal cancer (CRC). This is believed to be the first study to construct such a system. We found that the scoring system accurately distinguished survival differences among patients, which contributed to the individual management of patients with BM from CRC.

Citation: Quan JC, Guan X, Ma CX, Liu Z, Yang M, Zhao ZX, Sun P, Zhuang M, Wang S, Jiang Z, Wang XS. Prognostic scoring system for synchronous brain metastasis at diagnosis of colorectal cancer: A population-based study. *World J Gastrointest Oncol* 2020; 12(2): 195-204
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/195.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.195

INTRODUCTION

According to the latest cancer statistics, the incidence and mortality of colorectal cancer (CRC) rank fourth and second, respectively[1], and CRC is a severe threat to human health. Metastasis is the leading reason for treatment failure and cancer-associated death[2]. Around 25% of CRC patients develop metastases at the time of diagnosis[3]. Metastatic sites include the liver, lung, bone, and brain, but compared with lung and liver metastases, CRC brain metastasis (BM) is uncommon, with an incidence of only 0.3%-9%[4]. Despite being uncommon, however, once BM occurs, patients have a poor prognosis, with a median survival of 2-9.6 mo[5-10]. In addition to poor prognosis, BM is often accompanied by neurological symptoms such as headache, nausea, and hemiparesis, which often lead to poor quality of life. In view of the poor prognosis and quality of life, more attention should be paid to BM from CRC.

Reasonable treatment is helpful in improving the prognosis of patients, and accurate prognostic evaluation is also important to guide therapy, and the two complement each other. However, the survival of some patients with BM is different in clinical practice. Thus, establishing a scoring system to accurately distinguish the survival differences and then choose individualized treatment is crucial. At present, although there have been some studies on the prognostic analysis of BM from CRC, these studies are mostly limited to single institution-based data[11-13], and contradictory views still exist[14-20].

BM from CRC includes synchronous and metachronous BM. Previous studies have shown that synchronous BM account for only 3.4%-43% of total BM[6,21]. Therefore, compared to overall BM, synchronous BM is rarer, and the inadequate number of cases also limits in-depth research. At present, only a few studies have focused on the analysis of synchronous BM. To date, there is no prognostic scoring system specifically for synchronous BM from CRC; therefore, the disease is not adequately understood.

In the present study, we aimed to comprehensively evaluate the prognostic factors of synchronous BM at diagnosis of CRC by means of the Surveillance, Epidemiology, and End Results (SEER) database. On this basis, we constructed a scoring system to

accurately predict survival.

MATERIALS AND METHODS

Study population

We performed a retrospective study using the SEER database. The study included patients with synchronous BM from CRC between 2010 and 2014. In the present study, synchronous BM was defined as BM at the time of CRC diagnosis. Patients were excluded if the absence or presence of BM was unknown. In addition, patients who died from other causes, or were alive with no survival time were also excluded, as were those with appendix malignancies and no evidence of primary tumor. In total, 371 patients were evaluated.

The following clinicopathological variables were included: Age (< 60, 60-74, ≥ 75 years); race (white, black, other); gender; primary tumor site (colon or rectosigmoid/rectum); tumor grade (well/moderately differentiated, or poorly differentiated/undifferentiated); histological type (adenocarcinoma, mucinous carcinoma, signet ring-cell carcinoma, or other); carcinoembryonic antigen (CEA) level (negative or positive); T stage (T1/T2 or T3/T4); N stage (N0 or N1/N2); and survival time. Data for liver, lung and bone metastases were obtained from the SEER database. To clarify the relationship between liver, lung and bone metastases and BM, the status of extracranial metastasis to liver, lung or bone was also analyzed as a variable.

To stratify the prognosis of patients with synchronous BM, we developed a scoring system based on the independent prognostic factors, and 0, 1 or 2 points were assigned to each significant variable. The scoring system was formed by summing the points of each prognostic factor, and the scoring system finally classified patients into different prognostic subgroups. This study was approved by the Ethics Committee of Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China.

Statistical analysis

Survival curves were evaluated using the Kaplan-Meier method and compared with the log-rank test. Cox proportional hazards models were used to determine the prognostic factors of patients with BM. Factors that were significant in univariate analysis were included in the multivariate analysis for determination of the final independent prognostic factors. In further analysis, a scoring system was used to stratify the prognosis of patients with BM into different subgroups, and the median survival of different subgroups was compared, hazard ratio (HR) and 95%CI were calculated. $P < 0.05$ was deemed to be significant. All analyses were performed by SPSS version 20.0 (IBM, Armonk, NY, USA).

RESULTS

Patient characteristics

The present study included 371 patients with synchronous BM from CRC. Two hundred and seventy patients had concomitant liver ($n = 199$), lung ($n = 177$) or bone ($n = 81$) metastases. The probability of concomitant liver, lung or bone metastases was 53.6%, 47.7% and 21.8%, respectively. The detailed patient characteristics are shown in Table 1.

Survival and prognostic factors of patients with BM

The median overall survival of patients with BM was 5 mo, with a 1-year survival rate of 27.0% and 2-year survival rate of 11.2%. Figure 1A shows the survival curves for patients with BM and non-BM (data from non-BM were not shown in this study). The results of univariate analysis revealed that age, CEA level, and extracranial metastasis to liver, lung or bone are significant factors affecting the survival of patients with BM (Table 2). However, no significant differences were found in terms of race, gender, primary tumor site, tumor grade, histological types, T stage and N stage. When the above significant variables were included in the multivariate analysis, age, CEA level, and extracranial metastasis to liver, lung or bone were independent prognostic factors (Table 3). Patients aged 60-74 years ($P = 0.012$), and ≥ 75 years ($P < 0.001$) had shorter survival compared with those aged < 60 years. Survival of CEA-positive patients was shorter than that of CEA-negative patients ($P = 0.020$). Similarly, patients with concomitant liver, lung or bone metastases were significantly associated with poorer prognosis ($P = 0.023$).

Table 1 Characteristics of patients with brain metastasis from colorectal cancer, *n* (%)

Variable	No. of patients
Age (yr)	
< 60	144 (38.8)
60-74	157 (42.3)
≥ 75	70 (18.9)
Race	
White	295 (79.5)
Black	45 (12.1)
Other	31 (8.4)
Gender	
Male	199 (53.6)
Female	172 (46.4)
Primary tumor site	
Colon	215 (58.0)
Rectosigmoid/rectum	112 (30.2)
Unknown	44 (11.9)
Tumor grade	
Well/moderately differentiated	152 (41.0)
Poorly/undifferentiated	101 (27.2)
Unknown	118 (31.8)
Histology types	
Adenocarcinoma	320 (86.3)
Mucinous carcinoma	13 (3.5)
Signet ring-cell carcinoma	10 (2.7)
Other	20 (5.4)
Unknown	8 (2.2)
CEA level	
Negative	46 (12.4)
Positive	206 (55.5)
Unknown	119 (32.1)
T stage	
T1/T2	57 (15.4)
T3/T4	164 (44.2)
Unknown	150 (40.4)
N stage	
N0	119 (32.1)
N1/N2	172 (46.4)
Unknown	80 (21.6)
Liver metastasis	
No	166 (44.7)
Yes	199 (53.6)
Unknown	6 (1.6)
Lung metastasis	
No	184 (49.6)
Yes	177 (47.7)
Unknown	10 (2.7)
Bone metastasis	
No	276 (74.4)
Yes	81 (21.8)
Unknown	14 (3.8)
Extracranial metastasis to liver, lung or bone	
No	97 (26.1)
Yes	270 (72.8)

Unknown	4 (1.1)

CEA: Carcinoembryonic antigen.

Construction of the scoring system

To accurately stratify the survival of different patients with BM, we constructed a scoring system of 0-4 (Table 4). For example, patients aged < 60 years (0 point), CEA-negative patients (0 point), and absence of extracranial metastasis to liver, lung or bone (0 point) scored 0. Patients aged ≥ 75 years (2 points), CEA-positive patients (1 point), and presence of extracranial metastasis to liver, lung or bone (1 point) scored 4. According to the scoring system, the patients were divided into three prognosis subgroups: group I (score 0-1), group II (score 2-3), group III (score 4). The median survival was 14 mo for group I, 5 mo for group II, 2 mo for group III, and the differences were statistically significant ($P < 0.001$). The survival curves for the three subgroups are shown in Figure 1B. Compared with group I patients, group II-III patients had significantly poorer survival (group II, $P = 0.001$; group III, $P < 0.001$), similarly, the prognosis of group II patients was significantly better than that of group III ($P = 0.003$) (Table 5).

DISCUSSION

Understanding the prognostic factors of BM is crucial for assessing survival and guiding treatment. However, the current prognostic factors for BM from CRC have not reached consensus[14-20]. The reason for the above contradictions may be due to the differences in variables and sample sizes in different studies. Little is known about synchronous BM from CRC, which is mainly because of the small number of cases. Compared with the single-center, small-sample studies, population-based research can make up for the above limitations and may better reflect the state of the disease. Therefore, in order to understand the disease better, we performed a population-based retrospective analysis. We analyzed the prognosis of 371 patients with synchronous BM and consequently constructed a prognostic scoring system. The system was based on three independent prognostic factors: Age, CEA level, and extracranial metastasis to liver, lung or bone. Our results confirmed that the prognosis of different patients was significantly different, and the scoring system accurately classified patient survival. To our knowledge, we are the first group to construct a scoring system specifically for synchronous BM from CRC.

BM from CRC occurs in the late stage of CRC, and is often associated with extracranial metastases such as liver, lung and bone at diagnosis. This is also one of the factors leading to poor prognosis[10,12]. In this study, 72.8% of patients had concomitant liver, lung or bone metastases, which was significantly associated with poorer prognosis. This result confirmed the findings of Gu *et al*[10], who analyzed 93 patients with BM. Median survival in the presence and absence of extracranial metastasis was 7 and 13 mo, respectively. The prognosis of patients with extracranial metastasis was worse than those without extracranial metastasis. Matsunaga *et al*[12] reached the same conclusion that the presence of extracranial metastasis worsened prognosis. These results suggest that extracranial metastases are an important prognostic factor in patients with BM from CRC.

CEA is a common tumor marker and is often used for CRC diagnosis and postoperative follow-up monitoring. Moreover, the prognostic value of CEA in BM from CRC has been verified by other researchers[6,22]. Consistent with previous studies, our study showed that CEA is an independent prognostic factor, and median survival was 5 and 8 mo for patients with CEA-positive and CEA-negative disease, respectively. The survival of CEA-positive patients was significantly shorter than that of CEA-negative patients.

Age is another important prognostic factor. In our study, patients were divided into three age groups: < 60, 60-74 and ≥ 75 years. We found that patients aged < 60 years had the best prognosis, followed by those aged 60-74 years, and those aged ≥ 75 years had the worst prognosis. Consistent with our findings, Yang *et al*[23] classified patients into five age groups: < 40, 40-49, 50-59, 60-69 and ≥ 70 years; they confirmed that the prognosis of patients aged ≥ 70 years was significantly worse than in those aged < 40 years. Similarly, Farnell *et al*[14] also emphasized the value of age in predicting the prognosis of BM. These studies suggested that older patients tend to have poorer prognosis than young patients with BM from CRC. Therefore, with the increase in the aging population, it is necessary to pay more attention to elderly patients.

As reported in previous studies[24], patients with cancer at the same stage often have

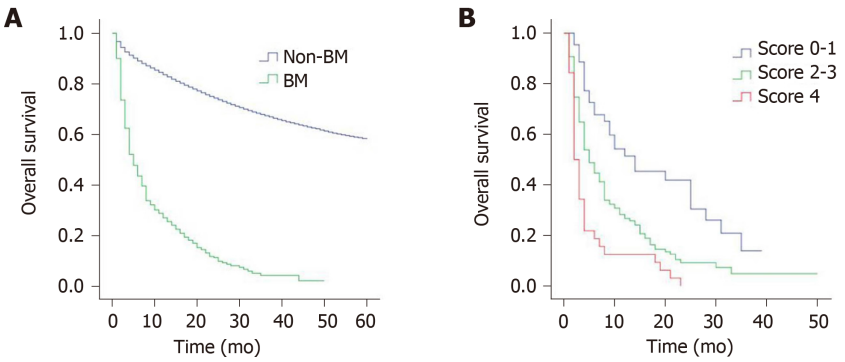

Figure 1 Kaplan-Meier survival curves for patients. A: Kaplan-Meier survival curves for patients with brain metastasis and non-brain metastasis (Log rank $P < 0.001$); B: Kaplan-Meier survival curves for patients with different scoring groups (Log rank $P < 0.001$). BM: Brain metastasis.

different prognoses. Similarly, CRC patients with BM also face the same problem. Kim *et al*[22] conducted a single-center study of 107 CRC patients with BM. They developed a graded prognostic assessment and divided the patients into three prognostic subgroups with a median survival of 2.3, 4.3 and 12.7 mo. Their results showed that the prognosis of patients with different grades differed significantly. However, in their study, there was no clear distinction between synchronous and metachronous BM. Therefore, the prognosis of synchronous BM is not clear. Unlike that study, our study specifically focused on synchronous BM, and our scoring system divided the patients into three subgroups with scores ranging from 0-1 to 4, with a median survival of 14, 5 and 2 mo, respectively. We found that the higher the score, the worse the prognosis. Patients with scores of 0-1 had the best prognosis, with a median survival of up to 14 mo. However, the prognosis was worst in patients with a score of 4, and their median survival was only 2 mo. Therefore, in clinical practice, the prognosis of patients with BM should not be generalized, and individualized survival evaluation should be made based on the patient's own situation.

Our study had some limitations. Firstly, the SEER database only provides information on the presence or absence of BM at initial diagnosis; thus, our study only assessed patients with BM at initial presentation of CRC. Patients who developed BM later in the disease course could not be commented upon in our analysis. Secondly, information regarding the Karnofsky performance status, number of BM, detailed treatment of BM and molecular markers was not provided in the SEER database; therefore, these factors were not included in our study. In the future, a large multicenter study is needed to confirm the value of these variables in synchronous BM.

In conclusion, this study confirmed the prognostic factors of synchronous BM from CRC and constructed a prognostic scoring system. The scoring system more accurately distinguished the prognostic differences among different patients and can be used as an effective prognostic predictive tool to help clinicians quickly and conveniently predict survival.

Table 2 Univariate analysis of prognostic factors in patients with brain metastasis from colorectal cancer

Variable	Median survival (mo)	HR	95%CI	*P* value
Age (yr)				
< 60	8	1	-	-
60-74	4	1.398	1.082-1.807	0.010
≥ 75	3	2.434	1.787-3.315	< 0.001
Race				
White	5	1	-	-
Black	6	0.990	0.703-1.395	0.955
Other	4	1.309	0.889-1.926	0.172
Gender				
Male	4	1	-	-
Female	6	0.932	0.743-1.169	0.543
Primary tumor site				
Colon	5	1	-	-
Rectosigmoid/Rectum	6	0.849	0.656-1.098	0.211
Unknown	3	1.451	1.025-2.053	0.036
Tumor grade				
Well/moderately differentiated	8	1	-	-
Poorly/undifferentiated	5	1.185	0.895-1.569	0.236
Unknown	4	1.527	1.170-1.994	0.002
Histology types				
Adenocarcinoma	5	1	-	-
Mucinous carcinoma	8	0.761	0.416-1.394	0.377
Signet ring-cell carcinoma	4	0.923	0.451-1.888	0.826
Other	4	1.314	0.814-2.122	0.264
Unknown	3	2.416	1.133-5.149	0.022
CEA level				
Negative	8	1	-	-
Positive	5	1.964	1.331-2.896	0.001
Unknown	4	2.149	1.431-3.225	< 0.001
T stage				
T1/T2	4	1	-	-
T3/T4	6	0.942	0.669-1.326	0.731
Unknown	4	1.125	0.798-1.585	0.502
N stage				
N0	5	1	-	-
N1/N2	6	0.952	0.733-1.238	0.715
Unknown	4	1.239	0.907-1.691	0.178
Extracranial metastasis to liver, lung or bone				
No	6	1	-	-
Yes	4	1.400	1.071-1.831	0.014
Unknown	7	1.544	0.566-4.269	0.392

CEA: Carcinoembryonic antigen; HR: Hazard ratio.

Table 3 Multivariate analysis of prognostic factors in patients with brain metastasis from colorectal cancer

Variable	HR	95%CI	*P* value
Age (yr)			
< 60	1	-	-
60–74	1.395	1.075-1.809	0.012
≥ 75	2.497	1.818-3.430	< 0.001
CEA level			
Negative	1	-	-
Positive	1.613	1.078-2.413	0.020
Unknown	1.865	1.229-2.829	0.003
Extracranial metastasis to liver, lung or bone			
No	1	-	-
Yes	1.383	1.045-1.831	0.023
Unknown	1.280	0.461-3.551	0.636

CEA: Carcinoembryonic antigen; HR: Hazard ratio.

Table 4 Scoring system

Variable	Point
Age (yr)	
< 60	0
60-74	1
≥ 75	2
CEA level	
Negative	0
Positive	1
Extracranial metastasis to liver, lung or bone	
No	0
Yes	1

CEA: Carcinoembryonic antigen.

Table 5 Median survival of patients with brain metastasis according to different scoring groups

Variable	*n*	Median survival (mo)	HR	95%CI	*P* value[1]
Group I (score 0-1)	44	14	1	-	-
Group II (score 2-3)	172	5	2.050	1.363-3.083	0.001
Group III (score 4)	32	2	3.721	2.225-6.225	< 0.001

[1]Group II *vs* Group III: $P = 0.003$ (Hazard ratio = 0.551, 95%CI: 0.374-0.812). HR: Hazard ratio.

ARTICLE HIGHLIGHTS

Research background

Synchronous brain metastasis (BM) from colorectal cancer (CRC) is rare, and the prognosis is poor. However, only a few studies have focused on the analysis of synchronous BM, and there is no prognostic scoring system specifically for synchronous BM from CRC to date. Therefore, more studies on synchronous BM from CRC are needed.

Research motivation

We comprehensively evaluated the prognostic factors of synchronous BM, and further constructed a scoring system to accurately predict survival.

Research objectives

This study was designed to confirm the clinical value of the prognostic scoring system for synchronous BM at diagnosis of CRC.

Research methods
We retrospectively studied patients with synchronous BM from CRC using the Surveillance, Epidemiology, and End Results database. The Kaplan-Meier method was used to assess the median survival time, and Cox proportional hazards models were used to determine the independent prognostic factors. A scoring system was constructed to stratify the patients into different subgroups, and the survival differences among different subgroups were compared.

Research results
The results showed that age, carcinoembryonic antigen level and extracranial metastasis to liver, lung or bone were independent prognostic factors. A scoring system based on the three independent prognostic factors classified the patients into three prognostic subgroups: group I (score 0-1), group II (score 2-3), and group III (score 4). The median survival was 14 mo for group I, 5 mo for group II, and 2 mo for group III, and there were significant differences in prognosis among the groups ($P < 0.001$).

Research conclusions
This study is the first to construct a scoring system specifically for synchronous BM from CRC, and we confirm that the scoring system accurately distinguishes the survival differences among different patients.

Research perspectives
The scoring system can be used as an effective prognostic predictive tool to help clinicians quickly and conveniently predict survival.

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2020 February 15; 12(2): 205-218

DOI: 10.4251/wjgo.v12.i2.205

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Observational Study

Neuropathy experienced by colorectal cancer patients receiving oxaliplatin: A qualitative study to validate the Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity scale

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Author contributions: Kaiser K, Shaunfield S and Cella D contributed to study conception and design; Kaiser K, Shaunfield S, Lyleroehr M, Lacson L, Kircher S and Corona M contributed to data acquisition, data analysis and data interpretation; Kaiser K contributed to writing of the article; Kaiser K, Lyleroehr M, Shaunfield S, Lacson L, Corona M, Kircher S, Nittve M and Cella D contributed to editing, reviewing, and final approval of the manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Northwestern University.

Informed consent statement: The patients provided written consent before participation in the study.

Conflict-of-interest statement: The authors declare that they have no

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Abstract

BACKGROUND

Although oxaliplatin is widely established as a standard treatment in colorectal cancer (CRC), oxaliplatin-induced neuropathy has emerged as a prominent dose-limiting side effect associated with quality of life decrements. Ongoing monitoring and management of neuropathy is important for CRC patient quality of life and adherence to treatment. Therefore, a validated self-reported measure of neuropathy would aid in the management and assessment of oxaliplatin-induced neuropathy in clinical practice and research. We sought to evaluate the content validity of the 13-item Functional Assessment of Cancer Therapy/Gynecologic Oncology Group- Neurotoxicity subscale (FACT/GOG-Ntx) for CRC patients receiving oxaliplatin.

AIM

To understand the neuropathy experiences of CRC patients and assess content validity of the FACT/GOG-Ntx.

METHODS

Semi-structured concept elicitation and cognitive debriefing interviews were conducted with 31 CRC patients experiencing peripheral neuropathy from current or previous oxaliplatin treatment. Interview data were analyzed using a

conflict of interest.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared according to the STROBE Statement-checklist of items.

Manuscript source: Unsolicited Manuscript

Received: October 7, 2019
Peer-review started: October 7, 2019
First decision: October 18, 2019
Revised: October 29, 2019
Accepted: January 6, 2020
Article in press: January 6, 2020
Published online: February 15, 2020

P-Reviewer: Ananthakrishnan N, Trkulja V
S-Editor: Ma YJ
L-Editor: A
E-Editor: Qi LL

constant comparative approach, and data were mapped to the FACT/GOG-Ntx to assess content validity.

RESULTS

Mean age of the sample was 54 (range 34-82). The sample was primarily Caucasian (84%) and consisted of nearly equal numbers of men and women. Participants described 28 unique neuropathy symptoms; hand tingling (experienced by 87% of respondents); feet tingling (81%); hand numbness (68%); and feet numbness (84%) were most frequently mentioned. Neuropathy symptoms occurring on the feet were most often identified as most bothersome by participants. Eleven of the 13 FACT/GOG-Ntx items exhibited moderate to strong evidence of content validity. Two items related to trouble hearing and ringing in the ears had weak support; however, these items represent severe neuropathy and could be useful for a patient reported outcome measure.

CONCLUSION

The FACT/GOG-Ntx represents the key neuropathy experiences of CRC patients treated with oxaliplatin.

Key words: Neuropathy; Colorectal cancer; Patient reported outcomes; Quality of life

Core tip: Colorectal cancer patients report significant impairment in dexterity, mobility, and balance due to neuropathy. Because prevention and treatment options for oxaliplatin-induced neuropathy are limited ongoing monitoring and management of neuropathy is important for patient quality of life and treatment adherence. A validated self-reported measure of neuropathy would aid in the management and assessment of neuropathy. This study examined the content validity of the Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity scale for colorectal patients with oxaliplatin-induced neuropathy; the measure was found to have content validity for this population.

Citation: Kaiser K, Lyleroehr M, Shaunfield S, Lacson L, Corona M, Kircher S, Nittve M, Cella D. Neuropathy experienced by colorectal cancer patients receiving oxaliplatin: A qualitative study to validate the Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity scale. *World J Gastrointest Oncol* 2020; 12(2): 205-218
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/205.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.205

INTRODUCTION

Although oxaliplatin is widely established as a standard treatment in colorectal cancer (CRC), oxaliplatin-induced neuropathy has emerged as a prominent dose-limiting side effect associated with quality of life decrements[1,2]. CRC patients report significant impairment in their activities of daily living because of their neuropathy, including difficulty with tasks requiring fine motor skills and dexterity, mobility, and balance[3]. Oxaliplatin induced neuropathy may be acute or chronic[1-7]. Acute neuropathy typically occurs within 1-2 d of the first oxaliplatin infusion[1]. Acute symptoms tend to resolve spontaneously within one week and typically do not necessitate dose reductions; however, symptoms may return with subsequent administration of oxaliplatin[1,8,9]. Chronic neuropathy develops gradually over time and is related to the cumulative oxaliplatin dose[2,5,8,10-12]. Although symptoms can resolve within six months of treatment[13], there are several reports of chronic neuropathy lasting two years or more[8,10,14,15]. Prevention and treatment options for oxaliplatin-induced neuropathy are limited; approaches include scheduled drug holidays, magnesium and calcium infusions, and pharmacologic interventions including anti-depressant and anti-epileptic agents[1].

Ongoing monitoring and management of neuropathy is important for patient quality of life and adherence to treatment. A validated self-reported measure of neuropathy would aid in the management and assessment of oxaliplatin-induced

neuropathy in clinical practice and research. The Functional Assessment of Cancer Therapy/Gynecologic Oncology Group- Neurotoxicity subscale (FACT/GOG-Ntx) is a 13-item subscale of the FACT-G that was developed with input from the Eastern Cooperative Oncology Group (ECOG), the Gynecologic Oncology Group (GOG), and the National Surgical Adjuvant Breast and Bowel Project. The FACT/GOG-Ntx includes the previously validated 11-item FACT/GOG neurotoxicity subscale[16-20], which assesses sensory symptoms (*e.g.*, numbness, tingling, and discomfort in hands and feet), motor symptoms (*e.g.*, trouble walking; buttoning buttons), and ototoxicity (*e.g.*, ringing or buzzing in ears). With the introduction of oxaliplatin, and its unique cold hypersensitivity, two new items were written by investigators from National Surgical Adjuvant Breast and Bowel Project. We sought to examine the content validity of the 13-item FACT/GOG-Ntx for CRC patients receiving oxaliplatin.

MATERIALS AND METHODS

Content validity of the 13-item FACT/GOG-Ntx subscale was assessed via concept elicitation and cognitive debriefing interviews with CRC patients experiencing oxaliplatin-induced neuropathy. Trained interviewers used a semi-structured guide that was informed by literature and guides from prior work to assess content validity of PRO measures[21-24]. The study protocol was reviewed and approved by the Northwestern University Institutional Review Board; all participants provided informed consent.

Participants

Patients were recruited from the Robert H. Lurie Cancer Center and the CRC Alliance (https://www.ccalliance.org/) in 2017. Eligible patients were age 18 and older, had a diagnosis of CRC (any stage), were receiving or had received oxaliplatin, and were experiencing peripheral neuropathy. Patients with a cognitive impairment or those experiencing neuropathy from other causes were excluded. Participants were interviewed in-person or via phone, and were compensated for their time.

Concept elicitation

After providing basic demographic information, patients were asked to list their neuropathy symptoms. Additional details were gathered on each symptom, such as when the symptom began, frequency of the symptom, if it co-occurred with other neuropathy symptoms, and its impact on functioning. Patients rated each symptom as it pertained to their health-related quality of life on a 0 to 10 scale, where 0 = not at all important and 10 = extremely important. Lastly, patients were asked to share which neuropathy symptom was most bothersome, and why.

Cognitive debriefing

After the concept elicitation interview, patients completed the FACT/GOG-Ntx 13 plus 6 additional items (Table 1). The FACT/GOG-Ntx measures the severity and impact of symptoms of neurotoxicity, such as numbness, discomfort, or trouble with motor skills, over the past 7 d. Responses are selected from a scale of 0 ("not at all") to 4 ("very much"). The 6 additional items were written to test whether numbness and tingling should be combined in one item, as in the current version of the FACT/GOG-Ntx (items NTX1 and NTX2), or divided into separate items (additional items 1-4, Table 1). Additional items 5-6 tested whether "discomfort" or "pain" best fit the patients' neuropathy experiences. After the patient completed the measure and the 6 additional items, the interviewer conducted a cognitive interview with the patient using a structured interview guide based on the work of Willis[25] to assess patients' understanding of the measure's instructions, items, and response options. Patients were asked to state each item in their own words, describe how they arrived at their response, and indicate each item's relevance to their experience.

Statistical analysis

Interviews were audiotaped, transcribed verbatim, and transcripts were de-identified. A list of neuropathy symptoms that patients reported during concept elicitation was compiled and redundant symptoms were removed; this condensed symptom list formed the basis of a codebook. Two experienced qualitative researchers analyzed the concept elicitation data systematically using a constant comparative approach[26]. The researchers met regularly to review data for each code. Saturation-the point at which no new, relevant data emerges[27,28]-was tracked using a saturation tracking table. Summaries of the data for each code were written, highlighting terminology used by patients and their experiences. These summaries were mapped to the FACT-GOG/Ntx content. The mapping process aimed to highlight (1) dimensions of

Table 1 Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity scale items and additional items used in cognitive interviews

FACT/GOG-Ntx Items
NTX1. I have numbness or tingling in my hands
NTX2. I have numbness or tingling in my feet
NTX3. I feel discomfort in my hands
NTX4. I feel discomfort in my feet
NTX5. I have joint pain or muscle cramps
HI12. I feel weak all over
NTX6. I have trouble hearing
NTX7. I get a ringing or buzzing in my ears
NTX8. I have trouble buttoning buttons
NTX9. I have trouble feeling the shape of small objects when they are in my hand
An6. I have trouble walking
NTX10. I have pain in my hands or feet when I am exposed to cold temperatures
NTX11. I have difficulty breathing when I am exposed to cold temperatures
Alternate items tested in cognitive interviews
I have numbness in my hands. (Add-1)
I have tingling in my hands. (Add-2)
I have numbness in my feet. (Add-3)
I have tingling in my feet. (Add-4)
I have pain in my hands. (Add-5)
I have pain in my feet. (Add-6)

neuropathy identified by patients and covered by FACT/GOG-Ntx; (2) FACT-GOG-Ntx content that does not align with CRC patients' experiences with oxaliplatin-induced neuropathy; and (3) experiences with oxaliplatin-induced neuropathy not represented by the FACT/GOG-Ntx[29]. Although content validity assessment primarily relied on the concept elicitation data, we also utilized the literature and cognitive interview data.

RESULTS

Sample

Sociodemographic and clinical characteristics of the concept elicitation sample (n = 31) are shown in Table 2. Our sample was primarily Caucasian (n = 26, 84%) and consisted of nearly equal numbers of men and women. Approximately one-third of the sample (n = 11, 35%) were currently receiving treatment.

Concept elicitation results: Patient reported symptoms of neuropathy

In response to the question, "Please describe all of the neuropathy side effects that you are currently experiencing," patients reported over 60 side effects (*i.e.*, symptoms of neuropathy). After redundant categories were removed, 28 unique concerns remained (Table 3). Saturation of patient-reported neuropathy symptoms occurred at interview number 31. The most frequently mentioned symptoms were associated with the hands and feet: hand tingling (experienced by n = 27, 87% of respondents); feet numbness (n = 26, 84%); feet tingling (n = 25, 81%); and hand numbness (n = 21, 68%). Additionally, 74% (n = 23) of participants reported cold sensitivity in their hands or feet, which they described as feelings of shock, stinging, or pain upon touching cold items. Neuropathy symptoms affecting the feet were most frequently identified as most bothersome (Table 4). Seven of the 28 (25%) respondents to this question stated that numbness in the feet was most bothersome. For example, patient 014 said, "My feet have been most bothersome, because with the numbness, you know, especially if I'm getting tired, I can't feel my feet and sometimes I just find it difficult to start walking in the morning." Five of 28 (18%) stated that discomfort/pain in the feet was most bothersome.

Cognitive interview results

Twenty-nine patients participated in a cognitive interview. Due to time constraints,

Table 2 Characteristics of patient sample (*n* = 31), *n* (%)

Patient characteristic	Number of participants
Mean age (range, yr)	54 (34-82)
Gender	
Male	15 (48.4)
Female	16 (51.6)
Education	
High school grad/GED	5 (16.1)
Some college/Technical degree/AA	5 (16.1)
College degree (BA/BS)	9 (29.0)
Advanced degree (MA, PhD, MD, JD)	12 (38.7)
Race	
Caucasian	26 (83.9)
African-American	3 (9.7)
Asian	1 (3.2)
Mixed race	1 (3.2)
ECOG status (self-reported)	
0	5 (16.1)
1	13 (41.9)
2	7 (22.6)
3	5 (16.1)
4	1 (3.2)
Currently on therapy	
Yes	11 (35.5)
No	20 (64.5)
Mean (raw score) FACT/GOG-Ntx-13 subscale score (range)	18.8 (7-38)

ECOG: Eastern Cooperative Oncology Group; FACT/GOG-Ntx: Functional Assessment of Cancer Therapy/Gynecologic Oncology Group- Neurotoxicity subscale.

not all patients completed the entire cognitive interview. Thus, the sample size varies slightly across questions, as noted below. Twenty-eight patients provided feedback on the questionnaire instructions; all 28 (100%) said the instructions were clear. For every item, 100% of respondents (28 of 28) indicated that they were confident or very confident in their response to the item. All items were interpreted in ways that were consistent with the intended meaning (results not shown). Twenty-three of 28 participants (82%) said they thought of a specific time period when answering questions; the most common time period referenced by this group were the past 7 d (12 of 23 participants, 52%) or the current day (7 of 23 participants, 30%). Patients were also asked if their responses would have been different if the instructions said to think of the last 24 hours. The majority of patients (18 of 28, 64%) said no. Almost all participants (27 of 29, 93%) reported that the instrument captured their experiences with treatment-related neuropathy. Cognitive interview results related to the relevance of individual items is provided below and in Table 5, as part of the content validity assessment.

Content validity for FACT-GOG-Ntx items

Items NTX1 - I have numbness or tingling in my hands: Numbness of the hands was commonly described as a lack of feeling or sensation that affected activities of daily living such as buttoning buttons, feeling and holding objects, and writing (Supporting quotations are shown in Table 5). Tingling of the hands was commonly described as a feeling of pins and needles, stabbing, or prickling. Patients reported that the numbness or tingling of the hands was often a constant sensation. During the cognitive interview, 26 of 28 patients (93%) said that item Ntx1 was relevant to their experience.

Item NTX2 - I have numbness or tingling in my feet: Patients described numbness in their feet as a lack of feeling or sensation. Tingling was commonly described as a feeling of electricity, vibration, or being asleep. Patients reported that their numbness or tingling was often a constant sensation that impaired mobility and affected daily

Table 3 Patient reported symptoms of neuropathy and impact on health-related quality of life (*n* = 31), *n* (%)

Symptom	Number of patients who listed the symptom	Importance for health-related quality of life, 0 = not at all important, 10 = extremely important mean (range)	Symptom definition
Hand tingling	27 (87.0)	4.9 (0-10)	Feeling of pins and needles, stabbing, or prickling in one's hands
Numbness - feet	26 (83.8)	6.0 (0-10)	Lack of feeling or sensation causing discomfort in one's feet
Tingling - feet	25 (80.6)	5.8 (0-10)	Feeling of pins and needles, stabbing, or a prickling sensation in one's feet
Cold sensitivity - hands/feet	23 (74.1)	6.7 (1-10)	Feeling of shock, stinging, or pain upon touching cold items with hands or feet
Numbness – hands	21 (67.7)	5.6 (0-10)	Lack of feeling or sensation in one's hands
Discomfort/pain - feet	14 (45.1)	7.3 (3-10)	Aches (sometimes throbbing), or pain when standing on one's feet
Impaired fine motor skills	13 (41.9)	7.2 (3-10)	Trouble buttoning buttons, grasping, or holding objects with one's hands
Discomfort/pain - hands	12 (38.7)	7.3 (3-9)	Achy, stinging, or stiff sensation in one's hands
Joint pain/muscle cramps	7 (22.5)	6.4 (3-10)	Feeling of stiffness, pain, or aches in joints, hand fatigue, or cramps in one's legs or feet
Cold sensitivity - eating/drinking	6 (19.3)	5.6 (2-10)	Feeling as if sharp objects are scratching one's throat, causing pain when swallowing cold food or drink
Trouble walking	6 (19.3)	6.8 (2-10)	Difficulty feeling feet when on the floor, resulting in stumbling or clumsiness when walking
Discomfort/pain - other body parts	5 (16.1)	7.6 (3-10)	Various sensations covered including clenched jaw, heavy sensation of the eyelid, leg pain or spasms
Abnormal sensation - foot	4 (12.9)	6.3 (4-8)	Feeling as if a small object (*e.g.*, walnut) is beneath one's foot. Affects balance and walking
Cold sensitivity - other body part	4 (12.9)	4.3 (3-7)	Internal feeling of body coldness (as opposed to on the surface). Spasms or twitching of one's face, eyes, or chest. when exposed to cold temperatures
Cold sensitivity - breathing	3 (9.6)	8.0 (7-9)	Sensitivity to cold temperatures affecting one's nose (burning) or throat (closure, spasm, choking)
Numbness - legs	2 (6.4)	5.0 (5)	Feeling of prickling or general sense of fatigue in one's legs or calves
Abnormal sensations – other body part	2 (6.4)	3.0 (1-5)	Less common abnormal bodily sensations include feeling of coldness inside one's head and heavy sensation of the eyelids
Burning feet	2 (6.4)	2.0 (0-4)	Burning sensation in one's feet causing discomfort when touched

Side effects listed by 1 patient each: Ringing ears, Blurry vision, Numbness – tongue, Swelling hands, Discomfort/pain – general, Tingling – legs; Abnormal sensation – eyelid, Numbness – lips, Spasms – chest, Twitching face, Abnormal sensations - other body part.

activities. Twenty-five of 26 (96%) responding patients said the item was relevant to their experience.

Including numbness or tingling within the same item: We asked patients, "In your experience, do hand/foot numbness and tingling go together?" For hands, 18 of 27 patients (67%) stated that numbness and tingling went together. For feet, 20 of 26 patients (77%) stated that numbness and tingling went together. Patient 025 explained, "I'll be trying to go to sleep at night…if I move my feet or I touch anything, it comes back-that tingling and that numbness." During the cognitive interview, 2 of

Table 4 Neuropathy symptoms identified as most bothersome by patients (*n* = 28), *n* (%)

Symptom/issue identified as most bothersome	Number of patients who chose that symptom/issue
Numbness- feet	7 (25.0)
Discomfort/pain- feet	5 (17.8)
Cold sensitivity- eating/drinking	4 (14.2)
Cold sensitivity- hands/feet	3 (10.7)
Tingling- feet	3 (10.7)
Loss of hand function	2 (7.1)
Numbness- hands	2 (7.1)
Abnormal sensation- foot	1 (3.5)
Tingling- hands	1 (3.5)
Trouble walking	1 (3.5)
Muscle spasms- feet and legs	1 (3.5)

28 patients (7%) thought that the concepts of numbness and tingling should be separated into two questions. For both NTX1 and NTX2, 100% of patients (28 of 28) indicated they were confident or very confident in their ability to provide a response to the items as they were written; none of the patients found the questions to be confusing. When asked which questions were difficult to answer, patient 031 identified the additional questions 3-4, which separated numbness and tingling in the feet, as most difficult to answer: "Some of the neuropathy questions (were difficult) where you had separate sensations but they should go together, like tingling and numbness."

Item NTX3 - I feel discomfort in my hands: Over one third of patients (12 of 31, 39%) listed hand discomfort or pain as a symptom of their neuropathy during concept elicitation. Hand discomfort tended to interfere with everyday activities or "basically anything that involved my hands" (patient 033). Twenty-five of 28 (89%) cognitive interview participants said item NTX3 was relevant to their experiences.

Item NTX4 - I feel discomfort in my feet: Eighteen of 31 participants (58%) listed symptoms consistent with discomfort in the feet, including aches, pain, burning sensations, and abnormal sensations. Discomfort in the feet affected activities such as standing and driving. Patients also described feeling as if a small object was in their shoe or under their foot, which affected balance and walking. Twenty-five of 28 (89%) cognitive interview participants said the item was relevant to their experiences.

Discomfort *vs* pain: Patients overwhelmingly stated that "discomfort" was more consistent with neuropathy experienced in their hands than "pain" (23 of 28 patients, 82%). Likewise, 21 of 28 patients (75%) said that "discomfort" was more consistent with peripheral neuropathy experienced in their feet than "pain". Patients described discomfort as general, more constant, less severe and less likely to interrupt daily activities than pain. Pain was described as throbbing, hurting, more severe, and more likely to stop daily activities. According to patients: "Pain came sometimes but discomfort was always there." (PT 011) "I have more discomfort than pain." (PT 019) "Because my experience is numbness and tingling which is not painful, doesn't hurt, (it is) just annoying." (PT 023) "It is irritating as opposed to hurting." (PT 025).

Mean responses on the discomfort and pain items are shown in Figure 1. Thirteen of 28 patients (46%) had identical responses to the items referencing discomfort in the hands and pain in the hands. Of the 15 patients (54% of the sample of 28) whose responses were different, all 15 reported more hand discomfort than hand pain. Moreover, of these 15 patients, 11 (73%) reported "not at all" in response to "I have pain in my hands" while reporting levels of hand discomfort ranging from "a little bit" to "very much". Fewer patients (11 of 28, 39%) had identical scores for foot discomfort and foot pain. Sixteen of the 17 (94%) patients with differing scores reported more foot discomfort than pain. We investigated the significance of differences in mean responses using two-tailed *t*-tests assuming equal variance. The findings revealed significant group differences for NTX3 and Add-5 ($P = 0.007$) and NTX4 and Add-6 ($P = 0.016$).

Item NTX5 - I have joint pain or muscle cramps: Seven of 31 participants (23%) listed symptoms consistent with joint pain or muscle cramps. These symptoms were impactful to patient quality of life. For example, according to patient 023, "It was my

Table 5 Summary of support for the Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity scale items in patient concept elicitation data

Instrument content		Content validity support from patient interviews	Example quotations from patient concept elicitation interviews	Recommendation
NTX1	I have numbness or tingling in my hands	Strong	"It felt like there was a coating of wax over my hands" (PT 031). "It's like a dead feeling and you don't really have like complete-like if you're trying to pick up something, like a dime or something, you might not realize that you have it or don't have it" (PT 003). "Well, first it was my fingertips. And they were numb and tingly" (PT 006).	Retain the item. Strong support in concept elicitation data, cognitive interview data, and extant literature.
NTX2	I have numbness or tingling in my feet	Strong	"Just I can't feel (sometimes) I can't feel like if my feet are…cold or hot, I don't know, I just it's just numb, you know" (PT 026). "The best way I can describe it is walking on rice with pieces of broken glass in it. Yeah, I guess that's the best I way I can describe the tingling. It's constant, yeah...Fuzzy maybe feeling. Needle, sharp needle pain, because it's kind of a combination of those. So it's like a needle" (PT 031). ""It's mainly in my feet. I have tingling in my toes. A slight numbness that runs up past my ankles" (PT 011).	Retain the item. Strong support in concept elicitation data, cognitive interview data, and extant literature.
NTX3	I feel discomfort in my hands	Strong	"I can't open them [fingers]. There's probably like I said maybe- I mean they're almost open but they can't go flat, and if I try it, it will hurt more…a dull ache" (PT 008).	Retain the item. Strong support in concept elicitation data, cognitive interview data, and extant literature.
NTX4	I feel discomfort in my feet	Strong	"They just ache. All up and down, they ache, they hurt, it's uncomfortable, it's nagging…it hurts" (PT 018). "…...a tightness like you have a really, really tight shoe on. Like something really heavy is on your feet and you can't get it off. It feels like an intense weight. Your foot is being smashed" (PT 019). "If you're walking you feel like maybe there's something in your shoe or like something, did you step on something? And you really didn't. It's just an odd feeling...even like barefoot it will sometimes feel like you stepped on a sock or something" (PT 003).	Retain the item. Strong support in concept elicitation data, cognitive interview data, and extant literature.

NTX5	I have joint pain or muscle cramps	Moderate	"It's overall hand fatigue, it's joint pain. It's almost like repetitive motion, like I find that if I was out in a tractor all day and just the act of spinning the steering wheel constantly as I went across the field and operating the levers and everything on the tractor, that I needed to get my compression gloves on to do some compensating for that. And then at the end of the day my hands are just really, really fatigued" (PT 013).	Retain the item based on cognitive interviews and literature support as a severe symptom of oxaliplatin-induced neuropathy.
HI12	I feel weak all over	Moderate	"My feet and legs are always cold. And then I guess you could call weakness and I guess chronic pain in my feet and legs, lower legs" (P T 031).	Retain the item based on cognitive interviews and literature support as a symptom of oxaliplatin-induced neuropathy.
NTX6	I have trouble hearing	Weak	--	Retain the item based on cognitive interviews and literature support as a severe symptom of oxaliplatin-induced neuropathy.
NTX7	I get a ringing or buzzing in my ears	Weak	"I had ringing in my ears, but…I haven't noticed it in the past week. And the last oxaliplatin was June 19th, so I think it took about a month probably after oxaliplatin for the ringing in my ears to settle down" (PT 005).	Retain the item based on cognitive interview and literature support as a severe symptom of oxaliplatin-induced neuropathy.
NTX8	I have trouble buttoning buttons	Strong	"As the button is concerned, I had a very hard time grasping them and getting them through the button holes. It's a very frustrating and annoying task. I just kept on fumbling with them and not being able to properly grasp the buttons, and I would have to ask my wife to actually come in and button up my shirt" (PT 033).	Retain the item. Strong support in concept elicitation data, cognitive interview data, and extant literature.
NTX9	I have trouble feeling the shape of small objects when they are in my hand	Strong	"It takes me longer to do a lot of things. Even like if I'm reaching in my pocket to get something and there's multiple things in the pocket it's more difficult to go by feel on what I've grabbed" (PT 035).	Retain the item. Strong support in concept elicitation data, cognitive interview data, and extant literature.
An6	I have trouble walking	Strong	"I have to be hyper vigilant about stepping over sticks and watching my balance…There are times when I would say I trip over my own feet because I will step funny because of lack of sensation. So I have to be conscious of walking and making sure that I'm planting my feet squarely to avoid stumbling" (PT 013).	Retain the item. Strong support in concept elicitation data, cognitive interview data, and extant literature.

NTX10	I have pain in my hands or feet when I am exposed to cold temperatures	Strong	"It's just a…ultra-sensitivity to cold, anything, it was, it's like anything colder than my body temperature would either cause like pain or I couldn't hold, like in my hands, I wouldn't be able to hold things...And then for my feet…if I go into cold water or if I'm outside in the cold and my feet seem to get cold faster first before anything else, so...the cold sensitivity would be like touching something extremely hot, like you…your body reacts to pull away, you know. And then for my feet, it's actually painful when they get cold, when they're exposed to cold" (PT 012).	Retain the item. Strong support in concept elicitation data, cognitive interview data, and extant literature.
NTX11	I have difficulty breathing when I am exposed to cold temperatures	Moderate	"As the temperature of the air started to change, to breathe in was difficult-it became kind of painful even" (PT 006). "I would have to cover up my mouth and nose because if I breathed the cold air; it was like somebody was trying to strangle me. My throat would close up and it's like somebody had little daggers or needles they were sticking in my throat. It's a very horrible experience" (PT 033).	Retain the item based on cognitive interviews and literature support as a symptom of oxaliplatin-induced neuropathy.

calves that were cramping and it was, oh, I don't think I'd call it severe, but it was at least moderate to severe cramping. And it was pretty painful." Over half of cognitive interview participants (15 of 28, 54%) reported the item as relevant to their experience.

Item HI12 - I feel weak all over: Five of 31 concept elicitation participants (16.1%) mentioned feeling weak. Feeling weak was used to describe neuropathy symptoms in the feet, hands, legs, and feet. Others noted weakness when describing the impact of neuropathy symptoms, such as difficulty walking. For example, "Sometimes I have weakness in general, like I'll be walking and I feel like I'm going to trip" (patient 010). Twenty-two (78.6%) of cognitive interview participants said the item as relevant to their experience.

Item NTX6 - I have trouble hearing: Trouble hearing was not spontaneously mentioned as a neuropathy symptom in the concept elicitation data. Some support for this item came from the cognitive interviews; three of 28 (11%) participants reported the item "I have trouble hearing" as relevant. Of the 25 who said the item was less relevant, 22 did not have trouble hearing, and three reported having hearing problems prior to beginning treatment.

Item NTX7 - I get a ringing or buzzing in my ears: Ringing or buzzing in the ears was mentioned spontaneously by one patient in the concept elicitation interview. A quarter of cognitive interview participants (7 of 28, 25%) reported the item as relevant to their experiences. Of the 21 (75%) who said it was not relevant, 20 did not experience ear ringing or buzzing, and one was unsure if his ear ringing was a neuropathy symptom.

Item NTX8 - I have trouble buttoning buttons: Seven of 31 participants (23%) described limited fine motor function during concept elicitation that affected their ability to button buttons. The process of buttoning buttons was described as time consuming and frustrating, causing some to require assistance or avoid wearing clothing with buttons. "It (buttoning buttons) took more time and it was frustrating having fine motor function limited" (patient 020). A majority of cognitive interview participants (25 of 28, 89%) reported the item as relevant to their experience.

Item NTX9 - I have trouble feeling the shape of small objects when they are in my

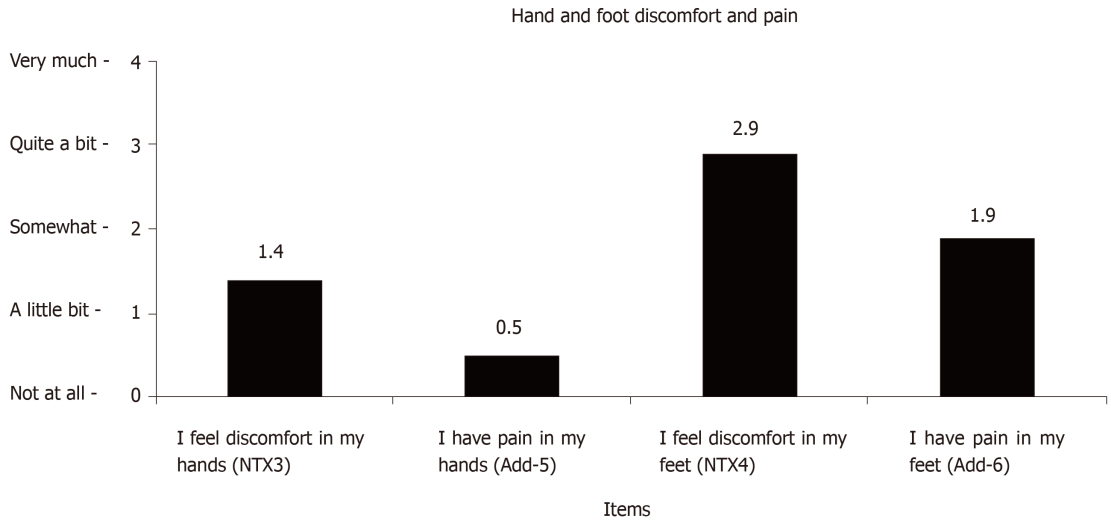

Figure 1 Mean scores of hand and foot discomfort and pain items.

hand: Ten of 31 concept elicitation participants (32%) listed symptoms consistent with trouble feeling the shape of small objects in their hand, including difficulties feeling small objects that resulted in problems grasping or holding on to small objects. Furthermore, 24 of 28 cognitive interview participants (86%) reported the item as relevant to their experience.

Item An6 - I have trouble walking: Six of 31 concept elicitation participants (19%) mentioned trouble walking because of their neuropathy. "Because of the tingling and numbness in my feet it's hard for me to find my balance, to figure out where my feet are in the ground. So I tend to be a little, you know, wobbly…kind of unsure footing" (patient 011). Patients' comments about trouble walking ranged from fear of falling, practicing caution while walking, and having to limit or avoid activities, such as exercise. Twenty-four of 28 (86%) cognitive interview participants stated that the item was relevant to their experience.

Item NTX10 - I have pain in my hands or feet when I am exposed to cold temperatures: Twenty-three of 31 concept elicitation participants (74%) reported hand or feet pain when exposed to cold temperatures. Some likened the pain to being shocked, "If I touch cold things, I get little zings going through my fingers. It intensifies if it's colder. Like if I pull something out of the freezer, sometimes I have to drop it because it's too cold" (patient 010). Patients reported limiting exposure to cold items and temperatures because of the pain and discomfort. All 28 (100%) cognitive interview participants reported the item as relevant to their experiences.

Item NTX11 - I have difficulty breathing when I am exposed to cold temperatures: Three of 31 concept elicitation participants (10%) listed difficulty breathing when exposed to cold temperatures as a symptom of neuropathy. They described feeling their throat close, throat spasms, or choking when exposed to cold temperatures. They also described feeling like needles were sticking in their throat. While relatively few patients listed difficulty breathing when exposed to cold temperatures in the concept elicitation interview, those who reported the symptom rated its importance to quality of life high (mean score=8). Moreover, 16 of 28 (57%) cognitive interview participants reported the symptom as relevant to their experience.

Coverage of all patient-reported concepts on the instrument: To ensure that the instrument adequately covers symptoms of importance to patients, we considered whether symptoms reported by at least 20% of the sample (Table 3) during concept elicitation were represented in the FACT/GOG-Ntx. Nine symptoms fit this criteria: hand tingling, feet tingling, hand numbness, feet numbness, cold sensitivity in the hands or feet, discomfort in the feet, discomfort in the hands, impaired fine motor skills, and joint pain or muscle cramps. Each of these symptoms is included in the FACT/GOG-Ntx.

DISCUSSION

In this qualitative study with 31 CRC patients experiencing peripheral neuropathy, tingling and/or numbness of the hands and feet were the most commonly experienced peripheral neuropathy symptoms. Additionally, almost 3 out of 4 participants reported cold sensitivity in their hands or feet. Neuropathy symptoms affecting the feet were most bothersome to patients.

The qualitative concept elicitation data, in combination with data from cognitive interviews and the literature, provide moderate to strong support for the content validity of 11 the 13 items of the FACT/GOG-Ntx-13. Two items - "I have trouble hearing" (NTX6) and "I get a ringing of buzzing in my ears" (NTX7), had limited support in our data. However, limited support is not surprising given that hearing impairment is a symptom of severe neuropathy, and oxaliplatin therapy may be discontinued or reduced prior to impacting hearing. We recommend retaining these items as indicators of severe neuropathy.

Patients related more with the term "discomfort" than "pain" when reporting neuropathy in their hands and feet. Quantitative responses to items with either "discomfort" or "pain" showed that patients report higher levels of discomfort relative to pain. These results are consistent with the original selection of the term "discomfort" over "pain" owing to the observation that discomfort is reported earlier than pain in the trajectory of emerging neuropathy. These findings also highlight the need for developers of other patient reported outcome measures to consider whether pain or discomfort is the best concept for their particular population or condition. We also considered whether including "numbness or tingling" in a single item was problematic due to the possibility that such items would be "double-barreled." Most patients reported experiencing the two symptoms together. Patient comments suggest that numbness and tingling tend to appear, increase, and decrease in similar ways; future work should confirm this observation. Most patients did not find the items on "numbness or tingling" in hands or feet to be confusing, although they did understand the distinction between the two symptoms. Only 2 patients (7.1%) stated that the concepts should be separated into two questions. Adding questions to the Ntx-13 that separate numbness and tingling is not likely to improve the measure or change responses to any measurable degree. Patients most often considered the last 7 days when responding to the questionnaire. The 7 d recall period is consistent with other PRO measures recommended for use in clinical oncology[30].

This study has a number of strengths, including a relatively large concept elicitation sample and a cognitive interview protocol that closely examined patient interpretation of key concepts. Our methods are consistent with Food and Drug Administration Guidance and other published guidelines for assessing content validity[29,31].

ARTICLE HIGHLIGHTS

Research background

A content valid assessment of neuropathy is needed for clinical research among colorectal cancer (CRC) patients receiving oxaliplatin. The authors assessed the content validity of the Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity (FACT/GOG-Ntx) scale among CRC patients who had received oxaliplatin. The measure exhibited good content validity. Moreover, patients reported that foot neuropathy was most bothersome for them. This study is significant because the authors provide evidence that the FACT/GOG-Ntx is suitable for use in clinical trials and other research studies of this population.

Research motivation

This study examines the neuropathy experiences of CRC patients and the appropriateness (*i.e.*, content validity) of a patient reported outcome measure of neuropathy. These topics are important to examine because patient reported outcome measures are needed to assess drug side effects in clinical trial settings.

Research objectives

The main objective was to test the content validity of FACT/GOG-Ntx. This objective was realized; the measure was found to have content validity and can be used in future research and clinical practice.

Research methods

The authors used semi-structured patient interviews to assess the FACT/GOG-Ntx. Semi-structured interviews entail using a set list of questions, administered by a trained interviewer. Interviews typical contain a combination of closed-ended and open-ended questions. By using pre-planned and spontaneous probing questions, the interviewer is able to gather a detailed description of the key topics from the perspective on the interviewee.

Research results

The qualitative concept elicitation data, in combination with data from cognitive interviews and the literature, provide moderate to strong support for the content validity of 11 the 13 items of the FACT/GOG-Ntx-13. Two items - "I have trouble hearing" (NTX6) and "I get a ringing of buzzing in my ears" (NTX7), had limited support in our data. However, limited support is not surprising given that hearing impairment is a symptom of severe neuropathy, and oxaliplatin therapy may be discontinued or reduced prior to impacting hearing. The authors recommend retaining these items as indicators of severe neuropathy.

Research conclusions

The FACT/GOG-Ntx has content validity for CRC patients receiving oxaliplatin. Patients related more with the term "discomfort" than "pain" when reporting neuropathy in their hands and feet. The FACT/GOG-Ntx has content validity for CRC patients receiving oxaliplatin. This study builds upon the body of evidence supporting the use of the FACT/GOG-Ntx in future research and clinical practice.

Research perspectives

Existing patient reported outcome measures can be tested for their validity in new, specific populations. The authors anticipate continued advancement in the use of patient reported measures in clinical research and in drug development. Future work on the use of patient reported outcomes measures in clinical practice is best suited for a combination of patient-focused, qualitative research and large, quantitative surveys to assess measurement properties.

ACKNOWLEDGEMENTS

We are grateful to the CRC Alliance (https://www.ccalliance.org/) for their assistance in recruiting patients to this study.

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2020 February 15; 12(2): 219-227

DOI: 10.4251/wjgo.v12.i2.219

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Observational Study

Clinical value evaluation of serum markers for early diagnosis of colorectal cancer

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Author contributions: Song WY, Zhang PJ, and Zhang R designed the study; Song WY, Zhang X, and Zhang Q performed the research; Song WY, Zhang PJ , and Zhang R analyzed the date; Song WY wrote the paper; Zhang PJ and Zhang R revised the manuscript for final submission; Song WY and Zhang X contributed equally to this study; Zhang PJ and Zhang R are the co-corresponding authors.

Supported by National Key R and D Program of China, No. 2016YFC0106604; National Natural Science Foundation of China, No. 81502591.

Institutional review board statement: The study was reviewed and approved by the Peking University Cancer Hospital and Institute review board.

Informed consent statement: All study participants or their legal guardian provided written informed consent prior to study enrollment.

Conflict-of-interest statement: We declare that we have no financial or personal relationships with other individuals or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature in any product, service and/or company

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Abstract

BACKGROUND

Early screening for colorectal cancer (CRC) is important in clinical practice. However, the currently methods are inadequate because of high cost and low diagnostic value.

AIM

To develop a new examination method based on the serum biomarker panel for the early detection of CRC.

METHODS

Three hundred and fifty cases of CRC, 300 cases of colorectal polyps and 360 cases of normal controls. Combined with the results of area under curve (AUC) and correlation analysis, the binary Logistic regression analysis of the remaining indexes which is in accordance with the requirements was carried out, and discriminant analysis, classification tree and artificial neural network analysis were used to analyze the remaining indexes at the same time.

RESULTS

By comparison of these methods, we obtained the ability to distinguish CRC from healthy control group, malignant disease group and benign disease group. Artificial neural network had the best diagnostic value when compared with binary logistic regression, discriminant analysis, and classification tree. The AUC of CRC and the control group was 0.992 (0.987, 0.997), sensitivity and specificity were 98.9% and 95.6%. The AUC of the malignant disease group and benign group was 0.996 (0.992, 0.999), sensitivity and specificity were 97.4% and 96.7%.

CONCLUSION

Artificial neural network diagnosis method can improve the sensitivity and

that could be construed as influencing the position presented in or the review of the manuscript.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement – checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Manuscript source: Unsolicited manuscript

Received: December 18, 2019
Peer-review started: December 18, 2019
First decision: January 13, 2020
Revised: January 17, 2020
Accepted: February 8, 2020
Article in press: February 8, 2020
Published online: February 15, 2020

P-Reviewer: Snowdon VK, Takamatsu S, Voigt M
S-Editor: Zhang L
L-Editor: A
E-Editor: Ma YJ

specificity of the diagnosis of CRC, and a novel assistant diagnostic method was built for the early detection of CRC.

Key words: Colorectal cancer; Serum; Index; Biomarker; Artificial neural network

Core tip: We aimed to combine the serum index together by several multiparameter method, such as, the binary logistic regression, discriminant analysis, classification tree and artificial neural network analysis. Finally, a multiparameter diagnostic model based on artificial neural network which showed better diagnostic value was built for the early detection of colorectal cancer.

Citation: Song WY, Zhang X, Zhang Q, Zhang PJ, Zhang R. Clinical value evaluation of serum markers for early diagnosis of colorectal cancer. *World J Gastrointest Oncol* 2020; 12(2): 219-227
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/219.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.219

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer[1] and the fourth major cause of cancer-related deaths worldwide. CRC has a high incidence and high mortality rate and is a public health burden in most industrialized countries. In recent years, the incidence of CRC in Asia is rising rapidly[2]. In eastern Asia, the incidence of countries, such as China, Japan, South Korea and Singapore, has increased two to four times in recent decades. Among Asian ethnic groups, the incidence of CRC in China is significantly higher than that of other ethnic groups. According to the 2003 China Cancer Database, CRC is one of the three fastest growing morbidity cancers[1].

CRC is a common malignant tumor in the gastrointestinal tract. Early symptoms are not obvious. As cancer increases, it shows changes in bowel habits, blood in the stool, diarrhea, alternating diarrhea or constipation, local abdominal pain and other symptoms. In the advanced stage, CRC shows anemia and body weight loss or other systemic symptoms. A typical CRC is developed from a focal change in benign, precancerous polyps. These polyps are local growths or the accumulation of abnormal cells in the intestinal mucosa that protrude into the intestinal lumen[3]. With time, the dividing cells in these polyps may accumulate enough genetic changes to gain the ability to invade the intestinal wall, which is a hallmark of CRC that may eventually become more susceptible to change and spread to regional lymph nodes, eventually spread to distant transfer sites[4]. This multistep development process accumulates over time and allows early precancerous polyps to be screened and tested before the average risk of CRC is cancerous, which may lead to a dramatic decline in the incidence of CRC[5].

Clinical screening for CRC involves (1) Colonoscopy. Many studies have confirmed that colonoscopy, as a useful screening tool, can reduce the incidence of CRC by 76% and mortality by 65%[6]; (2) Sigmoidoscopy. Compared with colonoscopy, sigmoidoscopy has the advantages of low cost, short preparation time and no need for sedation[7]; (3) Computed tomography colon imaging; (4) FOBT and fecal immunochemical tests. These tests have advantages of low cost, noninvasiveness and good tolerance. FOBT and fecal immunochemical tests are widely used on a global scale, but are also susceptible to food, drugs and other factors, and the stool collection is inconvenient, resulting in a high false positive rate[8]; and (5) Screening for biomarkers: (a) Carcinoembryonic antigen (CEA); (b) Circulating tumor cell; (c) Circulating tumor DNA/RNA; and (d) Abnormal DNA methylation[9].

Early screening for CRC plays an important role in combating and controlling the growth of CRC morbidity and mortality worldwide[10]. However, the currently available screening methods are severely inadequate because of their high cost and cumbersome preparation procedures that ultimately result in a low participation rate. People are often reluctant to use colonoscopy[11]. Therefore, developing an unconventional method of testing based on blood biomarkers as the first test procedure may be the ideal method. This method will make it possible to identify high-incidence individuals among the general public. Colonoscopy will become the

second type of examination and continue to screen high-incidence populations. This strategy will encourage participation rates and will help achieve the goal of early screening for CRC and will reduce the globally expected increase in CRC incidence[12]. Blood-based screening experiments attract the public because of their noninvasive and low patient harm features. This screening is easy to perform and can be repeated in shorter time intervals, which in turn leads to higher participation rates[13].

In this study, we conducted a retrospective analysis. Through *t* test, ROC curve analysis, binary logistic regression analysis, and simultaneous use of discriminant analysis, classification tree and artificial neural network analysis of multiple methods combined detection were used to determine the tumor marker diagnostic value for detection of CRC.

MATERIALS AND METHODS

Patients selection

The serum samples of the patients involved in this study were from blood samples of patients and confirmed by imaging and pathology. According to the blood collection record, all serum biochemical and immunological indexes of the CRC disease group and the healthy control group were counted from the medical records of each subject for the subsequent statistical analysis. As shown in Table 1, these analyses included 300 cases of colorectal benign polyps and 350 cases of malignant colorectal cancer (166 cases in early stage and 136 in late stage, 48 cases unconfirmed). All patients had clear imaging and pathological diagnosis and did not receive radiotherapy, chemotherapy or other immunotherapy before surgery. A total of 360 patients in the healthy control group received physical examination, were examined by tumor markers and imaging examinations, had no diseases related to the study, and the tumor markers and imaging examinations were all qualified. The serum index in the hospital was collected and used to analyze.

Statistical analysis

Statistical analysis was performed on comparing colorectal cancer and healthy controls, malignant disease group and benign disease group. Data from various indexes of colorectal cancer and healthy control groups as well as malignant disease groups and benign disease groups were compared by *t* test. The diagnostic value was evaluated by the area under the curve of the ROC curve, and the cutoff value was determined by the Youden index. The combination of indexes is analyzed by statistical methods, such as binary logistic regression analysis, discriminant analysis, classification tree and artificial neural network. All data were statistically analyzed by SPSS (version 20.0, SPSS Inc. Chicago, IL) software. All statistical tests were bilateral, and $P < 0.01$ was considered statistically significant.

RESULTS

Significant analysis and ROC curve analysis of CRC and healthy control group, malignant disease group and benign disease group

There were significant differences in 32 indexes between CRC and healthy controls. There were significant differences in 37 indexes between the malignant disease group and the benign disease group. The ROC curves were performed on 36 indexes with significant differences in colorectal cancer and healthy controls and 42 indexes with significant differences between the malignant disease group and the benign disease group. Among these indexes, 32 indexes of colorectal cancer and healthy control group had *P* values < 0.01. There were 37 indexes in the malignant disease group and the benign disease group with *P* values < 0.01.

Among these results, the largest area under the curve in CRC and healthy control group were for RDV and CEA, with area under the curve values of 0.781 and 0.846, respectively. When the RDV cutoff value was 12.625, the sensitivity and specificity were 61.7%, 82.2%; when the CEA cutoff value was 1.915, the sensitivity and specificity were 81.4%, 71.7%, respectively. The largest area under the curve in the malignant disease group and the benign disease group were for CRP and H-FABP, with area under the curve values of 0.798 and 0.762, respectively. When the CRP cutoff value was 0.145, the sensitivity and specificity were 62.0%, 89.7%; when the H-FABP cutoff value was 1.965, the sensitivity and specificity were 73.7%, 88.3%, respectively.

Table 1 The clinical characteristics of patients with colorectal cancer, *n* (%)

	Malignant (*n* = 350)	Benign (*n* = 300)	Controls (*n* = 360)
Age, yr			
< 40	10 (2.86)	16 (5.33)	63 (17.5)
40-60	149 (42.57)	143 (47.67)	258 (71.67)
≥ 60	191 (54.57)	141 (47)	39 (10.83)
Gender			
Male	217 (62)	200 (66.67)	212 (58.89)
Female	133 (38)	100 (33.33)	148 (41.11)
T			
T1-2	67 (19.14)		
T3-4	235 (67.14)		
Lymph node			
Yes	165 (47.14)		
No	119 (34)		
Metastasis			
Yes	30 (8.57)		
No	320 (91.43)		
0	2 (0.58)		
TNM Stage			
I	56 (16)		
II	108 (30.86)		
III	106 (30.29)		
IV	30 (8.57)		
Non	48 (13.7)		

Binary logistic analysis of CRC and healthy control group, malignant disease group and benign disease group

Seventy percent of the data from 32 indexes in CRC and healthy controls and 37 indexes in the malignant disease group and benign disease group were used for the establishment of a binary logistic regression analysis model. As shown in the Figure 1A, the area under the curve for the CRC and the healthy control group was 0.989 (0.982, 0.995). When the cutoff value was 0.479, its sensitivity and specificity were 90.2% and 90.1%, respectively. As shown in the Figure 1B, the area under the curve of the malignant disease group and the benign disease group was 0.929 (0.901, 0.958), and when the cutoff value was 0.329, its sensitivity and specificity were 98.4% and 95.7%, respectively. Binary logistic regression analysis was more effective in distinguishing colorectal cancer from healthy controls than in malignant and benign disease groups.

Discriminant analysis of CRC and healthy control group, malignant disease group and benign disease group

Seventy percent of the data from 32 indexes in CRC and healthy controls and 37 indexes in malignant disease groups and benign disease groups were used to establish a discriminant analysis model. As shown in Figure 2A, the area under the curve for CRC and healthy controls was 0.961 (0.946, 0.977), and when the cutoff value was 0.33, its sensitivity and specificity were 92.2% and 89.3%, respectively. As shown in Figure 2B, the area under the curve for the malignant disease group and the benign disease group was 0.973 (0.960, 0.986), and when the cutoff value was 0.467, its sensitivity and specificity were 91% and 94.8%, respectively. Discriminant analysis differentiated between the malignant disease group and the benign disease group better than the CRC and healthy control group.

Classification tree analysis of CRC and healthy control group, malignant disease group and benign disease group

The 70% data for 32 indexes in the CRC and healthy control groups and the 37 indexes in the malignant disease group and the benign disease group were established by the classification tree model, and 30% of the data were used for model validation. As shown in Figure 3A, the prediction accuracy rate of the healthy control group was

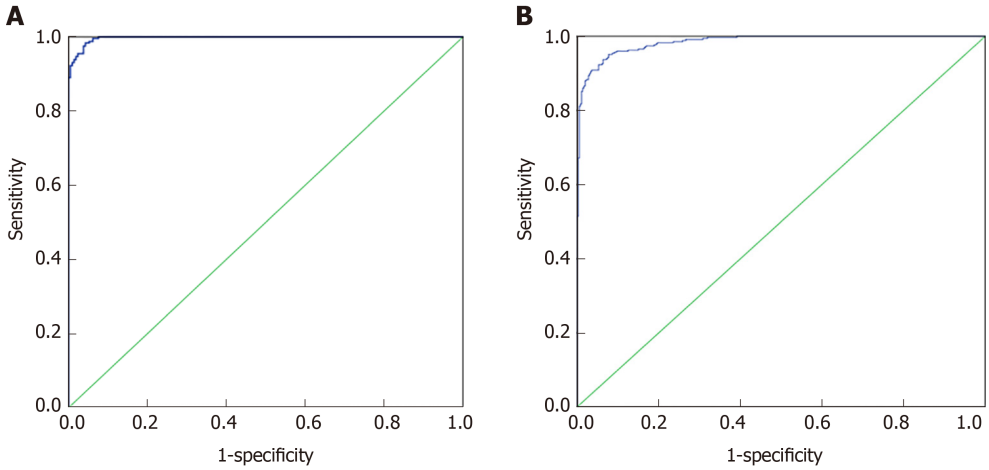

Figure 1 Binary Logistic analysis of colorectal cancer and healthy control group, malignant disease group and benign disease group. A: ROC curve of the binary logistic regression analysis model of the colorectal cancer and healthy control group, respectively; B: ROC curve of the binary logistic regression analysis model of the colorectal cancer and healthy control group.

85.5%, the prediction accuracy rate of the CRC group was 77.1%, and the overall prediction accuracy rate was 81.3%. The area under the curve of CRC and the healthy control group was 0.924 (0.905, 0.944), and when the cutoff value was 0.4324, its sensitivity and specificity were 83.7% and 85.3%, respectively. As shown in Figure 3B, the predictive accuracy rate was 82.8% in the benign disease group, 75.2% in the malignant disease group, and 78.8% in the overall prediction rate. The area under the curve of the malignant disease group and benign disease group was 0.922 (0.903, 0.941), and when the cutoff value was 0.564, its sensitivity and specificity were 80.6% and 86%, respectively. The classification tree analysis distinguished the CRC from the healthy control group basically in the same way as the malignant disease group and the benign disease group.

Artificial neural network analysis of CRC and healthy control group, malignant disease group and benign disease group

Seventy percent of the data from 27 indicators in CRC and healthy controls and 30 indicators in the malignant disease group and benign disease group were used for artificial neural network model establishment, and 30% of the data were used for model validation. As shown in Figure 4A, the prediction accuracy rate of the healthy control group was 88.6%, the prediction accuracy rate of the CRC group was 84.8%, and the overall prediction accuracy rate was 87.8%. The area under the curve of CRC and the healthy control group was 0.992 (0.987, 0.997), and when the cutoff value was 0.065, its sensitivity and specificity were 98.9% and 95.6%, respectively. As shown in Figure 4B, the predictive accuracy rate was 90.0% in the benign disease group, 88.9% in the malignant disease group, and 89.4% in the overall prediction. The area under the curve of the malignant disease group and benign disease group was 0.996 (0.992, 0.999), and when the cutoff value was 0.443, its sensitivity and specificity were 97.4% and 96.7%, respectively. The effect of artificial neural network analysis on CRC and the healthy control group was basically the same as that of the malignant disease group and the benign disease group, but the prediction accuracy rate was higher than that of the classification tree method.

Through comparison of these four methods, we obtained the ability to distinguish colorectal cancer from healthy control group, malignant disease group and benign disease group: Artificial neural network > binary logistic regression > discriminant analysis > classification tree.

DISCUSSION

Colorectal cancer is the second most common cancer disease in women and the third most common cancer in men. The number of new cases worldwide was estimated as 1.2 million in 2008, and the deaths were approximately 600000[14].

Tumor markers can be present in cells, tissues, blood, and feces and can thus be qualitatively or quantitatively detected by related techniques[15]. With the development of molecular diagnosis, lots of novel detection method have been developed[16-20]. Tumor markers can be an important tool for cancer detection and

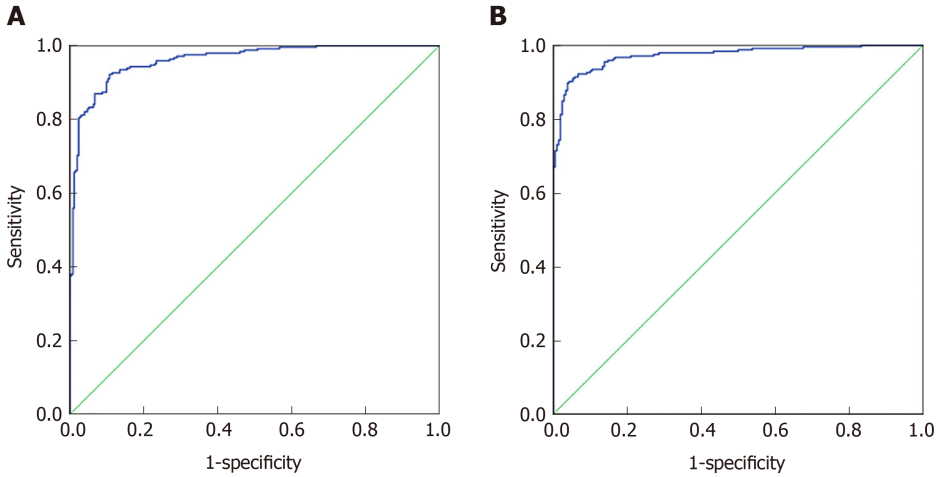

Figure 2 Discriminant analysis results of colorectal cancer and healthy control group, malignant disease group and benign disease group. A: ROC curve of the discriminant analysis model of the colorectal cancer and healthy control group, respectively; B: ROC curve of the discriminant analysis model of the colorectal cancer and healthy control group.

patient prognosis. Gene mutations are the main factor in the development of CRC, and many discoveries have been made in recent years. APC, VEGF, Septin9 and other DNA in feces, blood and other biological fluids can be used as the primary detection and prognostic indicator[21-25]. In addition to genetic alterations such as mutations, microsatellite instability[26,27] and hypermethylation of tumor suppressor genes in promoter regions have also been extensively studied[28,29]. MicroRNAs and their putative target gene dysregulation may affect the development of colorectal cancer[30]. Protein markers, such as IMP3[31,32] and COX-2[33], have attracted much attention in CRC screening, and their concentrations may be related to CRC[34]. There are very few tumor markers that simultaneously satisfy high sensitivity and specificity, mainly because tumor markers are difficult to distinguish between benign diseases and malignant diseases when the levels are elevated[35].

A large number of studies have found that the clinical significance of detecting the increase[15] in the level of a single tumor marker is very limited. Therefore, people have improved the diagnostic value of tumor markers by two methods: continuous detection and joint detection. Continuous testing is used in the detection of malignant tumors, but it is mainly used for the detection of therapeutic effects and early diagnosis of prognosis. Joint detection is a very promising as an early detection method for malignant tumors by detecting multiple indicators to improve the sensitivity and specificity of tumor marker diagnosis, such as binary logistic regression analysis, discriminant analysis, classification tree analysis, and artificial neural network, which have improved the shortcomings of tumor markers that are difficult to simultaneously meet the sensitivity and specificity. Several indicators are combined, and statistical methods are used to improve the diagnostic value of tumor markers.

In this study, the diagnostic value of the combined diagnostic analysis for distinguishing between healthy controls and disease groups was superior to that of single-index tests; the diagnostic value of combined diagnostic analysis for distinguishing between benign disease groups and malignant disease groups was significantly better than the single-index test. Through comparison of these four methods, we obtained the ability to distinguish colorectal cancer from healthy control group, malignant disease group and benign disease group: Artificial neural network > binary logistic regression > discriminant analysis > classification tree.

However, there are still some limitations in our study. First, the sample size in our study was relatively small, and it may affect the results of our study. Second, although we have built a multiparameter diagnostic model, and it has better diagnostic value when compared with the conventional biomarker, but the diagnostic model has not been validated. Third, the diagnostic value should be performed on multi-center and larger sample size to validate its diagnostic value.

In conclusion, through multiparameter joint diagnostic analysis, we found that the combined diagnosis method can improve the sensitivity and specificity of the diagnosis, but some joint diagnosis methods may not be improved. Therefore, the optimal strategy is determined by comparing various joint diagnosis methods, followed by verification of the sample and confirmation of its value for use.

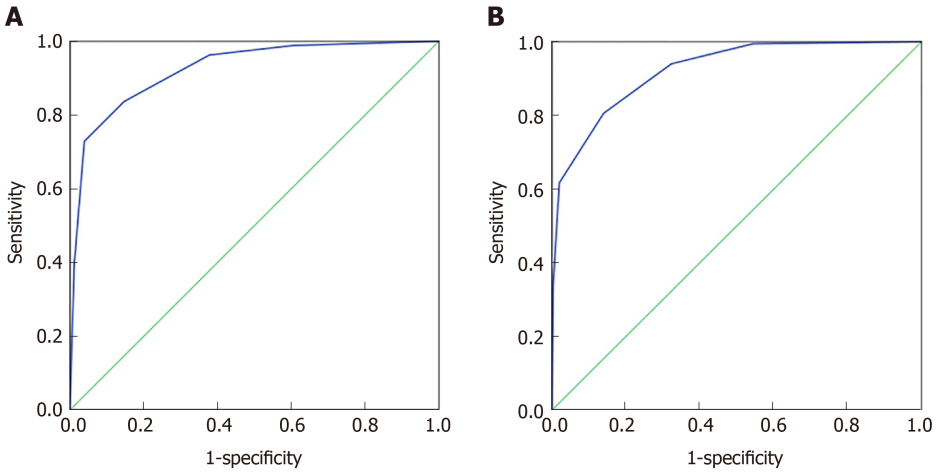

Figure 3 Classification tree analysis results of colorectal cancer and healthy control group, malignant disease group and benign disease group. A: ROC curve of the classification tree analysis model of the colorectal cancer and healthy control group, respectively; B: ROC curve of the classification tree analysis model of the colorectal cancer and healthy control group.

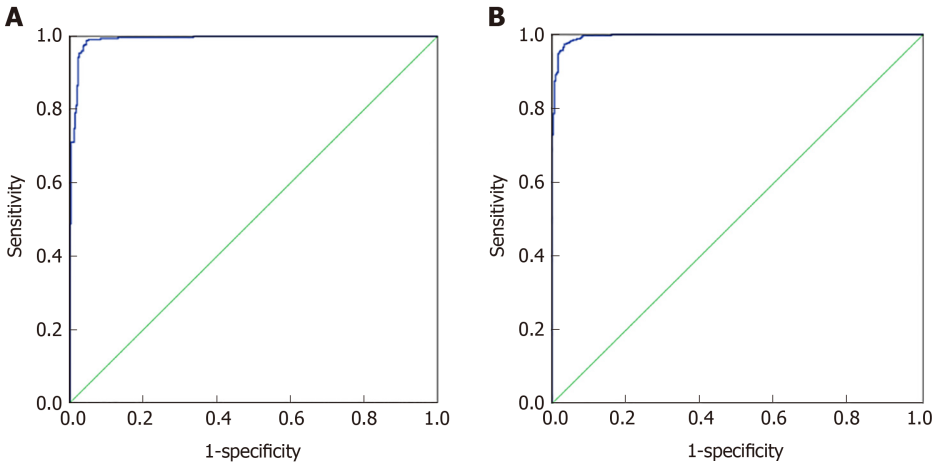

Figure 4 Artificial neural network analysis results of colorectal cancer and healthy control group, malignant disease group and benign disease group. A: ROC curve of the artificial neural network analysis model of the colorectal cancer and healthy control group, respectively; B: ROC curve of the artificial neural network analysis model of the colorectal cancer and healthy control group.

ARTICLE HIGHLIGHTS

Research background

Early screening for colorectal cancer (CRC) is important in clinical practice. However, the currently methods are inadequate because of high cost and low diagnostic value.

Research motivation

Blood-based screening method attract the public because of their noninvasive, and multiparameter method was demonstrated to increase the diagnostic value.

Research objectives

We aimed to conduct a retrospective analysis. By multiparameter methods combined detection were used to determine the tumor marker diagnostic value for detection of CRC.

Research methods

350 CRC, 300 colorectal polyps and 360 normal controls were enrolled. Combined with the results of area under curve, the binary Logistic regression analysis, and discriminant analysis, classification tree and artificial neural network were used to analyze the diagnostic value.

Research results

For distinguishing CRC from healthy control group, malignant disease group and benign disease group. Artificial neural network had the best diagnostic value when compared with the other methods. The area under the curve of CRC and the control group was 0.992 (0.987, 0.997),

sensitivity and specificity were 98.9% and 95.6%. The area under the curve of the malignant disease group and benign group was 0.996 (0.992, 0.999), sensitivity and specificity were 97.4% and 96.7%.

Research conclusions

Artificial neural network diagnosis method can provide a novel assistant diagnostic method was built for the early detection of CRC.

Research perspectives

Although we have built a multiparameter diagnostic model, the sample size was relatively small, and the diagnostic model has not been validated. Multi-center and larger sample size to validate its diagnostic value.

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World J Gastrointest Oncol 2020 February 15; 12(2): 228-236

DOI: 10.4251/wjgo.v12.i2.228

ISSN 1948-5204 (online)

SYSTEMATIC REVIEWS

Yttrium-90 radioembolization for unresectable hepatic metastases of breast cancer: A systematic review

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Author contributions: Feretis M and Solodkyy A wrote the paper, Feretis M and Solodky A read and approved the final manuscript.

Conflict-of-interest statement: All the authors declare that they have no competing interests.

Manuscript source: Invited manuscript

Received: October 8, 2019
Peer-review started: October 8, 2019
First decision: November 11, 2019
Revised: December 19, 2019
Accepted: January 6, 2020
Article in press: January 6, 2020
Published online: February 15, 2020

P-Reviewer: Koukourakis GV
S-Editor: Zhang L

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Abstract

BACKGROUND

Liver metastases secondary to breast cancer are associated with unfavourable prognosis. Radioembolization with ytrrium-90 is an emerging option for management of liver metastases of breast cancer when other systemic therapies have failed to achieve disease control. However, unlike the case of other liver tumours (colorectal/melanoma metastases/cholangiocarcinoma), its role in the management of breast liver metastases is yet to be elucidated.

AIM

The aims of this systematic review were to (1) assess the effect of radioembolization with yttrium-90 on tumour response; and (2) to estimate patient survival post radioembolization.

METHODS

The review was performed using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses. A systematic literature search was performed using the PubMed and EMBASE databases from January 2007 to December 2018. The initial search yielded 265 reports which were potentially suitable for inclusion in this review. Studies published in English reporting at least one outcome of interest were considered to be suitable for inclusion. Conference abstracts; case reports, animal studies and reports not published in English were excluded from this review. Data was retrieved from each individual report on the name of primary author, year of publication, patient demographics, type of microspheres used, radiation dose delivered to tumour, duration of follow-up, disease control rate (%), tumour response, and overall patient survival.

RESULTS

The final number of studies which met the inclusion criteria was 12 involving 452 patients. There were no randomized controlled trials identified after the literature

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search. The age of the patients included in this review ranged from 52 to 61 years. The duration of the follow up period post-radioembolization ranged from 6 to 15.7 mo. The total number of patients with breast metastases not confined to the liver was 236 (52.2%). Cumulative analysis revealed that radioembolization with yttrium-90 conferred tumour control rate in 81% of patients. Overall survival post-radioembolization ranged from 3.6 to 20.9 mo with an estimated mean survival of 11.3 mo.

CONCLUSION
Radioembolization with ytrrium-90 appears to confer control of tumour growth rate in most patients, however its effect on patient survival need to be elucidated further. Furthermore, quality evidence in the form of randomized trials is needed in order to assess the effect of radioembolization in more depth.

Key words: Breast cancer; Liver metastases; Yttrium-90; Radioembolization; Survival

Core tip: This is the first systematic review on the subject of liver radioembolization with yttrium-90 for breast metastases. Our paper reports cumulative findings of the 12 studies included on two important outcomes that of tumour response to embolization and patient survival. The paper summarises the current evidence available in the field and also makes recommendations for future areas of research in clinical practice.

Citation: Feretis M, Solodkyy A. Yttrium-90 radioembolization for unresectable hepatic metastases of breast cancer: A systematic review. *World J Gastrointest Oncol* 2020; 12(2): 228-236
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/228.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.228

INTRODUCTION

Breast cancer (BC) is the most common cancer in women and is associated with a life-time risk of incidence of 10%-15%[1,2]. However, the presence of *BRCA1* or *BRCA2* genes increases the life-time risk to 50%[3]. Breast cancer metastases will develop in up to 50% of patients with bone (85%), liver (50%), and lungs (20%) being the commonest sites[4,5]. The average 5-year survival rate for patients with breast cancer is 90% but if the cancer has spread to a distant part of the body, the 5-year survival rate drops dramatically to 27%[6]. Median survival for patients with liver metastases is generally very poor ranging from 4-21 mo[7,8].

The treatment options available for patients with liver metastases are limited. Palliative systemic chemotherapy is the commonest approach to metastatic breast cancer aiming to prolong survival. Resection of liver metastases in breast cancer has not been widely adopted perhaps due to the presence of multi-segmental liver disease at the time of diagnosis[9].

Transarterial radioembolization with yttrium-90 (TARE) microspheres offers an alternative radiotherapy option in the management of primary and secondary intrahepatic tumours[10]. Yttrium-90 microspheres are injected into the hepatic artery feeding the tumour and emit radiation at a local level. The advantage of TARE, in contrast to non-selective radiotherapy, is the ability to deliver high dose radiation to the tumour with minimal collateral damage to the normal liver parenchyma[11]. Liver radioembolization with yttrium-90 has been previously used to manage unresectable intrahepatic cholangiocarcinoma, colorectal and melanoma liver metastases[12-14]. The role of TARE in the management pathway of breast liver metastases is yet to be elucidated.

The purpose of this review was to systematically review the literature on the role of TARE in the management of breast liver metastases and summarise all evidence available on treatment response and patient survival. The primary outcomes of interest of this study were (1) to assess tumour response to TARE; and (2) to estimate overall patient survival following TARE as reported in the literature.

MATERIALS AND METHODS

The Preferred Reporting Item for Systematic Reviews and Meta-analyses statements were followed to conduct this systematic review[15].

Literature search

Published English-language manuscripts were considered for review and inclusion in this study. A systematic literature search was performed in PubMed and EMBASE databases from January 2007 to December 2018. The following search terms were used in order to identify the relevant bibliography: "yttrium" or "yttrium-90" or "Y90" or "radio-embolization" and "breast". All full text studies, and abstracts identified were screened independently by the two authors in order to identify those concerning transarterial radio-embolization with yttrium-90 (TARE) of breast liver metastases. The PubMed function" related studies" was used to broaden the search and the reference list of all potentially relevant studies was analysed.

Inclusion and exclusion criteria

Studies published in the English-language reporting at least one of the primary outcomes of interest were included in this review. Conference abstracts; case reports, animal studies and reports not published in English were excluded from this review. The final decision regarding study eligibility for inclusion in this review was reached by mutual agreement between the two authors.

Data extraction and outcomes of interest

Data of interest from each study were extracted using standardised data collection database. The following information was extracted from each study: Name of primary author, year of publication, patient demographics, duration of follow-up, disease control rate (%), tumour response, type of spheres used and overall patient survival. Data was extracted by each of the two authors independently for data validation purposes.

Statistical analysis

Descriptive statistics (absolute frequencies, percentages and mean or median values) were used to report study and patient data. Due to the high heterogeneity among studies and the lack of randomized controlled trials, performing a meta-analysis was not deemed to be appropriate.

RESULTS

Studies included

The literature search initially yielded 265 reports from January 2007 to December 2018. After screening the titles and abstracts of the reports identified a total number of 12 cohort studies were included in this systematic review (Figure 1). There were no randomized trials identified after the literature search.

Demographics and treatment procedures

The total number of patients originating from the 12 studies included was 452. Patient baseline demographic characteristics from the reports included in this review are summarised in Table 1. The age of the patients included in this review ranged from 52 to 61 years[16-27]. Data on the number of patients with extra-hepatic disease present at the time of radioembolization was available in 9/12 studies[17-19,21-25,27]. The total number of patients with breast metastases not confined to the liver was 236 (52.2%). The type of microspheres used to deliver the radioembolization to the hepatic metastases was clearly identifiable in 10 studies[16-19,21-24,26,27]. Currently there are two types of commercially available yttrium-microspheres. Resin microspheres (SIR-spheres, SIRTex Medical Limited, Sydney, Australia) were used in six studies whereas glass microspheres (TheraSphere, MDS, Nordion Inc., Ottawa, Canada) were used in 2 studies. In 2 of the studies included patients received treatment by a combination of resin and glass microspheres[26,27]. Data on the dose of yttrium-90 delivered to the patients was extractable from 9 studies[16,17,19,21-23,25-27]. The radiation dose delivered to the hepatic metastases varied from 0.8-2.1GBq (Table 1).

Tumour response and survival

The duration of the follow up period post-radioembolization was reported clearly in 4 studies (range 6-15.7 mo)[17,19,21,22]. Data on tumour/disease response to radioembolization and patient survival is summarised in Table 2. Data on tumour response to Ytrrium-90 treatment was retrievable from 11 studies included in this

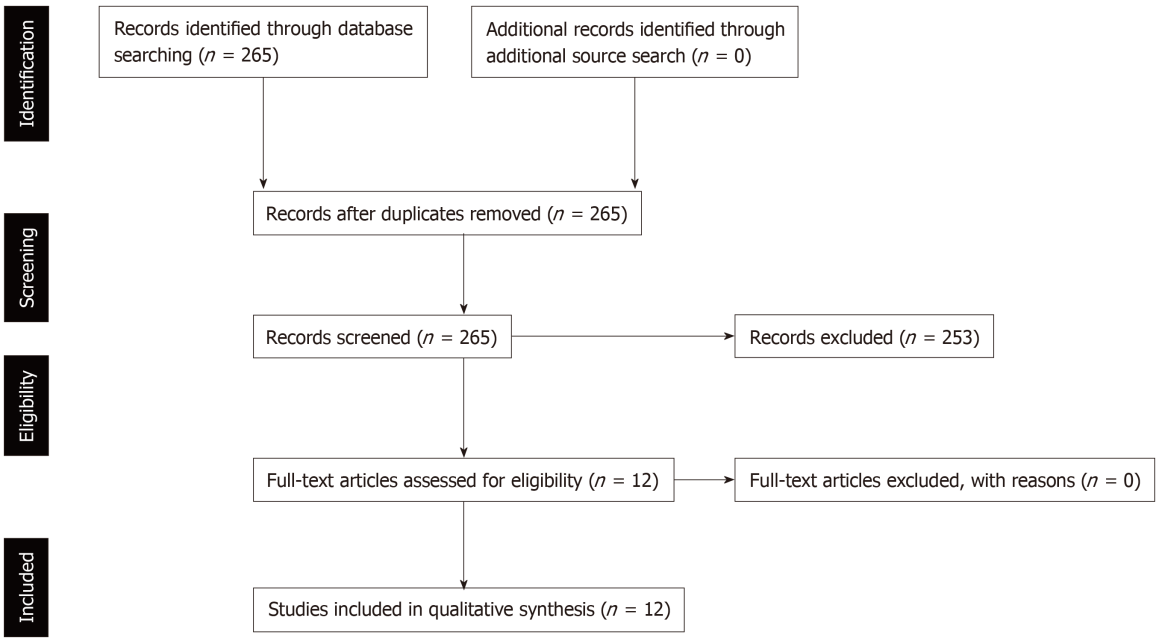

Figure 1 The Preferred Reporting Item for Systematic Reviews and Meta-analyses flow diagram of the studies included in this review.

review[16,17,19-27]. Tumour response to radioembolization, defined as tumour appearance on follow-up versus baseline imaging, was described in all 12 studies included [data available on 357/452 subjects, (81%)]. Tumour response was evaluated using the Response Evaluation Criteria in Solid Tumours (RECIST, *n* = 7 studies), the modified Response Evaluation Criteria in Solid Tumours (mRECIST, *n* = 1 study) or the World Health Organization (WHO) classification method (*n* = 2 studies)[28-30]. Two further studies did not provide information on the criteria used to assess response to treatment[18,25]. In summary, according to the WHO/RECIST criteria, patients are sub-categorized in four groups when comparing post treatment imaging with baseline imaging for up to two target lesions: (1) Complete response (CR) if all lesions disappear; (2) Partial response (PR) if the sum of the longest diameters decreases at least 30%; (3) Stable disease (SD) if neither partial response or progressive disease is present; and (4) Progressive disease (PD) if the sum of the longest diameters increases by at least 20%[28-30]. Following radioembolization, disease control rate, calculated as the sum of CR + PR + SD, was achieved in 282 patients (77%, Table 2). Post-radioembolization imaging revealed CR in 30 subjects (8.2%, data available from 5 studies); PR in 113 subjects (30.8%, data available from 9 studies); SD in 94 subjects (26%, data available from 8 studies) and PD in 49 subjects (13.4%, data available from 10 studies). Patient survival post- radioembolization was reported in 9 studies. Overall survival post-radioembolization ranged from 3.6 to 20.9 mo with an estimated mean survival of 11.3 mo.

DISCUSSION

In this report the relevant medical literature was systematically reviewed and the results of 12 studies are summarised. The primary outcomes of this review were survival and radiological response to radioembolization with Yttrium-90 microspheres for inoperable breast liver metastases. In summary, data from the studies included has demonstrated that radioembolization of breast cancer liver metastases with yttrium-90 confers a disease control rate of 81% with an estimated mean survival of 11.3 mo.

The development of liver metastases from breast cancer is associated with poor prognosis. Hepatic resection is a potential treatment option for patients, but unfortunately in the vast majority of cases the disease is unresectable at the time of diagnosis of liver metastases[31]. Other liver-directed therapies have been previously attempted for liver-only disease with the primary aim of palliating and prolonging survival. These treatments include radiofrequency and microwave ablation[32,33], transarterial chemoembolization[34] and stereotactic body radiotherapy[35]. Despite employing these treatment modalities, the reported median survival in patients with liver metastases remains poor ranging from 5-12 mo[8,36].

Table 1 Patient and radioembolization characteristics of the original reports included in this review

Ref.	Number of patients with breast cancer	Mean age (yr)	Number of patients with extrahepatic disease	Type of microsphere used	Activity infused (GBq)
Bangash *et al*[16], 2007	27	52	N/A	Glass	2.05
Coldwell *et al*[17], 2007	44	58	29 (66%)	Resin	2.1[1]
Stuart *et al*[18], 2008	7	N/A	1 (14%)	Resin	N/A
Jakobs *et al*[19], 2008	30	58	17 (57%)	Resin	1.9
Cianni *et al*[20], 2010	32	N/A	N/A	N/A	N/A
Haug *et al*[21], 2012	58	58	38 (65%)	Resin	1.8
Saxena *et al*[22], 2014	40	54.4	24 (60%)	Resin	1.67
Gordon *et al*[23], 2014	75	53.7	58 (77%)	Glass	1.52
Bagni *et al*[24], 2015	17	59.2	10 (59%)	Resin	N/A
Fendler *et al*[25], 2016	81	61[1]	54 (67%)	N/A	1.6[1]
Pieper *et al*[26], 2016	44	56.1	N/A	Resin = 56, Glass = 13	1.35
Chang *et al*[27], 2018	30	55*	5 (17%)	Resin = 46, Glass = 3	0.8[1]

[1]Median value as reported in the original report. N/A: Data not available.

TARE with Yttrium-90 is an increasingly popular treatment choice in patients with unresectable liver involvement. It is a combination of embolization and radiotherapy techniques. During the procedure radioactive microspheres are injected *via* peripheral access into hepatic artery and due to their small size of 15-40 uM lodged into arteriolar level of liver vascular system. A high radiation dose can be delivered to the tumour itself saving healthy liver cells in comparison to external radiation technique. A previous structured review concluded that TARE for inoperable breast liver metastases, is well tolerated by patients especially when compared to the side effects associated with systemic chemotherapy[37]. The overall survival data retrieved from the studies included in this present review varied from 6 to 20.9 mo[17-19,21-27]. Although data from the studies included should be interpreted with caution due to the heterogeneity of the methodology in the reports included, the overall impression is that radioembolization is a promising option considering that over 50% of the total number patients included in this review had metastases beyond the liver at the time of TARE. Furthermore, survival data from one of the studies included, demonstrate that patients who have a complete or partial response to embolization treatment have a survival over 12 months compared to 3.6 months in those patients who failed to respond[17]. As an extension of the above one may speculate that radioembolization instead of being a monotherapy could have a synergistic role to systemic chemotherapy as it has been previously the case in colorectal liver metastases. In the context of colorectal liver metastasis, a previous randomized controlled trial demonstrated that the addition of radioembolization with ytrrium-90 to 5-fluorouracil treatment led to a significantly prolonged progression free survival and a non-statistically significant prolonged overall survival[13].

The response at a tumour level in the case of breast liver metastases to radioembolization has been a matter of debate in the literature. First of all the fact that, unlike the case of colorectal or uveal melanoma metastases which are often confined to the liver, breast cancer patients often have more extensive disease spread making radioembolization a modality less likely to succeed. However, it has been previously suggested that breast liver metastases are hypervascular compared to colorectal liver metastases which are described as hypovascular[38,39]. Therefore, the ratio between the number of spheres arriving at the level of the tumour versus the number of spheres arriving to healthy liver may be higher in the case of breast liver metastases making radioembolization an appropriate treatment modality for breast metastases. Data on tumour response to radioembolization could be retrieved from ten of the studies included in this review. However, interpretation of the data is limited by the use of different criteria (WHO *vs* RECIST) in the studies included[28,29]. Disease control rates varied from 48%-100% with an estimated mean response to TARE of 81%. The 2 studies[16,17] which used the WHO criteria to assess response to TARE reported disease response over 90%, whereas the rest of the studies reported disease control rates of 48%-100% based on the RECIST/mRECIST criteria[19-24,26,27]. The heterogeneity in the criteria used to assess tumour response rates, the inconsistency in the type of microspheres used and the different timings that post-TARE radiological

Table 2 Tumour response to radio-embolization and survival data as reported in the studies included

Ref.	Evaluable patients	Assessment criteria	Follow up (mo)	Tumour response rate (%)	Cases of CR/PR/SD/PD	Overall survival (mo)
Bangash *et al*[16], 2007	23	WHO	N/A	21/23 (91%)	CR = 9 (39%); PR = 12 (52%); SD = 2 (9%); PD = 0	N/A
Coldwell *et al*[17], 2007	36	WHO	14	34/36 (94.4%)	CR = 0; PR = 17 (47.2%); SD = 17 (47%); PD=2 (6%)	> 14 for those with CR/PR, 3.6 for those with SD/PD
Stuart *et al*[18], 2008	7	N/A	N/A	N/A	N/A	20.9[1]
Jakobs *et al*[19], 2008	23	RECIST	15.7	22/23 (97.2%)	CR = 0; PR = 14 (61%); SD = 8 (35%); PD = 1 (4%)	9.6
Cianni *et al*[20], 2010	32	RECIST	N/A	32/32 (100%)	CR = 14 (44%); PR = 11 (34%); SD = 7 (22%); PD = 0	N/A
Haug *et al*[21], 2012	43	RECIST	6	38/43 (88%)	CR = 0; PR = 11 (26%); SD = 27 (62%); PD = 5 (12%)	10.8
Saxena *et al*[22], 2014	38	RECIST	11.2[1]	27/38 (71%)	CR = 2 (5%); PR = 10 (26%); SD = 15 (39%); PD = 11 (29%)	13.6
Gordon *et al*[23], 2014	25	RECIST	N/A	21/25 (84%)	CR = 3 (12%); PR/SD = 18 (72%); PD = 4 (16%)	6.6[1]
Bagni *et al*[24], 2015	17	RECIST	N/A	17/17 (100%)	CR = 2 (12%); PR = 15 (88%); SD = 0; PD = 0	13.5
Fendler *et al*[25], 2016	56	N/A	N/A	29/56 (52%)	N/A	8[1]
Pieper *et al*[26], 2016	38	RECIST	N/A	27/38 (71%)	CR = 0; PR = 11 (29%); SD = 16 (42%); PD = 11 (29%)	6
Chang *et al*[27], 2018	29	mRECIST	N/A	14/29 (48%)	CR = 0; PR = 12 (40%); SD = 2 (0.6%); PD = 15 (50%)	12.9

[1]Median value as reported in the original study. N/A: Not available; WHO: World Health Organization; RECIST: Response Evaluation Criteria in Solid Tumours; mRECIST: Modified Response Evaluation Criteria in Solid Tumours; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

surveillance was perfromed along with the retrospective nature of the studies identified, make it difficult to draw safe conclusions on the efficacy of TARE in disease control and necessitate the need for more quality evidence to be produced. Nevertheless, the results appear to be encouraging with an estimated mean disease control rate of over 80%. A recent systematic review on the role of TARE in disease control rate in cases of unresectable liver metastases secondary to melanoma reported a median control rate of 73.6%[14]. The findings of this review were promising and highlight the need for more quality evidence to explore the role of TARE either as a monotherapy or synergistically with systemic therapies in the future.

There are some limitations in the findings reported by this systematic review. First of all the absence of randomized controlled trials and the retrospective nature of the reports included carries the risk of selection bias. Furthermore, there is heterogeneity between the studies included and no standardised reporting system on the control-rate of the disease post-radioembolization. Differences between studies included were the type of spheres used to deliver the treatment locally, the variable radiation dose, variable presence of extrahepatic disease, previous chemotherapy and the length of follow-up.

This review, despite its limitations, highlights the potentially beneficial role of radio-embolization with yttrium microspheres in cases with inoperable liver metastases secondary to breast cancer. However, future randomized trials are need comparing systemic chemotherapy, local radiation and transarterial chemoembolization in order to identify the most suitable treatment modality for patients with inoperable hepatic metastases secondary to breast cancer. Standardization of the method that radioembolization is delivered by and the reporting systems used would be highly desirable.

ARTICLE HIGHLIGHTS

Research background

Breast cancer liver metastases are associated with dismal prognosis. Previous reports in the literature on liver metastases secondary to melanoma or colorectal origin have shown promising results with the use of transarterial embolization. The aim of this review was to consolidate the evidence available in the literature on the use of transarterial embolization for management of breast liver metastases.

Research motivation

The aim of this review was to consolidate the evidence currently available on transarterial embolization for breast liver metastases in a systematic fashion. This relatively new technique is not widely available and its role in the management pathway of breast metastases has not been clearly described before. Patients with breast liver metastases have poor prognosis despite advances in chemotherapy and therefore transarterial embolization could be of benefit for those patients with advanced disease.

Research objectives

The main outcomes of interest were tumour response and patient survival following radioembolization with ytrrium-90 spheres.

Research methods

A systematic literature search was performed in PubMed and EMBASE databases from January 2007 to December 2018. The following search terms were used in order to identify the relevant studies of interest: "yttrium" or "yttrium-90" or "Y90" or "radio-embolization" and "breast".

Research results

The final number of studies which met the inclusion criteria was 12 involving 452 patients. There were no randomized controlled trials identified after the literature search. The age of the patients included in this review was ranged from 52-61 years. The duration of the follow up period post-radioembolization ranged from 6 to 15.7 mo. The total number of patients with breast metastases not confined to the liver was 236 (52.2%). Cumulative analysis revealed that radioembolization with yttrium-90 conferred tumour control rate in 81% of patients. Overall survival post-radioembolization ranged from 3.6 to 20.9 mo with an estimated mean survival of 11.3 mo.

Research conclusions

Radioembolization with ytrrium-90 appears to confer control of tumour growth rate in most patients. The effect on patient survival need to be elucidated further. The findings reported in this review are limited by the absence of randomized trials on the subject and the heterogeneity in the methodology of the studies included. It is therefore highly desirable for more quality evidence to be produced in order to assess mor accurately the role of radioembolization with yttrium-90.

Research perspectives

The findings of this review highlight the need for more quality evidence to be produced in the form of randomized controlled trials. Standardisation of types of spheres used, timing of imaging modalities and criteria used in order to assess the effect of radioembolization is required. Furthermore, the potentially synergistic role of radioembolization for patients on palliative chemotherapy should be evaluated as it may confer a significant impact on survival.

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World J Gastrointest Oncol 2020 February 15; 12(2): 237-247

DOI: 10.4251/wjgo.v12.i2.237

ISSN 1948-5204 (online)

CASE REPORT

Cryoablation combined with radiotherapy for hepatic malignancy: Five case reports

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Author contributions: Liu YE participated in the design of the subject and drafted the manuscript; Zong J, Chen XJ, Zhang R and Ren XC participated in the therapy for these five patients and the interpretation of data; Guo ZJ participated in the design of the subject and the interpretation of radiological data; Liu CX participated in the design of the subject and helped to the writing of the manuscript; Lin Q designed the subject and interpreted the data, who is responsible for the whole manuscript. All authors issued final approval for the version to be submitted.

Supported by Health Commission of Hebei Province, No. G2018068.

Informed consent statement: Consent was obtained from the patient for publication of this report and any accompanying images.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

CARE Checklist (2016) statement: The authors have read the CARE Checklist (2016), and the guidelines from the check list have been adopted in the preparation of this

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Abstract

BACKGROUND

The survival of patients treated with monotherapy for hepatic malignancies is not ideal. A comprehensive program of cryoablation combined with radiotherapy for the treatment of hepatic malignancies results in less trauma to the patients. It may provide an option for the treatment of patients with advanced hepatic malignancies.

CASE SUMMARY

We reported 5 cases of advanced-stage hepatic malignancies treated in our hospital from 2017-2018, including 3 cases of primary hepatocellular carcinoma and 2 cases of metastatic hepatic carcinoma. They first received cryoablation therapy on their liver lesions. The procedure consisted of 2 freeze-thaw cycles, and for each session, the duration of freezing was 13-15 min, and the natural re-warming period was 2-8 min. Depending on the tumor size, the appropriate cryoprobes were selected to achieve complete tumor ablation to the greatest extent possible. After cryoablation surgery, intensity-modulated radiotherapy (IMRT) for liver lesions was performed, and the radiotherapy regimen was 5400 cGy/18f and 300 cGy/f. None of the 5 patients had adverse events above grade II, and their quality of life was significantly improved. Among them, 4 patients were free of disease progression in the liver lesions under local control, and their survival was prolonged; 3 patients are still alive.

manuscript.

Manuscript source: Invited manuscript

Received: May 21, 2019
Peer-review started: May 21, 2019
First decision: September 20, 2019
Revised: October 28, 2019
Accepted: December 13, 2019
Article in press: December 13, 2019
Published online: February 15, 2020

P-Reviewer: Hernanda PY
S-Editor: Dou Y
L-Editor: A
E-Editor: Qi LL

CONCLUSION
Our clinical practice demonstrated that cryoablation combined with IMRT could be implemented safely. The definitive efficacy for hepatic malignancies needs to be confirmed in larger-size sample prospective studies.

Key words: Hepatic malignancies; Primary hepatocellular carcinoma; Metastatic hepatic carcinoma; Cryoablation; Intensity-modulated radiotherapy; Case report

Core tip: The therapeutic efficacy of monotherapy for primary hepatocellular carcinoma (HCC) and secondary HCC is usually poor, and thus, combination therapy is needed. A treatment plan of cryoablation combined with radiotherapy is safe and effective and may result in survival benefits to patients.

Citation: Liu YE, Zong J, Chen XJ, Zhang R, Ren XC, Guo ZJ, Liu CX, Lin Q. Cryoablation combined with radiotherapy for hepatic malignancy: Five case reports. *World J Gastrointest Oncol* 2020; 12(2): 237-247
URL: https://www.wjgnet.com/1948-5204/full/v12/i2/237.htm
DOI: https://dx.doi.org/10.4251/wjgo.v12.i2.237

INTRODUCTION

Hepatic malignancies include primary hepatocellular carcinoma (HCC) and secondary hepatocellular HCC[1]. Primary HCC is one of the most common malignancies seen in clinical practice. Hepatic resection and orthotopic liver transplantation are considered radical treatments for HCC, while surgery is the first-line treatment for primary HCC. In China, most patients with HCC also have liver cirrhosis, and most have already reached the intermediate or advanced stages of HCC at the time of diagnosis; moreover, only approximately 20%-30% of patients have an opportunity for hepatic resection. Currently, sorafenib is one of the standard drugs used to treat advanced-stage HCC, but the median overall survival rate is only 6.5 mo[2]. Additionally, the liver is one of the most common sites for metastatic tumors. When a tumor has metastasized to the liver, the patient is already at an advanced stage and has a poor prognosis. Currently, the treatment effect on such patients is not ideal, and new local and systemic treatments are needed. Local ablation therapy, which has been widely utilized in recent years, is associated with less trauma and definite therapeutic effect, which offers the opportunity of radical treatment to some patients with hepatic malignancies who cannot or who are unable to tolerate hepatic resection.

Local ablation therapy directly targets tumors under the guidance of medical imaging technology. This is a treatment method that directly kills tumor tissue by local adoption of physical or chemical methods. It mainly includes radiofrequency ablation (RFA), microwave ablation, cryoablation, high-intensity focused ultrasound (HIFU) ablation, and percutaneous ethanol injection, among others. Among them, cryoablation has been increasingly applied to the local ablation of hepatic malignancies due to its advantages such as causing minimal damage to the great vessels, low incidence of pain, and controllable iceball formation.

The principle of cryoablation is based on the gas throttling effect (Joule–Thomson principle)[3], which states that after a high-pressure gas flows through a small orifice, it expands rapidly in the expansion space and absorbs the surrounding heat; this significantly reduces the surrounding temperature. Therefore, the physical destruction of tumor tissue and cells is achieved through freeze-thaw cycles. The mechanisms of cryoablation can be divided into freezing damage, thawing damage, microvascular damage, and immunomodulatory mechanisms. Generally, it is thought that the threshold temperature that induces cell death is -40 °C[4]. After repeated freeze-thaws of tumor cells, the cells burst and the cell membrane dissolves, which promotes the release of hidden antigens in the cell and stimulates the body to produce antibodies. With the death of tumor cells, the immunosuppressive state of the tumor on the body is removed. Therefore, the body's anti-tumor immunity is enhanced, and the immune-destroying effect on the tumor cells is activated.

To achieve complete and sufficient targeted tumor destruction, the tumors were frozen until the iceball extended approximately 3-5 mm beyond the tumor margin, which can be accurately monitored by imaging techniques such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI)[5-6].

The Asia-Pacific clinical practice guidelines for the treatment of primary HCC recommends the following[7]: Local ablation is suitable for Child-Pugh class A or B patients with 3 or fewer tumors, each 3 cm or less in diameter. RFA is the first-line image-guided percutaneous ablation technique that is recommended. Many studies have shown that for the local ablation of primary HCC, cryoablation is as effective as RFA. A multicenter randomized controlled trial of 360 patients with primary HCC showed that for lesions less than or equal to 4 cm and lesions less than or equal to 2 cm, both cryoablation and RFA achieved similar therapeutic effects[8]. The 1-, 3-, and 5-year overall survival rates were 97%, 67%, and 40% for cryoablation, respectively, and 97%, 66%, and 38% for RFA, respectively (P = 0.747). The 1-, 3-, and 5-year tumor-free survival rates were 89%, 54%, and 35% in the cryoablation group, respectively, and 84%, 50%, and 34% in the RFA group, respectively (P = 0.628).A recent meta-analysis compared the therapeutic efficacy of cryoablation and RFA in patients with hepatic malignancies and 7 articles that met the inclusion criteria were included[9]. The meta-analysis showed an almost equal mortality of at least 6 mo, and no significant difference was observed in local tumor progression between the 2 groups. The studies discussed above showed that the therapeutic efficacy of cryoablation and RFA was similar for early-stage primary HCC.

Cryoablation is also one of the major therapies for unresectable HCC. In 2003, Xu *et al*[10] reported the use of cryoablation in 105 masses from 65 patients with HCC. Among the 41 patients who were followed-up for more than 1 year, 32 patients (78%) were alive despite tumor recurrence, 7 patients (10.8%) died due to disease recurrence, and 3 patients (5%) died of non-cancer-related diseases. Chen *et al*[11] applied cryoablation to treat unresectable HCC and found that the 1- and 3-year overall survival rates were 81% and 60%, respectively, while the 1-and 3-year disease-free survival rates were 68% and 21%, respectively. The 1- and 3-year overall survival rates of patients with recurrent HCC were 70% and 29%, respectively, while the 1-and 3-year disease-free survival rates were 54% and 8%, respectively.

Similarly, cryoablation is also effective for metastatic hepatic tumors. Chang *et al*[12] reported that for the 19 patients who underwent cryoablation for liver metastases after gastrectomy for primary gastric cancer, the median overall survival was 16.0 mo, the median local tumor progression-free survival was 8.0 mo, and the 1-, 2-, and 3-year overall survival rates were 78.9%, 43.4%, and 21.7%, respectively. The patients' quality of life also improved after cryoablation therapy (P < 0.05) and no severe complications occurred. In summary, cryoablation is suitable for both primary and secondary HCC and is safe and effective for the treatment of advanced-stage hepatic malignancies.

Intensity-modulated radiation therapy (IMRT) technology has become increasingly advanced and can simultaneously effectively protect normal hepatic tissue and deliver a high dose of radiation to the targeted area of HCC to improve therapeutic efficacy; this confirms the status of radiotherapy in the treatment of HCC[13,14].

The application of radiotherapy in the comprehensive treatment of HCC has gradually increased, especially for further improvement of poor efficacy after local treatment [for example, after transcatheter arterial chemoembolization (TACE)] or to target residual tumor at the margins of lesions. Radiotherapy can improve therapeutic efficacy of unresectable primary HCC treatment, improve the local control, and at the same time, effectively protect normal hepatic tissue and improve patient prognosis[13].

Radiotherapy causes irreversible damage to the DNA of tumor cells in the irradiation field and induces tumor cell death through apoptosis, necrosis, and autophagy, among other mechanisms[15,16]. It also promotes the release of tumor-related antigens[17], increases the production of cytokines, alters the tumor microenvironment, and activates the body's immune system to initiate an anti-tumor immune response. Postow *et al*[18] proposed the "Abscopal Effect", that is, a phenomenon related to local radiotherapy and the regression of metastatic cancer distant from the radiation site, which may be related to activation of the immune system. The mechanism of action may be that radiotherapy induces tumor cells to release a large amount of antigen in a short period of time; T lymphocytes are then activated after APC presentation and activated T lymphocytes (cytotoxic lymphocytes) can then act on primary and metastatic tumor cells.

Studies have shown that cryotherapy can sensitize dendritic cells to enhance their antigen presenting ability and promote their secretion of IL-4, IL-12, and other cytokines; cryoablation can also promote T and B cell proliferation and activation and can induce the body's immune system to play an anti-tumor role. Sidana *et al*[19] proposed the model of cryoimmunotherapy, that is, cryotherapy combined with other

immunotherapy treatment to enhance the immunostimulating response. While cryoablation controls the primary tumor, it also enhances the body's anti-tumor immune response to effectively control tumor recurrence and metastasis.

The results of the study by Mu *et al*[20] showed that the therapeutic efficacy of combination therapy of cryoablation and chemotherapy drugs for patients with advanced HCC was significantly better than that of cryoablation alone. The overall survival rate of the patients increased significantly. In addition, the overall survival rate of patients with early use of the combined multiple treatment plan was significantly better than that of patients who used monotherapy or who delayed the use of combination therapy. Studies have shown that cryoablation combined with immunotherapy could improve the median survival duration of patients and can play an anti-tumor role. This suggests that cryoablation combined with multiple other therapies can achieve fair therapeutic efficacy. Radiotherapy can activate the immune system, and hence, it has an anti-tumor function. In theory, cryoablation combined with IMRT may have a synergistic effect to enhance efficacy.

The effective freezing range of cryoablation should be 1 cm beyond the margin of the tumor[21], that is, the surgical resection margin. In theory, all tumor tissues can be inactivated with no remaining residual tumor cells. Only through this method can significant efficacy be achieved. However, in clinical practice, cryoablation therapy may not be able to inactivate all tumor cells due to the tumor location, insertion pathway, the tumor blood supply and surrounding great vessels, and many other factors. Therefore, residual tumor cells can easily appear around the formed iceball. The postoperative supplementary treatment can effectively kill the minimal residual lesions and improve therapeutic efficacy.

IMRT can effectively solve the problem of residual tumor that forms around the iceball after cryoablation therapy. For tumor tissues with an abundant blood supply, residual tumor may be present after cryoablation. Therefore, IMRT administration at this time can effectively kill the residual tumors. IMRT is therefore a beneficial supplement after cryoablation. It is well known that hypoxic cells comprise a high proportion of tumor cells in the tumor center and that they are resistant to radiotherapy, while cryoablation can effectively kill the central area of the tumor that is relatively abundant with anaerobic cells. Therefore, cryoablation combined with IMRT may play a synergistic and complementary role, which could improve the local control rate of liver lesions. To the best of our knowledge, cryoablation combined with IMRT is rarely reported, and for the first time, we report the clinical cases of this combination therapy.

CASE PRESENTATION

Case 1

Chief complaints: We treated a 59-year-old male patient with liver metastases from colon cancer.

History of present illness: The patient underwent radical resection of colorectal cancer in December 2014. Postoperative pathology: Differentiated adenocarcinoma of the colon and liver metastasis were found after surgery. The XELOX chemotherapy regimen was administered for 4 cycles. Resection of the liver metastasis was performed on April 2, 2015. In July 2016, the carcinoembryonic antigen (CEA) level was elevated and new metastatic lesions were observed in the liver. In August 2016, TACE was performed for 1 cycle, and 8 cycles of capecitabine monotherapy were given, followed by stable efficacy evaluation. On May 25, 2017, the left hepatic lobe containing the metastatic tumor grew larger, and the disease progressed. Hepatic arteriography + chemoembolization was performed once, FOLFOX4 chemotherapy was given for 1 cycle and FOLFIR chemotherapy was given for 3 cycles, and the disease progressed again after second-line treatment. On August 29, 2017, hepatic arteriography + embolization was performed once.

History of past illness: There was no significant past medical history or family history of malignancy.

Physical examination upon admission: Physical examination of the patient showed no apparently positive signs.

Laboratory examinations: CEA 246.14 ng/mL, sugar antigen 19-9 128.02 U/mL.

Imaging examinations: The metastatic lesion in the left lobe of the liver was larger than that after the previous treatment.

Case 2

Chief complaints: We treated a 45-year-old male patient with primary HCC with hepatic metastatic and formation of a right branch of the portal vein.

History of present illness: In March 2014, the patient was diagnosed with primary HCC, which was located near the great vessels and could not be surgically resected. TACE was performed twice in March 2014 and on April 14, 2014, and HIFU ablation was performed on May 11, 2014. The third TACE treatment was performed in June 2014, and in July 2014, liver radiotherapy was performed 16 times with a total radiation dose 4800 cGy/16f. On May 17, 2017, new liver lesions were found with an increased alpha-fetoprotein (AFP) level of 888 IU/mL. The patient underwent liver CT on July 24, 2017, which revealed right hepatic cancer and right portal vein thrombi formation. Therapeutic efficacy evaluation: Disease progression.

History of past illness: This patient had a history of hepatitis B-associated cirrhosis for 20 years and was untreated. A history of hypertension for 5 years.

Physical examination upon admission: The patient had hepatic tenderness.

Laboratory examinations: On May 17, 2017, Alpha fetoprotein increased to 1000IU/mL (upper limit of detection value in our hospital).

Imaging examinations: On July 24, 2017, liver enhanced CT scan: Right HCC, right portal vein thrombus formation.

Case 3

Chief complaints: A 41-year-old female diagnosed with primary HCC.

History of present illness: On March 16, 2017, right lobe liver cancer was diagnosed by both liver MRI and CT with an AFP level of 259IU/mL. Two TACE treatments were performed on April 1, 2017, and May 11, 2017.

History of past illness: This patient had a history of hepatitis B-associated cirrhosis for 10 years and was untreated.

Physical examination upon admission: Physical examination of the patient showed no apparently positive signs.

Laboratory examinations: On July 25 2017, Alpha fetoprotein was 22.34 IU/mL.

Imaging examinations: Enhanced abdominal CT suggested that the lesion at the top of the right lobe of HCC changed after interventional surgery.

Case 4

Chief complaints: A 61-year-old male diagnosed with primary HCC, with multiple liver metastases, cirrhosis, and ascites.

History of present illness: On December 20, 2015, he diagnosed with primary HCC by MRI and CT, with multiple liver metastases, cirrhosis, and ascites. TACE was performed 4 times on December 9, 2015, January 18, 2016, February 14, 2016, and May 17, 2016. Hepatic encephalopathy occurred on November 11, 2016, and improved after treatment. TACE was given 4 times successively on February 9, 2017, May 4, 2017, June 14, 2017, and August 21, 2017.

History of past illness: This patient had a history of hepatitis B-associated cirrhosis for more than 20 years and was untreated.

Physical examination upon admission: Physical examination of the patient showed no apparently positive signs.

Laboratory examinations: On August 17, 2017, Alpha fetoprotein increased to 376.47 IU/mL and saccharide antigen 19-9 was 62.38 U/mL.

Imaging examinations: Abdominal CT showed postoperative changes of lesions in the right lobe of the liver, cirrhosis, portal hypertension, and open abdominal collateral vessels.

Case 5

Chief complaints: A 61-year-old female was diagnosed with Spinal canal invasion after thoracolumbar fibrosarcoma surgery (T12L1) multiple intrahepatic metastasis.

History of present illness: On December 13, 2014, the patient underwent posterior

lumbar laminectomy for intraspinal tumors (extramedullary subdural) and adnexal tumors. Local tumor recurrence occurred 3 mo after surgery, and the tumor at the recurrence site was controlled after three-dimensional conformal radiotherapy and HIFU ablation. MRI findings on May 15, 2017: New metastatic lesions in the liver. Ultrasound-guided liver space occupying biopsy pathology (May 26, 2017, pathology no. 1901664): Consistent with fibrosarcoma metastasis to the liver; the disease had progressed again. Three TACE treatments and HIFU ablation of the metastatic liver lesions were given. Therapeutic efficacy evaluation: Stable. On January 5, 2018, the patient was reexamined by enhanced CT: A rich blood supply was observed around the liver metastatic lesions, which was indicative of tumor recurrence. TACE treatment was performed once on January 17, 2018. However, no further treatment was given due to personal reasons. Reexamination of upper abdominal MRI + enhancement in June 2018: Progression of liver metastasis.

History of past illness: This patient had a history of hypertension for 12 years.

Physical examination upon admission: Physical examination of the patient showed no apparently positive signs.

Laboratory examinations: The serum chemistries and complete blood count was normal.

Imaging examinations: MRI scan of liver suggested the progression of liver metastases.

FINAL DIAGNOSIS

Case 1

Radical resection of colon cancer with multiple hepatic metastases.

Case 2

(1) Primary HCC with hepatic metastasis of portal vein thrombus formation; (2) Decompensated period of cirrhosis after chronic viral hepatitis Band; and (3) Hypertension.

Case 3

(1) Primary HCC; and (2) Chronic viral hepatitis band liver cirrhosis with an enlarged spleen.

Case 4

(1) Primary HCC with multiple intrahepatic metastasis; and (2) Decompensated period of cirrhosis after chronic viral hepatitis Band, Celiac effusion, hepatic encephalopathy.

Case 5

(1) Spinal canal invasion after thoracolumbar fibrosarcoma surgery (T12L1) multiple intrahepatic metastasis; and (2) Hypertension.

TREATMENT

Case 1

On September 19, 2017, hepatic metastatic tumor cryoablation was performed. Before the procedure, a raster and spiral CT was used for guidance and localization. After determining the insertion point and insertion angle, routine sterilized drape was used in the operative area. Two cryoprobes 2.4 mm in diameter were selected and inserted at the predetermined location on the lesion under CT guidance, and then cryotherapy was initiated. Two freeze-thaw cycles were used in the cryotherapy process. Freezing occurred for 15 min during the first cycle, which was followed by natural rewarming for 2 min; freezing occurred for 15 min during the second cycle. No adverse events (AEs) such as pneumothorax and hemorrhage were encountered during the surgery. From October 25, 2017, IMRT for metastatic liver tumors was performed. Radiotherapy regimen: 5400 cGy/18f and 300 cGy/f.

Case 2

On July 25, cryoablation for the hepatic liver lesion was performed: 2 cryoprobes 1.7 mm and 2.4 mm in diameter were selected and inserted at the predetermined location

on the lesion under CT guidance, and then cryotherapy was initiated. Two freeze-thaw cycles were performed in the cryotherapy process. Freezing occurred for 15 min during the first cycle, which was followed by natural rewarming for 8 min; freezing occurred for 15 min during the second cycle. The surgery went well and only a small degree of pneumothorax occurred. On September 24, 2017, HIFU ablation of the right portal vein thrombi was performed. On October 17, 2017, liver lesion radiotherapy was initiated with a total dose of radiotherapy of 5400 cGy/18f and 300 cGy/f.

Case 3

On July 25, 2017, cryoablation of the hepatic lesions was performed under local anesthesia: 2 cryoprobes 1.7 mm and 2.4 mm in diameter were selected and inserted at the predetermined location on the lesion under CT guidance, and then cryotherapy was initiated. Two freeze-thaw cycles were used in the cryotherapy process. Freezing occurred for 15 min during the first cycle, which was followed by natural thawing for 8 min; freezing occurred for 15 min during the second cycle. The surgery went smoothly, and only a small degree of pneumothorax occurred. On September 5, 2017, she began radiotherapy for the lesion in the right lobe of the liver. Radiotherapy regimen: 5400 cGy/18f and 300 cGy/f. The radiotherapy was completed on September 28, 2017.

Case 4

Cryoablation of hepatic lesions was performed on September 3, 2017: 4 cryoprobes 2.4 mm in diameter were selected and inserted at the predetermined location on the lesion under CT guidance, and then cryotherapy was initiated. Two freeze-thaw cycles were used in the cryotherapy process. Freezing occurred for 15 min during the first cycle, which was followed by natural thawing for 8 min; freezing occurred for 15 min during the second cycle. The surgery went smoothly, and a small degree of pleural effusion was observed on the right side. On October 24, 2017, this patient began radiotherapy for the hepatic lesion. Radiotherapy regimen: 5400 cGy/18f and 300 cGy/f. The radiotherapy was completed on November 26, 2017.

Case 5

Cryoablation of the hepatic tumor was performed on July 5, 2018: 2 cryoprobes 1.7 mm in diameter were selected and inserted at the predetermined location on the lesion under CT guidance, and then cryotherapy was initiated. Two freeze-thaw cycles were used in the cryotherapy process. Freezing occurred for 13 min during the first cycle, which was followed by natural rewarming for 5 min; freezing occurred for 13 min during the second cycle. The surgery was performed without incident. On August 6, 2018, radiotherapy of the liver lesions was initiated. Radiotherapy regimen: 5400 cGy/18f and 300 cGy/f; radiotherapy ended on August 30, 2018.

OUTCOME AND FOLLOW-UP

Case 1

CEA decreased to 126.63 ng/mL, and carbohydrate antigen 19-9 decreased to 76.62 U/mL. Postoperative oral monotherapy (tegafur chemotherapy) was administered for 1 cycle. Reexamination on June 2018: The lesions treated by cryoablation had no blood supply. but new liver lesions were observed. Overall evaluation: Disease progression.

On June 29, 2017, radioactive iodine-131 seed implantation was performed. On October 29, 2018, a new bone metastasis was found, and on November 6, 2018, TACE was performed. Clinical death occurred on March 13, 2019. The local control duration of the hepatic lesions was 17 mo and clinical death occurred 18 mo after cryoablation therapy.

Case 2

On April 14, 2018, a new pulmonary metastasis was found at reexamination and the disease was in progression. On April 25, 2018, pulmonary interventional perfusion chemotherapy (TAE) was performed once, and on June 10, 2018, cryoablation was performed on the lesion in the right lung. On June 22, 2018, new lesions were found in the periphery of the hepatic lesion treated with cryoablation. Evaluation: Disease progression. Death occurred on September 16, 2018. The local control time of the hepatic lesion was 11 mo, and clinical death occurred 14 mo after cryoablation therapy.

Case 3

AFP dropped to normal and she began treatment with oral entecavir (an anti-viral treatment) from September 28, 2017. She now lives a normal life and is still alive. The

liver lesion has been under control for 20 mo.

Case 4

Alphafetoprotein decreased during follow-up. He began treatment with oral entecavir (an anti-viral treatment) from November 26, 2017. After treatment, he lived completely independently and is still alive. The liver lesion has been controlled for 19 mo.

Case 5

Reexamination in October 2018: Liver cryoablation lesions were stable, but lesions in both lungs were increased. Overall evaluation: Disease progression. Oral treatment of anlotinib was given, and the Karnofsky Performance Score (KPS) was 80. After cryoablation therapy, the patient lived independently and is still alive. The liver lesion treated with cryoablation has been controlled for 9 mo.

A summary of each patient was shown on Table 1.

DISCUSSION

Most patients have already reached the intermediate or advanced stages of HCC at the time of diagnosis, and therefore, they lose the opportunity for radical surgery. In China, 85%-90% of liver cancer occurs as a result of post-hepatitis cirrhosis, and many patients cannot tolerate surgery. At the same time, the liver is also a common target organ for metastasis of some malignant tumors, such as colorectal cancer, breast cancer, pancreatic cancer, melanoma, and renal cancer, among others. Due to multiple metastatic lesions, the surgical resection rate is low, and the therapeutic effect is dismal.

Cryoablation has the characteristics of rapid rewarming, cold temperature freezing, and reversal of hot and cold. It can rapidly reduce the temperature of lesioned tissue to -140 °C with argon gas, which causes rapid freezing of the lesion site[1]. Then, it eliminates the lesion using heat *via* rapid thawing with helium gas. This type of therapy has advantages in destroying cancer cells while effectively preserving normal hepatic tissues; this therapy is also associated with a quick recovery, minimal trauma, and high reproducibility and is also simple to perform[3].

Rong *et al*[22] selected 866 patients with primary HCC who met the Milan criteria (single lesions less than or equal to 5 cm, multiple lesions less than or equal to 3 cm, and each lesion was less than or equal to 3 cm) for cryoablation. The complete ablation rate reached 96.1%, and the postoperative 1-, 3-, and 5- year survival rates were 98.6%, 80.6%, and 60.3%, respectively, but the corresponding local recurrence rates were 10.7%, 22.1%, and 24.2%, respectively. Yang *et al*[23] treated 300 primary HCC patients with cryoablation therapy, after which the therapeutic efficacy, safety, and complications were evaluated. In all, 165 of the patients had incomplete ablation, while 135 had complete ablation. The median follow-up time was 36.7 mo. For the patients with early-, intermediate-, and advanced-stage HCC, the postoperative 1-, 2-, and 3-year survival rates were 91%, 85%, and 65%,respectively,for early-stage HCC, while the rates were 87%, 62%, and 45%,respectively, for intermediate-stage HCC; the rates were 73%, 25%, and 12%, respectively, for advanced-stage HCC. The median survival duration for patients with early-, intermediate-, and advanced-stage disease was 45.7 ± 3.8 mo, 28.4 ± 1.2 mo and 17.7 ± 0.6 mo, respectively. One study included 124 primary HCC patients treated with cryoablation[24], including 16 with early-stage disease, 42 with intermediate-stage disease, and 66 with advanced stage disease. After cryoablation of the tumors, the serum level of AFP was reduced in 76 (82.6%) patients, and 205 (92.3%) of the 222 tumor lesions were diminished or unchanged. The median survival time was 31.3, 17.4, and 6.8 mo for those in the early, intermediate, and progressive stages, respectively. The above studies indicate that cryoablation is an effective treatment for both early-, intermediate-, and advanced-stage primary HCC.

Qian *et al*[25]reported 1-year survival rates of 80% and 46% for 34 patients with secondary and recurrent HCC, respectively, treated with cryoablation therapy. Littrup *et al*[26] performed cryoablation on a total of 370 tumors in 176 patients with metastatic liver cancer, with an average follow-up time of 1.8 years. The local tumor recurrence rates of colorectal cancer and non-colorectal cancer with liver metastasis were 11.1% and 9.4%, respectively. The average time to local recurrence of liver metastasis was 9.5 mo for colorectal cancer and 7.9 mo for non-colorectal cancer. Another study[27] with the results of long-term follow-up also confirmed that cryoablation was a safe and effective ablation technique for patients with liver metastases from colorectal cancer. In this study, 304 patients with liver metastases from advanced colorectal cancer were treated with cryoablation. 293 of them were analyzed. The median overall survival time was 29 mo, and the survival rates of 1, 3, 5 and 10 years were 87% 41.8%, 24.2%,

Table 1 Summary of patients

Case	Sex	Age (yr)	Final diagnosis	Treatment options before cryoablation	Cryoablation + radiotherapy		Continue treatment	Follow-up time (mo)	Local control time (mo)	Survival
					Cryoablation	Radiotherapy				
1	M	59	Radical resection of colon cancer with multiple hepatic metastases	Disease was progressive after third-line treatment for liver metastasis after colon cancer surgery	2 cryoprobes, total treatment time: 31 min	5400 cGy /18f, 300 cGy/f	Chemotherapy, radioactive iodine-131 seed implantation	18	17	No, died on March 13, 2019
2	M	45	Primary hepatocellular carcinoma with hepatic metastasis of portal vein thrombus formation	Disease was progressive after first line treatment	2 cryoprobes, total treatment time: 38 min	5400 cGy /18f, 300 cGy/f	TACE, Cryoablation for metastatic lung lesion	14	11	No, died on September 16, 2018
3	F	41	Primary hepatocellular carcinoma	Disease was stable after first-line treatment	2 cryoprobes, total treatment time: 38 min	5400 cGy /18f, 300 cGy/f	Entecavir	20	20	Yes
4	M	61	Primary liver cancer with multiple intrahepatic metastasis	Disease was progressive after TACE first-line treatment	4 cryoprobes, total treatment time: 35 min	5400 cGy /18f, 300 cGy/f	Entecavir	19	19	Yes
5	F	61	Spinal canal invasion after thoracolumbar fibrosarcoma surgery (T12L1) multiple intrahepatic metastasis	Disease was progressive after fifth - line treatment after palliative surgery	2 cryoprobes, total treatment time: 31 min	5400 cGy /18f, 300 cGy/f	The liver lesion was stable; anlotinib was taken orally	9	9	Yes

TACE: Transcatheter arterial chemoembolization.

13.3%, respectively. The median disease-free survival was 9 mo.

In summary, experimental and clinical applications show that cryoablation is safe and effective for the treatment of hepatic malignancies. Cryoablation is an effective and acceptable new local therapy for metastatic liver cancer. Moreover, iceball formation can be observed by the naked eye. Cryoablation has a very small effect on the surrounding great vessels and can be performed alone or in combination with other methods such as radiotherapy, chemotherapy, immunology, or surgery to better control the lesions.

Our clinical practice also demonstrated that cryoablation combined with IMRT for primary and secondary HCC is safe and effective and is well tolerated with minor AEs. Some patients had a small degree of pneumothorax and pleural effusion, but none had AEs above Grade II. The local control time of liver lesions ranged from 9-20 mo (we continued to follow-up patients who already had 9 mo of local control). Three patients are still alive, and the KPS scores are all 80 points or above. For these 5 patients with liver malignancies, as planned, we adopted the combination treatment strategy by using cryoablation followed by local radiotherapy. Serious complications did not occur and good clinical efficacy was achieved.

CONCLUSION

Our clinical practice demonstrated that cryoablation combined with IMRT could be implemented safely. The definitive efficacy for hepatic malignancies needs to be confirmed in larger-size sample prospective studies.

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