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REVIEW

Eukaryotic initiation factor 5A2 and human digestive system neoplasms

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Abstract

Eukaryotic initiation factor 5A2 (eIF5A2), as one of the two isoforms in the family, is reported to be a novel oncogenic protein that is involved in multiple aspects of many types of human cancer. Overexpression or gene amplification of *EIF5A2* has been demonstrated in many cancers. Accumulated evidence shows that eIF5A2 initiates tumor formation, enhances cancer cell growth, increases cancer cell metastasis, and promotes treatment resistance through multiple means, including inducing epithelial–mesenchymal transition, cytoskeletal rearrangement, angiogenesis, and metabolic reprogramming. Expression of eIF5A2 in cancer correlates with poor survival, advanced disease stage, as well as metastasis, suggesting that eIF5A2 function is crucial for tumor development and maintenance but not for normal tissue homeostasis. All these studies suggest that eIF5A2 is a useful biomarker in the prediction of cancer prognosis and serves as an anticancer molecular target. This review focuses on the expression, subcellular localization, post-translational modifications, and regulatory networks of eIF5A2, as well as its biochemical functions and evolving clinical applications in cancer, especially in human digestive system neoplasms.

Key words: Eukaryotic translation initiation factor 5A2; Hypusine modification;

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Core tip: Eukaryotic initiation factor 5A2 (eIF5A2) is one of only two cellular proteins that contain the unusual amino acid hypusine. eIF5A2 initiates tumor formation, enhances cancer cell growth, increases metastasis, and promotes treatment resistance through inducing epithelial–mesenchymal transition, cytoskeletal rearrangement, angiogenesis, and metabolic reprogramming. Isoform eIF5A2 represents a promising target for treatment of human digestive system cancer. Our objective was to consolidate the current literature to better understand the expression, subcellular localization, post-translational modifications, and regulatory networks of eIF5A2, as well as its biochemical functions and evolving clinical applications in human digestive system cancer.

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INTRODUCTION

In 2000, *EIF5A2* was first sequenced and isolated as a novel candidate oncogene from human chromosome 3q26.2[1,2]. Eukaryotic initiation factor 5A2 (eIF5A2) is one of only two eIF5A family members that undergo an unusual post-translational hypusine modification[3]. Unlike isoform eIF5A1, which is ubiquitously expressed, eIF5A2 protein is normally not detected and its mRNA is expressed in a tissue-dependent manner in human tissues[1]. eIF5A2 protein has been shown to be overexpressed in many cancers, including cervical cancer[4,5], ovarian cancer[6-8], colorectal cancer[9,10], gastric cancer[11,12], liver cancer[13,14], melanoma[15,16], lung cancer[17], nasopharyngeal carcinoma[18], bladder cancer[19,20] and esophageal squamous cell carcinoma (ESCC)[21]. Accumulated evidence shows that eIF5A2 plays important roles as a regulatory molecule in many biological processes, including tumor formation, cancer cell growth, metastasis, maintenance of cancer stem cells (CSCs) and treatment resistance through multiple means including epithelial–mesenchymal transition (EMT), cytoskeletal rearrangement, angiogenesis, and metabolic reprogramming.

In this article, we review eIF5A2-related studies, particularly those about the discovery, subcellular location, functions, upstream and downstream regulation, and modification of eIF5A2, as well as its role as a biomarker and its therapeutic potential for human digestive system cancer.

LITERATURE SEARCH

A literature search was conducted using PubMed Library for “eIF5A2”, “eIF-5A2”, “eIF-5A-2”, “eIF5A-2”, “EIF5A2”, “eukaryotic translation initiation factor 5A2”, “eukaryotic initiation 5A2” or “human eukaryotic initiation factor 5A2”.

PROPERTIES AND EXPRESSION

Human eIF5A2 is a small (approximately 17 kDa) universally conserved acidic protein that contains 153 amino acids and is encoded by *EIF5A2* gene, which is located on chromosome 3q26.2; a chromosomal region that is frequently amplified in several human cancers[2,3]. Multiple forms of *EIF5A2* mRNA (5.6, 3.8, 1.6 and 0.7 kb, with one at 3.8 kb being the major form) are the products of one gene with various lengths of 3′-untranslated region (UTR), resulting from the use of different polyadenylation (AAUAAA) signals in various human cancer cell lines[22]. In short, for the structure of eIF5A2, the C-terminal domain consists of a three-turn α-helix α2 and five strands of β7-β11 and the N-terminal domain is dominated by β-strands[23].

Unlike *EIF5A1*, which is ubiquitously expressed, *EIF5A2* is normally not detected and its mRNA is expressed in a tissue-dependent and cell-type-specific manner, and is mainly found in testes, parts of adult brain, human cancer tissues (such as primary ovarian cancers) and some cancer cell lines (such as SW480 and UACC-1598)[1,2,24]. Clement *et al*[3] described the identification of eIF5A2 protein in human colorectal (SW-480) and ovarian (UACC-1598) cancer cell lines, and were first to report that eIF5A2 has an important role in eukaryotic cell survival similar to that of the ubiquitous eIF5A1. Overexpression of *EIF5A2* and/or eIF5A2 protein is observed in several human cancer tissues and/or cell lines such as cervical cancer[4,5,25], ovarian cancer[7,8], colorectal cancer[9,10,26-28], gastric cancer[11,12,29,30], ESCC[21,31], liver cancer[13,14,32-35], nasopharyngeal cancer[18], oral squamous cell carcinoma[36,37], pancreatic cancer[38-40], non-small cell lung cancer[17,41-43], melanoma[15,16], bladder cancer[34,44,45], and breast cancer[46,47]. In contrast, eIF5A2 is not generally overexpressed in glioblastoma[48] and chronic myeloid leukemia[49]. These observations suggest that eIF5A2 overexpression is not an invariable hallmark of cancer. Pällmann *et al*[50] reported high levels of *EIF5A2* mRNA in brain, epididymis, lung, prostate and testis tissues of wild-type mice, as assessed by quantitative real-time polymerase chain reaction.

POST-TRANSLATIONAL MODIFICATIONS

Hypusine modification and activation of eIF5A2

In humans, isoforms eIF5A1 and eIF5A2 are the only two cellular proteins that experience a post-translational hypusination by two essential enzymatic steps involving deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH), which selectively catalyze the polyamine spermidine- to finish eIF5A hypusination[22,51-53]. eIF5A exists mainly as the fully hypusination form in mammalian tissues and cells[54]. First, the 4- aminobutyl moiety of spermidine are transferred to the -amino group of Lys50 to form a deoxyhypusine-containing intermediate by DHS[3,22,51,55]. Second, DOHH catalyzes the hydroxylation of the deoxyhypusine residue to generate hypusine-containing eIF5A and activates it[22,51]. It has been reported that the endogenous activity of DHS and/or DOHH appears to be insufficient for modification of the excess precursors of mature eIF5A2 and eIF5A1[22], and exogenously expressed eIF5A2 and eIF5A1 is largely unhypusinated, and can be hypusinated only when DHS and DOHH are coexpressed[56,57]. Therefore, transfection studies with eIF5A2 expression vectors, such as our previous study[11] and others[7,9,13,26,27,31], should be re-assessed by evaluating the real changes in the concentrations of the hypusinated eIF5A2 or its precursor to determine the true cause of the biological effects. Hypusine modification not only activates eIF5A2, but also regulates its subcellular localization. However, in contrast to DHS- and DOHH-mediated hypusination of eIF5A1, which is crucial for embryonic development as well as for viability in adult mice, the cancer-associated isoform eIF5A2 is dispensable for embryonic development and viability in adult organisms[50]. Future work will be needed to determine the contribution of hypusine biosynthetic enzymes of eIF5A2 in tumorigenesis and metastasis.

Acetylation modification

In addition to unique hypusination, eIF5A2 also undergoes reversible acetylation modification at Lys-47, like eIF5A1 does[56,57]. Histone deacetylase 6 and sirtuin 2 have been identified as the major deacetylases of eIF5A2[56]. Acetylation of eIF5A2 at Lys-47 plays an important role in its subcellular localization. It is also reported that acetylation of the hypusine side chain in the N-terminal domain by a key polyamine catabolic enzyme, spermidine/spermine-N1-acetyltransferase 1 (SSAT1) inactivates eIF5A, which suggests regulation of eIF5A activity by reversible acetylation/deacetylation at this site though SSAT1 catalysis[58].

Other modifications

eIF5A can be modified by phosphorylation[59,60], ubiquitination[61] and transglutaminylation[62], but clear effects on its activity have not been fully detected. eIF5A dephosphorylation is required for translation arrest in stationary phase cells[60]. Shang *et al*[61] reported that the carboxyl terminus of Hsc70-interacting protein (CHIP) functions as a negative regulator of eIF5A to mediate its ubiquitination for degradation. This was the first report on regulation of eIF5A protein stability *via* a protein degradation mechanism. It is likely, therefore, that the CHIP–eIF5A2 axis mediates ubiquitination of eIF5A2 for degradation in human cancers. The potential role of eIF5A2 in human cancer development and metastasis has been found in recent years; therefore, the importance of eIF5A2 post-translational modifications in its

oncogenic properties should be elucidated in the future.

SUBCELLULAR LOCALIZATION OF eIF5A2

The nuclear membranes force nucleocytoplasmic exchange to proceed through nuclear pore complexes (NPCs)[63]. The NPC permeability barrier -allows free passage to small molecules, while limiting larger molecules that approach or exceed a limit of > 30kDa in mass or > 5nm in diameter[64]. Most evidence demonstrates s that eIF5A2, as a shuttling protein, is responsible for regulating protein translation in the cytoplasm, and only a few studies have shown that it is located and works in the nucleus[15,21,65]. More studies are necessary to address its role in the nucleus. eIF5A2 has an invariably small molecular mass of only 17 kDa and can thus cross the NPC permeability barrier rapidly, even without the help of an importin. The nuclear export of eIF5A may be mediated by the nuclear exporter exportin (XPO)4, which belongs to the importin-β family of nuclear transporters, in a hypusine-dependent manner[66,67]. In addition, the N-terminal 19 amino acids of eIF5A serve as a signal for nuclear localization of eIF5A[68]. Knockdown of XPO4 in murine hepatoma cells leads to nuclear accumulation of eIF5A2 as well as eIF5A1[65].

Post-translational modifications including acetylation at Lys-47 and hypusination at Lys-50 of eIF5A2 direct its subcellular localization[56]. Acetylation acts as a molecular switch for eIF5A2, allowing it to exert distinct functions in the cytoplasm and nucleus. The acetylated form of eIF5A2 is primarily enriched in the nucleus, suggesting that acetylation at Lys-47 induces nuclear accumulation[56]. In addition, the study also showed that unhypusinated eIF5A2 is highly acetylated but is significantly deacetylated upon hypusination, implying crosstalk between acetylation and hypusination[56]. Hypusination can reduce acetylation in eIF5A2, leading to its localization in the cytoplasmic compartment where it is required for protein synthesis. Inhibition of the deacetylases or impaired hypusination increases acetylation of eIF5A2, leading to nuclear accumulation. These findings provide strong evidence that cytoplasmic location of eIF5A2 requires not only hypusination but also hypo-acetylation.

REGULATION OF *EIF5A2* EXPRESSION IN HUMAN DIGESTIVE SYSTEM NEOPLASMS

Although the mechanisms of *EIF5A2* gene upregulation in tumor cells are not clear yet, most researchers believe that the main reason is genomic instability caused by copy number variation. To date, *EIF5A2* has been frequently found, but not always, to be amplified in human cancers and cancer cell lines[2,8,10,17,19,21,69]. Although tumors that exhibit gene amplification typically exhibit high eIF5A2 expression, many have high eIF5A2 levels without gene amplification, and thus other mechanisms, such as transcriptional regulation and/or post-transcriptional regulation, must exist in eIF5A2 upregulation. It has been demonstrated that K-ras activation upregulates eIF5A2 expression as well as hypusination *via* transcriptional regulation during the early stages of pancreatic ductal adenocarcinoma (PDAC) progression[38]. Another study has reported that hypoxia increases *EIF5A2* RNA levels, at least in part *via* hypoxia-inducible factor (HIF)-1α in ESCC cells[21].

Many studies have demonstrated that miRNAs (miRs) target the 3′-UTR of cytoplasmic mRNA of *EIF5A2* to post-transcriptionally regulate mRNA and protein levels[70] (Table 1). *EIF5A2* is a putative target for miR-203, miR-30b, miR-9, miR-125b, miR-599 and miR-588, which are predicted by the bioinformatic algorithm TargetScan (www.targetscan.org). miR-203 suppresses growth and invasion of colorectal cancer cells (SW620 and LOVO), at least partly, by binding the 3′-UTR of *EIF5A2* and repressing *EIF5A2* expression at both the mRNA and protein levels[26]. miR-30b[29], miR-599[71] and miR-588[72] suppress gastric cancer cell metastasis *via* binding to the 3′-UTR of *EIF5A2* and repressing eIF5A2 expression. miR-125b inhibits tumorigenic properties of hepatocellular carcinoma (HCC) cells *via* suppressing eIF5A2 expression, through binding to the 3′-UTR of *EIF5A2*[73]. miR-9 enhances sensitivity to cetuximab in epithelial phenotype HCC cells through regulation of eIF5A2[74].

Zender *et al*[65] has reported that eIF5A2 is a key downstream effector of XPO4 in tumor inhibition, and XPO4 is a negative regulator of eIF5A2, which may play a role in inhibiting cell proliferation in the nucleus. In murine hepatoma cells, knockdown of XPO4 leads to accumulation of eIF5A1 and eIF5A2 in the nucleus[65]. The sonic hedgehog-GLI family zinc finger 1 signaling pathway upregulates eIF5A2 in pancreatic cancer cells[28]. Moreover, hypoxia can induce eIF5A2 upregulation and

Table 1 miRNA action in regulation of *EIF5A2* gene expression

miRs	Ref.	Materials	Function
miR-203	Deng *et al*[26]	CRC cells (SW620 and LOVO)	Suppressing growth and invasion *via* miR-203/EIF5A2 axis
miR-599	Wang *et al*[71]	GC cells (BGC823 and MKN-45)	Inhibiting metastasis and EMT *via* miR-599/EIF5A2 axis
miR-588	Zhou *et al*[72]	GC cells (MGC803)	Regulating invasion, migration and EMT *via* miR-588/EIF5A2 axis
miR-30b	Tian *et al*[29]	GC cells (AGS and MGC803)	Downregulation of EIF5A2 by miR-30b inhibits EMT
miR-9	Xue *et al*[74]	HCC cells (Hep3B and Huh7)	Enhancing sensitivity to cetuximab *via* miR-9/EIF5A2 axis
miR-125b	Tsang *et al*[73]	HCC tissue and cells	Inhibiting tumorigenic properties *via* miR-125b/EIF5A2 axis

CRC: Colorectal cancer; GC: Gastric cancer.

promote eIF5A2 translocation from the cytoplasm to the nucleus in ESCC cell lines (KYSE140, KYSE180, KYSE410, KYSE510 and EC109)[21].

FUNCTIONS OF eIF5A2 IN HUMAN DIGESTIVE SYSTEM NEOPLASMS

The cancer-associated isoform eIF5A2 is not essential for normal development and viability, which has been confirmed *in vivo*[50]. Accumulating evidence shows that eIF5A2 plays important roles in tumor proliferation[11], metastasis[13], EMT[9,11,13,28-29,35,75,76], cytoskeletal rearrangement[13], angiogenesis[21], metabolic reprogramming[14], maintenance of CSCs[31,77] and drug resistance[33,38,74,75,78-80]*via* its subsequent signaling pathways. Additionally, eIF5A2 is associated with survival of many digestive cancer patients[9,11,12,14,21,32] (Figure 1).

eIF5A2 and EMT

Over the past 10 years, many studies have evaluated the role of eIF5A2 in activating EMT in human cancer cells. Tang *et al*[13] first reported that eIF5A2 induces EMT; an important event in tumor invasion and metastasis that is chiefly characterized by upregulation of mesenchymal markers (Vimentin, fibronectin, E-cadherin and α-smooth muscle actin) and downregulation of epithelial markers (E-cadherin and β-catenin) in HCC. Shek *et al*[35] and Lou *et al*[75] confirmed that eIF5A2 enhances the aggressiveness of HCC cells by inducing EMT. Zhu *et al*[9] found that overexpression of eIF5A2 also promotes colorectal carcinoma cell aggressiveness by upregulating Metastasis-associated protein 1 through C-myc to induce EMT[76]. In addition, eIF5A2 induces EMT of other human digestive system neoplasms such as gastric cancer[11,29] and pancreatic cancer[28].

eIF5A2 and cytoskeletal rearrangement

In HCC, eIF5A2 stimulates rearrangement of the cytoskeleton through activation of the RhoA/Rac1 GTPase signaling pathway[13]. That study showed that overexpression of eIF5A2 in human liver LO2 cells provokes the formation of stress fibers and lamellipodia, without affecting expression level of Rho/Rac GTPase in the cells[13]. However, the precise mechanism underlying EIF5A2-mediated Rho-GTPase activation requires further investigation.

eIF5A2 and angiogenesis

Increased expression of eIF5A2, *via* hypoxia or gene amplification, contributes to angiogenesis in ESCC *via* the HIF-1α-mediated signaling pathway[21]. *In vitro* and *in vivo* assays have both indicated that eIF5A2 increases angiogenesis by enhancing matrix metalloproteinase 2 activity *via* activation of the p38 mitogen-activated protein kinase pathway, and eIF5A2 silencing increases tumor vessel wall continuity, increases blood perfusion, and improves tumor oxygenation in HCC[33].

eIF5A2 and metabolic reprogramming

A recent study reported that eIF5A2 triggers cellular metabolic reprogramming, including glucose metabolism, by promoting aerobic glycolysis and fatty acid biosynthesis *via* upregulation of *FASN* and *ACSS2* in human liver cancer cells[14].

eIF5A2 and maintenance of stemness of cancer cells

CSCs are suggested to be responsible for driving resistance to conventional therapies and for cancer metastasis and/or recurrence. It has been reported that eIF5A2

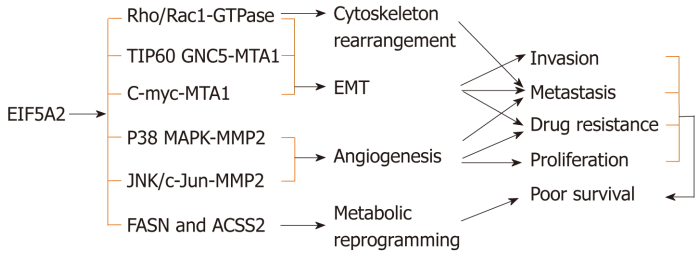

Figure 1 unctions and subsequent pathways of eukaryotic initiation factor 5A2 in human digestive system neoplasms. Overexpression of Eukaryotic initiation factor 5A2 (eIF5A2) induces epithelial–mesenchymal transition (EMT) by enhancing RhoA/Rac1-GTPase and ITP60 GNC5-MTA1 activity in hepatocellular carcinoma (HCC). Overexpression of *EIF5A2* also promotes colorectal carcinoma and gastric cancer cell aggressiveness by upregulating the C-myc/MTA axis to induce EMT. Increased expression of eIF5A2 contributes to angiogenesis in esophageal squamous cell carcinoma *via* the P38 MAPK/MMP2 pathway. eIF5A2 promotes cell proliferation and triggers cellular metabolic reprogramming in HCC cells, including glucose metabolism and fatty acid biosynthesis *via* upregulation of *FASN* and *ACSS2*. In HCC, eIF5A2 stimulates rearrangement of the cytoskeleton through activation of the RhoA/Rac1 GTPase signaling pathway. eIF5A2: Eukaryotic initiation factor 5A2; EMT: Epithelial–mesenchymal transition.

overexpression increases the stemness of ESCC cells (KYSE510)[31]. A recent study showed that eIF5A2 also contributes to the maintenance of HCC CSCs ($CD133^+$ HCC cells) *via* the c-Myc/miR-29b axis[77].

eIF5A2 and survival of patients

Overexpression of cytoplasmic eIF5A2 detected by immunohistochemistry is correlated with poor survival of patients with digestive system malignancies, including colorectal cancer[9], ESCC[21], gastric cancer[11,12] and liver cancer[14,32]. All these studies suggest that a high level of eIF5A2 expression in the cytoplasm is a potential prognostic indicator in many human cancers. However, a recent study demonstrated that nuclear eIF5A2 expression is also an independent prognostic marker in human melanoma[15]. Therefore, nuclear eIF5A2 may have the potential to serve as a therapeutic marker for some human cancers, and further study is needed to establish the subcellular localization of eIF5A2.

Role of eIF5A2 in treatment resistance of human digestive system neoplasms

Primary or secondary anticancer drug resistance is a clinical problem shared by both chemotherapy and targeted therapy. The development of resistance may be predicted from pre-existing genomic and proteomic profiles in patients[78]. eIF5A2 can be used as a biomarker for predicting drug resistance. N1-guanyl-1,7-diaminoheptane (GC7), an inhibitor of DHS, enhances the therapeutic efficacy of doxorubicin in epithelial HCC cells (Huh7, Hep3B and HepG2)[75,79] by preventing the doxorubicin-induced EMT through inhibition of eIF5A2 activation. GC7 can also enhance the sensitivity of oral cancer cells to cisplatin[37]. eIF5A2 promotes resistance to doxorubicin *via* regulation of EMT in colon cancer cells[27]. Downregulation of eIF5A2 increases tumor perfusion and reduces tumor hypoxia, thus increasing the chemosensitivity of HCC cells to 5-fluorouracil by remodeling tumor vessels[33]. eIF5A2 is significantly related to gemcitabine sensitivity in PDAC cells[38]. Recently, Xue *et al*[74] reported that eIF5A2 is associated with cytotoxicity of cetuximab in epithelial HCC cells[80]. A high level of eIF5A2 expression is related to drug resistance in many human digestive system cancers. However, other studies have shown no significant relationship between *EIF5A2* expression and effects of preoperative radiotherapy in human rectal cancer[81].

CONCLUSIONS AND PERSPECTIVES

Basic research and clinical evidence show that *EIF5A2* is a candidate oncogene and may be a key biomarker for the prognosis of various human digestive system cancers. There is growing evidence that inhibition of hypusination of eIF5A2 inhibits tumorigenesis. Hypusine modification of eIF5A by DHPS and DOHH forms an attractive platform for therapeutic intervention. Many studies have shown that GC7, as an inhibitor of DHS, enhances the sensitivity of drugs through inhibition of eIF5A2 activation in many kinds of human cancer cells[27,37,39,42,47,75,79,80,82,83]. However, hypusination takes place in all eukaryotic cells and has been shown to be necessary for proliferation of mammalian cell lines[52] and crucial for embryonic development as

well as viability in adult mice[50]. So, important questions remain regarding how to selectively target tumors and reduce adverse effects.

In contrast to *EIF5A1*, the of *EIF5A2* is limited to tissue such as testes and a few parts of the adult brain, but it is abundant in many human cancers. The eIF5A2 protein is associated with cancer metastasis by influencing the processes of EMT, angiogenesis, cytoskeletal rearrangement, and metabolic reprogramming. Thus, the isoform eIF5A2 represents a promising target for the treatment of malignant tumors. Moreover, in contrast to DHS or DOHH, the eIF5A2 isoform is not essential for embryonic development or for viability in an adult organism. So, we speculated whether eIF5A2, which is only expressed in a few tissues in the normal human body, but abundant in various tumor cells, might represent a better target for therapy. Therefore, we propose that specific inhibitors of eIF5A2 will exhibit selective toxicity toward eIF5A2-dependent cancer cells. Better understanding of the physiological and pathophysiological functions of eIF5A2 may lead to more effective management of many human digestive system cancers with high expression of *EIF5A2*, *via* early detection, precise prognostication, and molecular targeted treatment. A recent study demonstrated that Mg(II)-catechin nanocomposite particles (Mg(II)-Cat NPs) delivering siEIF5A2 inhibited bladder cancer cell growth *in vitro* and *in vivo*[45,84]. These results provide preclinical evidence for use of Mg(II)-Cat/siEIF5A2 combined therapeutic methods in cancer.

However, it is also clear that more researches are needed to clarify the underlying mechanisms that regulate eIF5A2 expression, for example, how does noncoding RNA regulate the UTR of *EIF5A2* and how is its promoter epigenetically modified. With regard to the downstream pathway, the exact mechanism of eIF5A2 in regulating its target and whether it can act as a transcriptional factor have not been elucidated.

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REVIEW

Role of circular RNAs in gastric cancer: Recent advances and prospects

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Abstract

Circular RNA (circRNA) is a newly discovered non-coding RNA with special structure, which is widely expressed in eukaryotic organisms and mainly located in the cytoplasm. circRNAs participate in gene regulation by working as miRNA sponges that block the inhibitory effect of miRNA on its target genes. In addition, circRNAs can bind to RNA binding proteins to regulate gene expression. Based on characteristics of stability, expression specificity and participation in gene regulation, circRNAs are expected to be biomarkers for early diagnosis of cancer or potential targets for cancer therapy. With the help of bioinformatics analysis, circRNA microarray analysis and high-throughput sequencing technology, more circRNAs were discovered to participate in the progression of gastric cancer (GC) over the past three years. This article gives an overview of these recent research focusing on the roles of circRNAs in GC and highlights the advances.

Key words: Circular RNA; Gastric cancer; Biomarker; Therapeutic target; Prognosis

Core tip: Gastric cancer (GC) is a common, worldwide malignant tumor with a poor prognosis. An increasing number of circRNAs was discovered to participate in the progression of GC. Therefore, exploring the function of circRNAs will help to achieve a better understanding of the pathogenesis of GC and identify new diagnostic biomarkers and therapeutic targets. This article gives an overview of the recent research focusing on the roles of circRNAs in GC and highlights the advances that were made over the past three years.

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INTRODUCTION

Gastric cancer (GC) is one of the most common human cancers. The number of new cases in 2018 was 1033701, which accounted for 5.7% of all new cancers, and the number of deaths from GC was 782685, which accounted for 8.2% of all cancer deaths; only behind lung cancer[1]. Although diagnostic and therapeutic techniques have been developing rapidly, the prognosis of GC remains poor[2]. The poor prognosis is partly due to an incomplete understanding of the molecular mechanisms of GC occurrence and development. Thus, it is critical to identify some new biomarkers and therapeutic targets to improve the diagnosis and treatment of GC.

Circular RNAs (circRNAs) are newly discovered endogenous non-coding RNAs (ncRNAs) that form covalently closed continuous loops with neither 5′ to 3′ polarity nor a polyadenylated tail[3]. They are generated from backsplicing of exons, introns, or both, and called exonic circRNAs, intronic RNAs and exon-intron circRNAs according to their components (Figure 1). Although circRNAs have been investigated for almost 40 years[4], significant attention has not been received until recent years[5]. circRNAs were found to participate in gene regulation by working as miRNA sponges that block the inhibitory effect of miRNA on its target genes[6], splicing of target genes[7] or interacting with RNA-binding proteins (RBPs)[8]. In addition, some circRNAs can even encode peptides[9,10]. circRNAs have been widely investigated in recent years because they play many important roles in proliferation, apoptosis and metastasis of cancer cells[11-15]. With the help of bioinformatics analysis, circRNA microarray analysis and high-throughput sequencing technology, more circRNAs was discovered to participate in the progression of GC[16-21]. Gu *et al*[20] conducted a circRNA microarray analysis to explore the difference in circRNAs expression between tumor and adjacent nontumorous tissues from six patients with GC. They showed that 440 circRNAs were expressed differently in tumor samples, including 176 upregulated and 264 downregulated circRNAs[20]. Exploration of GC-related circRNAs may provide a new insight into the diagnosis and treatment of GC. In addition, circRNAs are hardly degraded by RNA exonuclease or ribonuclease R[22], making them more stable in tissue or plasma. This feature makes circRNAs a potential use for biomarkers, prognosis predictors, and even therapeutic targets of GC.

We searched MEDLINE and PubMed before January 2019 using the following keywords: circular RNA, circRNA and gastric cancer. The inclusion criteria were as follows: (1) Studies associating circRNAs with GC samples or cancer cells, and discussing their potential use as biomarkers for diagnosis of the disease; (2) Studies associating circRNAs with biological functions or potential pathways in GC; and (3) Studies associating circRNAs with clinical significance of GC. This review highlights recent advances in circRNA in GC, especially focusing on their deregulation, biological function and clinical significance.

CIRCRNAS ACT AS MIRNA REGULATORS IN GASTRIC CANCER

As mentioned before, circRNAs primarily act as miRNA sponges to regulate gene expression. Most circRNAs contain a miRNA response element, which can bind with miRNAs and regulate their expression. At present, there are 13 circRNAs that act as miRNA regulators in GC. Some of these are downregulated and serve as tumor suppressors, while others are upregulated during carcinogenesis and serve as oncogenes. All these circRNAs are summarized in Table 1.

hsa_circ_0000993 is downregulated in GC. It can act as a miRNA sponge for miR-214-5p and inhibit the proliferation, invasion and migration of GC cells[23]. Has_circ_000146, a sponge for miR-548g, is significantly downregulated in GC cell lines and tissues, and negatively correlated with survival time of GC patients. Overexpression of has_circ_0001461 can inhibit proliferation, migration and invasion of GC cells. These effects can be reversed by overexpression of miR-548g, which can downregulate expression of runt-related transcription factor 1 (RUNX1). These results suggest that has_circ_0001461 acts as a tumor suppressor in GC cells by regulating the miR-548g/RUNX1 pathway[24]. Liu *et al*[25] reported that has_circ_0002320 level was

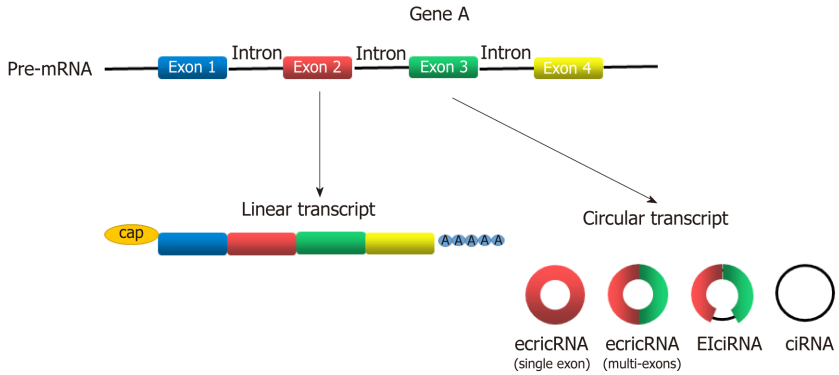

Figure 1 Classification of circRNAs according to their components. EcircRNAs are composed of single or multi-exons. In EIciRNAs, exons are circularized with introns "retained" between exons. CiRNAs are composed of introns.

significantly lower in GC tissues than in paired adjacent nontumorous tissues, and the survival time was shorter in GC patients with lower has_circ_0002320 level. By using fluorescence *in-situ* hybridization (FISH) in GC tissues, they found that has_circ_0002320 and miR-367-5p were colocalized in the cytoplasm. Overexpression of has_circ_0002320 upregulated expression of p27 Kip1 in GC cells and inhibited their growth and invasion, and these effects could be reversed by miR-367-5p mimics. These results demonstrate that has_circ_0002320 is a tumor suppressor in GC cells by targeting the miR-367-5p/p27 Kip1 pathway and provides a prediction of survival time in GC patients[25]. Hsa_circ_0027599 was significantly downregulated in GC patients and cells, and its overexpression inhibited proliferation and metastasis of GC cells. Moreover, hsa_circ_0027599 was verified to be a sponge of miR-101-3p.1 (miR-101) by bioinformatic technology and luciferase reporter assays. miR-101 can inhibit the expression of its target gene *PHLDA1* and promote proliferation of cancer cells. Conversely, overexpression of *PHLDA1* decreases the growth and migration of MKN-28 and HGC-27 GC cells. These results suggest that *PHLDA1* is regulated by circ_0027599/miR-101, which inhibits the growth and metastasis of GC cells[26]. Another study, which had different conclusions from the above, has shown that miR-101-3p is a tumor suppressor and overexpression of miR-101-3p inhibits proliferation and invasion of AGS GC cells[27]. Therefore, the functions of miR-101 needs more investigation. miR-630 is one of the newly discovered miRNAs, and its role in cancer has attracted increased attention. miR-630 is dysregulated in many tumors[28,29]. Direct interaction of miR-630 and circRNA_100269 was confirmed by dual-luciferase reporter assays. The level of miR-630 decreased significantly by circRNA_100269 overexpression, which inhibited proliferation of GC cells. These results suggest that the circRNA_100269/miR-630 axis plays an important role in the growth of GC cells[30]. A novel circRNA circ_101057, also termed as circLARP4, was shown downregulated in GC tissues by FISH analysis, and lower circLARP4 expression was associated with poor prognosis. Furthermore, circLARP4 inhibited biological behavior of GC cells[31]. These effects have also been seen in ovarian cancer[32]. circLARP4 was found to sponge miR-424-5p by bioinformatics analysis. miR-424-5p promotes proliferation and invasion of GC cells by targeting *LATS1* gene, and positively correlates with higher clinical stage and worse prognosis of GC patients[31]. However, the function of miR-424-5p is the opposite in breast cancer and esophageal squamous cell carcinoma. Wang *et al*[33] have reported that miR-424-5p acts as a tumor suppressor to regulate proliferation, invasion and migration of breast cancer cells by binding to the functional target Doublecortin Like Kinase 1[33]. Upregulation of miR-424-5p may prevent tumor invasion or metastasis[34]. circ-ZFR is a new circRNA that is markedly downregulated in tumor tissues compared with pair-matched adjacent nontumorous tissues. Moreover, expression of circ-ZFR is significantly lower in GC cell lines HGC-27, AZ521, and AGS than in gastric epithelial cell line GES1. circ-ZFR promotes cell cycle arrest and apoptosis in GC cells by sponging miR-107/miR-130a, and miR-107/miR-130a could bind to the 3′ untranslated region (UTR) of phosphatase and tensin homolog (PTEN)[35]. Many studies have demonstrated that PTEN could be targeted and regulated by miR-107 and miR-130a to influence activities of cancer cells[36,37]. All these results suggest that the circ-ZFR-miR-107/miR-130a-PTEN pathway plays an important role in the progression of GC.

One circRNA hsa_circ_0017639 that is derived from gene *SFMBT2*, also named circ-SFMBT2, shows higher expression level in GC tissues compared with adjacent nontumorous tissues, and is linked to higher tumor stages. The proliferation of GC

Table 1 Deregulated circRNA in gastric cancer: function and potential signaling pathway

circRNA	Deregulation	Function/clinical association	Gene/pathway affected	Ref.
hsa_circ_0000993	Downregulation	Inhibits proliferation, migration and invasion	miR-214-5p	[23]
has_circ_0001461	Downregulation	Inhibits proliferation, migration and invasion; correlates with the clinical stage	miR-548g, RUNX1 in the cytoplasm; YBX1 in the nucleus	[24]
has_circ_0002320	Downregulation	Inhibits proliferation and invasion; correlates with TMN stage and survival time	miR-367-5p, p27	[25]
hsa_circ_0027599	Downregulation	Inhibits proliferation and migration; correlates with TNM stage	miR-101, PHLDA1	[26]
circRNA_100269	Downregulation	Inhibits proliferation; correlates with histological subtype,node invasive number and overall survival time	miR-630	[30]
circRNA_101057	Downregulation	Inhibits proliferation and invasion; correlates with tumor size and lymphatic metastasis and overall survival time	miR-424, LATS1	[31]
circZFR	Downregulation	Inhibits proliferation and promotes apoptosis	miR-130a/miR-107, PTEN	[35]
hsa_circ_0017639	Upregulation	Promotes proliferation; correlates with TNM stage	miR-182-5p, CREB1	[38]
circRNA_0000284	Upregulation	Promotes proliferation; correlates with T stage	miR-124 and miR-29b, COL1A1, COL4A1 and CDK6	[42]
circRNA_001569	Upregulation	Promotes proliferation and inhibits apoptosis; correlates with tumor size, depth of invasion and clinical stage	miR-145, NR4A2	[44]
circPDSS1	Upregulation	Promotes proliferation and inhibits apoptosis;correlates with worse overall survival time	miR-186-5p, NEK2	[45]
circNF1	Upregulation	Promotes proliferation	miR-16, MAP7 and AKT3	[49]
ciRS-7	Upregulation	Promotes proliferation and inhibits apoptosis; correlates with TNM stage and poor overall survival time	miR-7, PTEN/PI3K/AKT pathway	[53]

RUNX1: Runt-related transcription factor 1; YBX1: Y-box binding protein-1; PHLDA1: Pleckstrin homology like domain family A member 1; LATS1: Large tumor suppressor kinase 1; PTEN: Phosphatase and tensin homolog; CREB1: cAMP response element binding protein 1; COL1A1: Collagen type I α1 chain; COL4A1: Collagen type IV α1 chain; CDK6: Cyclin-dependent kinase 6; NR4A2: Nuclear receptor subfamily 4 group A member 2; NEK2: NIMA related kinase 2; MAP7: Microtubule associated protein 7.

cells is significantly suppressed when circ-SFMBT2 is knocked down. Luciferase reporter assay revealed that miR-182-5p mimics induced a lower luciferase level in circ-SFMBT2 WT group than in the normal control group. Furthermore, it has been demonstrated that circ-SFMBT2 acts as a sponge of miR-182-5p to regulate expression of cAMP response element binding protein (CREB)1 and promotes proliferation of GC cells[38]. circHIPK3 (circRNA_0000284) that is derived from the homeodomain-interacting protein kinase-3 (*HIPK3*) gene sponges multiple miRNAs and serves as an oncogene in multiple cancers[39-41]. In GC tissues, circHIPK3 level is significantly higher than it in paired adjacent nontumorous tissues. Moreover, it negatively regulates expression of miR-29b/miR-124 and is associated with T stage of GC. Three candidate genes (*CDK6, COL1A1* and*COL4A1*) can be regulated by miR-29b and miR-124, suggesting that these genes may play important roles in GC though circHIPK3-miR-29b/miR-124 axes[42]. circRNA_001569 was firstly discovered to act as a positive regulator in cell proliferation and invasion of colorectal cancer[43]. Recently, it was found upregulated in tissues and cells of GC. circRNA_001569 overexpression significantly decreases expression of miR-145, while circRNA_001569 knockdown has the opposite effect. Moreover, circRNA_001569 knockdown decreases cell viability

dramatically and promotes apoptosis, but these effects of circRNA_001569 knockdown are reversed when cells are cotransfected with miR-145 inhibitor. The online microRNA.org predicted that miR-145 could bind with NR4A2 3′ UTR. miR-145 overexpression significantly decreased NR4A2 expression and cell viability, and promoted apoptosis. However, cotransfection with NR4A2 abolished the above effects[44]. All these results indicate that circRNA_001569 serves as an oncogene by regulating expression of the miR-145/NR4A2 axis. circPDSS1 was recently discovered to be highly expressed in GC tissue and cell lines. Patients with higher circPDSS1 expression have worse overall survival. CircPDSS1 knockdown significantly inhibits cell proliferation[45]. The expression of miR-186-5p, a tumor suppressor gene[46], is decreased by circPDSS1 overexpression. In luciferase reporter assays, luciferase activity was decreased by cotransfection of miR-186-5p mimics and wt-NEK2. This suggests that *NEK2*, an oncogene[47,48], is a target of miR-186-5p. Moreover, miR-186-5p inhibits *NEK2* expression, while miR-186-5p inhibitor reverses this effect[45]. In summary, circPDSS1/miR-186-5p/NEK2 pathway may play an important role in GC cancer progression, and may be a target for gene therapy. circNF1 is upregulated in GC tissues and cell lines. Functional studies have demonstrated that circNF1 serves as an oncogene and significantly promotes cell proliferation. Furthermore, luciferase reporter assays have shown that circNF1 acts as a sponge to miR-16, thereby affecting its downstream target mRNAs, AKT3 and MAP7[49]. ciRS-7 is a well-known circRNA due to its promotion of carcinogenesis in a variety of cancers[50-52]. In GC, the ciRS-7 level is significantly higher than in nontumorous tissues, and higher ciRS-7 is associated with worse survival. miR-7 overexpression increases expression of PTEN, decreases PI3K and Akt phosphorylation, and inhibits tumor growth, while ciR-7 attenuates these effects[53]. These results indicate that ciRS-7 might be a promising therapeutic target through modulation of mir-7/PTEN/PI3K/AKT pathway in GC.

CircRNAs ACT AS DIAGNOSTIC BIOMARKERS OF GASTRIC CANCER

The 5-year survival rate of early GC can exceed 92%[54,55]. However, if GC develops to a late stage, the survival rate is significantly decreased[56]. Therefore, stable and effective diagnostic markers for the early diagnosis of GC need to be identified. Over the past three years, many circRNAs have been found to have specific differences between GC and normal gastric tissue, and these differences have helped circRNAs to become potential markers of early diagnosis or predictors of prognosis[38,57-72]. All these circRNA are summarized in Table 2. We will cover in detail those circRNA with an area under the curve (AUC) > 0.75.

Hsa_circ_0000096 level was found to be lower in GC tissues and cell lines than paired adjacent nontumorous tissues and normal gastric epithelial cells. Furthermore, the cutoff value (ΔCt value) of hsa_circ_0000096 was 12.9 with an AUC of 0.82. Hsa_circ_0000096 was also linked to several clinicopathological features such as invasion and TNM stage[57]. Hsa_circ_0000181 levels in plasma from GC patients and tissues were significantly decreased compared with those from healthy individuals and paired adjacent nontumorous tissues. In addition, its level in plasma of GC patients was associated with differentiation and carcinoembryonic antigen (CEA) level. The AUC of hsa_circ_0000181 in plasma was 0.582 with a specificity of 20.6% and sensitivity of 99.0%. Moreover, hsa_circ_0000181 levels in GC tissues were associated with tumor diameter, lymphatic metastasis, distant metastasis, and carbohydrate antigen (CA)19- 9 level. The AUC of hsa_circ_0000181 in tissues was 0.756 with a specificity of 85.2% and sensitivity of 53.9%[58]. Hsa_circ_0000190 was firstly discovered to be downregulated in plasma and tissues samples from GC patients. Its levels in tissue were significantly associated with TNM stage and CA19-9 level. The AUC of hsa_circ_0000190 in tissue was 0.75. The sensitivity and specificity were 72.1% and 68.3%, respectively[59]. hsa_circ_0000520 expression was significantly downregulated in GC tissue, plasma and cell lines (BGC-823, MKN-45, AGS and MGC-803). In plasma, the AUC was 0.8967, and the sensitivity and specificity were 82.35% and 84.44%, respectively[60]. The limitation of this study was the small number of samples. There were only 56 paired GC tissues, 45 preoperative GC plasma and 17 healthy plasma samples used for analysis, thus indicating the need and necessity to expand the sample size to verify the efficacy of hsa_circ_0000520 as a biomarker for GC. Hsa_circ_0001895 levels were lower in 69.8% of GC tissues than in paired adjacent nontumorous tissues and were also downregulated in five GC cell lines (HGC-27, BGC-823, AGS, SGC7901 and MGC-803). In addition, its level was linked to tissue CEA expression, Borrmann type and cell differentiation. The AUC of hsa_circ_0001895 in tissue was 0.792. When the optimal cutoff value of hsa_circ_0001895

Table 2 Deregulated circRNA in gastric cancer: diagnostic or predictive biomarker

circRNA	Deregulation	Cut-off value (ΔCt)	AUC	Sensitivity	Specificity	Clinical association	Ref.
hsa_circ_0000096	Downregulation	12.9	0.82	-	-	Gender, invasion and TNM stage	[57]
hsa_circ_0001181	Downregulation	9.4	0.756	85.2%	53.9%	Tumor diameter, lymphatic metastasis, distal metastasis, and CA19-9 (tissue)	[58]
		7.27	0.582	20.6%	99%	CEA and differentiation (plasma)	
hsa_circ_0001190	Downregulation	6.83	0.75	72.1%	68.3%	Tumor diameter, TNM stage and CA19-9 (tissue)	[59]
		3.07	0.6	41.4%	87.5%	CEA (plasma)	
hsa_circ_0000520	Downregulation	-	0.6129	53.57%	85.71%	TNM stage (tissue)	[60]
		-	0.8967	82.35%	84.44%	CEA (plasma)	
hsa_circ_0000745	Downregulation	-	0.683	85.5%	45%	Tumor differentiation (tissue) and TNM stage (plasma)	[61]
hsa_circ_0001895	Downregulation	9.53	0.792	67.8%	85.7%	Tumor differentiation, Borrmann type, and tissue CEA	[62]
hsa_circ_0000164 9	Downregulation	0.227	0.834	71.1%	81.6%	Tumor differentiation	[63]
hsa_circ_002059	Downregulation	12.9	0.73	81%	62%	TMN stage, distal metastasis, gender and age	[64]
hsa_circ_0003159	Downregulation	12.31	0.75	85.2%	56.5%	Gender, distal metastasis, and TMN stage	[65]
hsa_circ_0006633	Downregulation	8.17	0.741	60%	81%	Distal metastasis and CEA	[66]
hsa_circ_0014717	Downregulation	12.14	0.696	59.38%	81.25%	Tumor stage; distal metastasis; CEA; CA199	[67]
has_circ_0066779	Downregulation	-	0.6726	90.3%	56.4%	TNM stage overall survival time	[68]
hsa_circ_0074362	Downregulation	12.17	0.63	84.3%	36.2%	CA19–9 and lymphatic metastasis	[69]
hsa_circ_0130810	Downregulation	1.443	0.7481	77.42%	68%	TNM stage and overall survival time	[70]
hsa_circ_0000467	Upregulation	-	0.79	70.5%	64.8%	TNM stage	[71]
hsa_circ_0017639	Upregulation	11.46	0.7585	80.56%	63.89%	TNM stage	[38]
hsa_circ_0066444	Upregulation	-	0.7328	70.75%	68.87%	Lymphatic metastasis	[72]

AUC: Area under the curve; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen.

was set at 9.53, the sensi-tivity and specificity were 67.8% and 85.7%, respectively[62]. Hsa_circ_0001649 is a well-known prognostic biomarker or tumor suppressor in multiple cancers[73-77]. Hsa_circ_0001649 levels in GC tissues were significantly lower than those in paired nontumorous tissues. The AUC was 0.834 with a sensitivity of 71.1% and specificity of 81.6%. Compared with plasma collected preoperatively, has_circ_0001649 level was significantly upregulated in plasma samples collected

postoperatively[63]. This suggests that has_circ_0001649 could be used as an index of postoperative follow-up. Whether the nonelevation or redecline of has_circ_0001649 level is related to poor prognosis or recurrence of GC needs further exploration. Compared to paired adjacent nontumorous tissues, hsa_circ_0003159 expression was recently found to be significantly downregulated in GC tissues. Moreover, its levels were negatively related to gender, distant metastasis, and TNM stage. The cutoff value was 12.31 with an AUC of 0.75. The sensitivity and specificity were 85.2% and 56.5%, respectively[65].

Hsa_circ_0000467 levels were significantly higher in GC tissue compared with adjacent nontumorous tissue. Moreover, its level in tissue was positively related to TNM stage. Similar results of hsa_circ_0000467 expression was obtained in AGS, MGC-803, HGC-27 and NUGC-3 compared with GES-1 cell lines. Furthermore, hsa_circ_0000467 knockdown markedly inhibited the proliferation, invasion and migration, and promoted apoptosis of GC cells *in vitro*. The AUC of hsa_circ_0000467 in plasma was 0.790. Its levels in plasma of the same patient obviously declined after surgery[71]. However, the small number of samples was a limitation in this study. More samples are needed to increase the accuracy. At present, none of the AUCs that were obtained using a single circRNA as a diagnostic marker for GC was > 0.9. Therefore, some scholars have suggested that the combined application of > 2 circRNAs may help to improve the accuracy of early diagnostic markers for GC. Li *et al*[78] reported that hsa_circ_0061276 and hsa_circ_0001017 were both downregulated in GC plasma and tissues. Patients with low plasma hsa_circ_0061276 or hsa_circ_0001017 levels had worse overall survival than those with high levels. The AUC of hsa_circ_0061276 and hsa_circ_0001017 in plasma was 85.1% and 84.9%, respectively. When these two plasma biomarkers of GC were used together for analysis, the AUC increased to 0.912, with a sensitivity of 84.7% and specificity of 96.6%[78]. Another similar study also found that the AUC was increased to 0.91 with the combination of hsa_circ_002509 and hsa_circ_0000096[57]. These are the top two highest AUC in all current research.

FUTURE PROSPECTS

Studies of circRNAs in GC are just at the beginning compared with coding RNAs, miRNAs and long ncRNAs. Although more functional circRNAs have been discovered and characterized in GC, most of the studies have focused on their relationship with pathological characteristics. For most of these circRNAs, their biogenesis, cellular location, and mechanism of regulation still need to be explored. In recent years, exosomes have been identified to play an important role in the progression of cancer[79]. One recent study showed that ciRS-133 was delivered into preadipocytes by exosomes derived from GC cells, promoting the transformation of preadipocytes into brown-like cells by suppressing miR-133 and activating *PRDM16*. Additionally, silence of ciRS-133 expression can reduce cachexia in tumor-implanted mice[80]. Therefore, exosome-delivered circRNAs are involved in white adipose tissue browning and play an important role in cancer-related cachexia. In the future, more in-depth studies about the roles of exosome-delivered circRNAs will help to prevent the occurrence of cachexia, improve the prognosis of GC, and prolong the survival time of patients. Some scholars have reported that circRNAs may participate in the process of epithelial–mesenchymal transition (EMT)[81,82], which plays a critical role in cancer metastasis[83]. Further studies on the regulation effect of circRNAs on EMT will be helpful to reveal the mechanism of circRNAs in cancer metastasis. Moreover, how to transfer circRNAs or si-circRNAs efficiently to the accurate lesion site without side effects needs to be resolved urgently for clinical applications. We hope that more basic research about circRNAs will be carried out with the advances in molecular biology and biological informatics technology to reveal the pathological and physiological functions of circRNAs, and to develop circRNA-based therapeutic strategies that can safely and successfully integrate into clinical practice.

CONCLUSION

Many circRNAs are dysregulated in GC tissues, plasma and cell lines. Moreover, their dysregulation is associated with clinicopathological features and prognosis of GC. By working as miRNA sponges or interacting with RBPs, these circRNAs regulate the expression of miRNAs and target proteins that are associated with cell proliferation, apoptosis, invasion and metastasis. Based on their characteristics of stability, expression specificity and participation in gene regulation, circRNAs are expected to be potential biomarkers for early diagnosis, prognostic predictors, and therapeutic

targets of GC.

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ORIGINAL ARTICLE

Basic Study

Plasma and wound fluid levels of eight proangiogenic proteins are elevated after colorectal resection

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Abstract

BACKGROUND

Colorectal resection is associated with 3-5 wk long elevations in the plasma levels of at least 11 proangiogenic proteins that may stimulate tumor angiogenesis post-surgery. The increases during the first week after surgery may be related to the acute inflammatory response; the cause(s) of the week 2-5 increases is unknown. The wounds are a possible source because of the important role that angiogenesis plays in the healing process. The main hypothesis of the study is that wound fluid levels of the proteins studied will be elevated well beyond plasma levels which, in turn, are elevated from preoperative baseline levels.

AIM

To determine plasma and wound fluid levels of 8 proangiogenic proteins after colorectal resection for cancer and benign pathology.

METHODS

Blood and wound fluid samples were taken simultaneously on postoperative (postop) day 1, 3, and later time points until wound drain removal in 35 colorectal cancer patients and 31 benign disease patients undergoing colorectal resection in whom closed wound drains had been placed in either the pelvis or the subcutaneous space of the abdominal incision. Postop plasma levels were compared to preop plasma and postop wound fluid levels (separate analyses for cancer and benign groups).

RESULTS

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Sixty-six colorectal disease patients were studied (35 cancer, 31 benign pathology). Most patients underwent minimally invasive surgery (open surgery in 11% of cancer and 6% of benign patients). The majority in the cancer group had rectal resections while in the benign group sigmoid or right colectomy predominated. Plasma levels of all 8 proteins were significantly elevated from baseline ($P < 0.05$) at all post-operative time points in the cancer group and at 90% of time points (29/32) in the benign group. Wound levels of all 8 proteins were 3-106 times higher ($P < 0.05$) than plasma levels at 87-90 percent of postop time points; of note, wound levels were more than 10 times higher at 47-50% of time points.

CONCLUSION
Plasma protein levels were elevated for 3 weeks after surgery; wound fluid levels were much greater than corresponding blood levels. Healing wounds may be the source of the plasma increases.

Key words: Effects of surgery; Colorectal resection; Colorectal cancer; Plasma protein levels; Wound protein levels; Angiogenesis

Core tip: Simultaneous postoperative (postop) measurement of plasma and wound fluid levels of 8 proangiogenic proteins for 3 wk after colorectal resection was carried out in 66 patients. Wound fluid protein levels were 3-106 times greater than postop plasma levels which, in turn, were significantly greater than preoperative plasma levels. Colorectal resection is associated with persistent systemic blood protein changes that might stimulate tumor angiogenesis and, thus, tumor growth in residual tumor deposits during the first month after surgery. It is hypothesized that the healing wounds are a major source of the added protein in the blood stream.

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INTRODUCTION

In a small percentage of cancer patients surgical excision of the primary tumor is associated with the rapid development of tumor recurrence(s) or the growth of existing metastases[1-7]. There is a sizable experimental literature regarding surgery's impact on tumors in the early postoperative (postop) period and numerous hypotheses proposed to account for the phenomenon of accelerated tumor growth in this time period[8,9]. Surgery-related immunosuppression and the elimination (*via* resection) of a metastasis suppressing protein generated by the primary tumor are two examples[10,11]. Recently, another mechanism has been proposed, namely the stimulation of angiogenesis in residual tumor deposits by persistent blood protein alterations[12].

Over the last decade it has been shown that minimally invasive colorectal resection (MICR) in colorectal cancer (CRC) patients is associated with persistent proangiogenic plasma protein changes that persist for 3 to 5 wk after surgery[12,13]. Prior investigators had noted only short lived plasma protein alterations that were attributed to the acute inflammatory and endocrine responses that follow major surgical trauma; these changes lasted hours or, at most, 3 days after MICR or major surgical trauma[14,15]. As regards the newly discovered long duration changes, thus far, a total of 11 proteins have been shown to be elevated for much of or all of the first postop month[12,13,16-21]. Interestingly, all of these proteins play a role in angiogenesis. It has also been shown that plasma from the second and third weeks after MICR stimulates endothelial cell (EC) proliferation, migration, and invasion in *in vitro* cultures; these results lend support to the hypothesis that the proangiogenic blood protein changes after surgery may promote tumor growth by stimulating tumor angiogenesis[22].

Of note, postop plasma from CRC patients who underwent open resection has been shown to have similar proangiogenic effects on *in vitro* EC cultures, thus both open and minimally invasive methods (MIS) are similar in this regard[21]. Finally, similar blood compositional changes and *in vitro* EC culture results have been noted in patients undergoing MICR for benign conditions such as diverticulitis or adenoma, thus, the indication for surgery does not appear to influence or be the source of these surgery related alterations[13].

The etiology of these persistent plasma protein changes is unknown. Because angiogenesis is central to wound healing and because during the first month after surgery the body is tasked with the job of healing both the intra-abdominal and the abdominal wall wounds, the authors hypothesized that the added protein in the bloodstream may originate in the healing wounds and then find its way into the circulation. Of note, previous investigators have noted elevated vascular endothelial growth factor (VEGF) levels in wound fluid (WFL) taken from mastectomy and other surgical patients[23-25]. The purpose of this study was to assess plasma and wound levels of 8 proteins that have proangiogenic effects in patients undergoing colorectal resection. The chosen proteins, all previously shown to have persistently elevated plasma levels after colorectal resection, are: VEGF, placental growth factor (PLGF), angiopoetin-2 (ANG-2), monocyte chemotactic protein-1 (MCP-1), chitinase 3 like protein-1 (CHI3L1), osteopontin (OPN), matrix metalloproteinase-2 (MMP2) and MMP3. Brief background information regarding the proangiogenic effects of these proteins follows.

VEGF, critical to angiogenesis, stimulates multiple early steps in neovascularization including EC proliferation, microtubule formation, invasion and migration. ANG-2 enhances VEGF's effects by destabilizing the connections between the endothelium and perivascular cells. ANG-2 does this by competitively binding to the Tie-2 receptor with a greater affinity than Ang-1 which, when bound to Tie-2 has anti-angiogenic effects[26,27]. PLGF primarily regulates the angiogenic switch under pathologic conditions[28], however, as regards non-pathologic neovascularization, by increasing the amount of VEGF available to bind to the key receptor VEGFR2 it maximizes VEGF's proangiogenic effects early in the process of vessel formation. MCP-1 is believed to mediate angiogenesis by recruiting proangiogenic protein producing macrophages and monocytes into wounds and tumors; MCP-1 also promotes EC migration, a critical early step in angiogenesis, by binding to C-C chemokine receptor 2 on the surface of EC's[29,30]. Human CHI3L1, also known as YKL-40, induces IL-8 and MCP-1 secretion through the extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase signal pathways[31]; these chemokines support macrophage recruitment and tumor angiogenesis. OPN is an integrin binding phosphorylated acidic glycoprotein that mediates cell-matrix and cell-cell communication[32,33]. OPN has been shown to enhance tumor progression and angiogenesis *via* the PI3K/AKT and ERK mediated pathways in association with VEGF[34,35]. MMP-2 is an extracellular matrix remodeling enzyme[36,37] that degrades type IV collagen[38] in the basement membrane which enables EC migration and tumor cell invasion[39,40]; it has also been shown to enhance VEGF release[41]. MMP-3 has been shown to support the process of epithelial-mesenchymal transition (EMT) during which epithelial cells loses adhesion, become invasive, and transition to the mesenchyme which is critical in wound healing, angiogenesis, and the initiation of cancer metastasis [42].

As stated above, the overriding goal of this study was to establish that the wounds are the likely source of the added protein and that plasma levels are persistently increased after surgery. Toward this end, simultaneous postop plasma and wound specimens were collected at multiple time points. Populations of cancer and benign pathology colorectal resection patients were assessed so as to determine if the surgical indication influenced the body's response. If the results support the hypothesis then it will have been can demonstrate that wound healing has potentially important, heretofore unknown, systemic manifestations. This information would provide insight into wound healing and may compel doctors to look for anti-cancer agents that could be given during the early postop period in an effort to negate these potentially tumor stimulatory conditions.

MATERIALS AND METHODS

Methods

This was an Institutional Review Board (IRB) approved prospective study. All colorectal patients undergoing elective MICR, regardless of the indication, who had consented preoperatively to participate in the Mount Sinai West Colorectal service's IRB approved general tissue and data banking protocol, in whom a Jackson Pratt (JP)

drain was placed in the abdominal cavity, pelvis, or subcutaneous space of the main extraction incision were eligible for the study (Institutional Review Board of the Mount Sinai School of Medicine, New York; IRB reference NO: GCO1: 16-2619 and Institutional Review Board of the Mount Sinai School of Medicine, New York; IRB reference NO: GCO1: 16-1863). Independent of this investigation, the authors have been investigating the use of subcutaneous wound drains to lower the incidence of superficial Surgical Site Infections (SSI's). Patients with JP drains were asked to consider participating in the study on postop day (POD) 1 by a Study Registered Nurse and interested patients were consented after they had been given a full explanation of the study and all questions had been answered. Prior consent to the tissue banking program was necessary such that a preoperative (preop) blood sample would be available to determine the baseline, pre-resection plasma protein levels. The tissue banking protocol allows the research dedicated blood samples to be used in IRB approved studies.

Plasma and WFL samples from 35 patents diagnosed with colorectal adenocarcinoma (rectal 21; colon 14; 21 male /14 female, mean age 63.6 ± 11.3 years) were collected and included into the study. The CRC stage distribution was: Stage 1, 10 (29%); Stage 2, 11 (31%); Stage 3, 12 (34%), and Stage 4, 0 (0%). The ethnic/race breakdown of the patients was as follows: Caucasian (40%), Hispanic (29%), African American (28%) and Asian (3%). In addition, a total of 31 patients with benign pathology who met the entry criteria (11 male/20 female, mean age, 57.3 ± 14.1 years) consented to participate in this study. The indications for surgery in the benign disease group were diverticulitis, 18 patients, 58%; benign neoplasm, 10, 32%; ulcerative colitis, 2, 7%; constipation, 1 (3.2%). The ethnicity/race breakdown was as follows: Caucasian (78%), Hispanic (12%), African American (7%) and Asian (3%) patients.

Sample collection

Blood samples and "WFL" samples from the JP suction device were simultaneously taken from patients on POD 1 and 3 as well as at the time of post discharge office appointments (provided the JP drain remained). Patients with high drain output were sent home with the JP drain(s) in place; in this subgroup later postop samples were obtained at the time of office visits. The initial office follow up appointment was usually between POD 7-13; however, some patients were seen between POD 14 and 21 as well. After hospital discharge it was not possible to collect the blood and WFL specimens on set postop days (for example, POD 7 or 14). Because late samples were obtained on different postop days the samples for each 7 day period were "bundled" together and considered as a single time point (POD 7-13, 14-20, *etc.*,). Blood samples, collected in heparin coated vacutainers, were collected at the same time the WFL samples were obtained and then promptly processed *via* centrifugation at 450 g after which the plasma fraction was stored in labeled 500 μL cryo storage vials at - 80 ^{o}C until the time of analysis. WFL samples, initially placed in sterile plastic containers, were processed promptly *via* centrifugation at 16000 g for 10 min at 6 ^{o}C after which the supernatant was divided into 0.5 mL aliquots that were stored in cryo vials at - 80 ^{o}C until the analysis was performed. Basic demographic, co-morbidity, operative, pathologic, and clinical data were obtained and recorded.

Exclusion criteria

Patients undergoing emergent surgery were not eligible. Also, HIV positive patients and those on immunosuppressive medications were not eligible.

Wound fluid and plasma analyses

WFL and plasma VEGF, PLGF, ANG2, MCP-1, CHI3L1, OPN, MMP2 and MMP3 levels were determined in duplicate *via* highly specific and sensitive commercially available Enzyme-Linked ImmunoSorbent Assays (ELISA) kits (R and D Systems, Quantikine kit numbers DVE00, DPG00, DANG20, DCP00, DC3L10, DOST00, MMP200 DMP300). The ELISA's used in the study were tested for precision (intra-assay precision and Inter-assay-precision), recovery, sensitivity and linearity by the vendor. Before analysis WFL samples were diluted 10-20 times and plasma samples diluted as per the manufacturer's recommendations for each protein. The plasma and WFL samples from each patient were analyzed on the same ELISA plate in duplicate for each protein and standards were included in each ELISA assay. As regards the frozen specimens, freeze thaw cycles were avoided in the utilization of the samples by storing the plasma in 500 μL aliquots and by performing several protein ELISA's on the same day such that a given vial of plasma or WFL was fully utilized once thawed. The ELISAs were read using an automated microplate reader (Synergy2; Bio-Tek Instruments, Inc., Winooski, VT, United States). Standard curves were generated on four parameter logistic curve fit and protein concentrations are reported as pg/mL or

ng/mL.

Statistics

As mentioned, the wound and blood samples after the first week were bundled into 7 days time periods and considered as single time points. Because some JP drains were removed prior to hospital discharge or at the first postop office visit, the "n" for the late bundled time points is notably smaller than for the POD 1 and 3 time points. The cancer and benign indication patient subgroups were assessed separately. Also, the intraperitoneal and subcutaneous WFL samples were considered both separately and together. For the preop *vs* postop plasma protein level comparisons, the data is reported as median and 95% confidence intervals and the Wilcoxon signed-rank match paired test was used. In regards to the postop plasma *vs* WFL comparisons, the results are reported as the median and 95% confidence intervals and the Mann and Whitney test was used. Plasma and WFL protein levels in figures are expressed as median and 75% quartile range. A P value < 0.05 was considered statistically significant. All data analysis was performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, United States). As the sample size varies for the POD 7-13 and PODS 14-20 time points, a separate preop results bar is included for each time point in the figures.

RESULTS

A total of 35 patients with colorectal adenocarcinoma (rectal 21, colon 14) and 31 patients with benign colorectal conditions in whom a JP drain was used in either the intraperitoneal or subcutaneous location were enrolled in this study. Table 1 provides the demographic and operative data as well as the length of stay for the cancer and benign patient groups. As regards surgical methods, most patients underwent laparoscopic-assisted resections (cancer, 63%; benign, 68%) while the rest underwent either a hand-assisted procedure (cancer, 26%; benign, 26%) or an open resection (cancer, 11%; benign, 6%). There were 2 conversions in the cancer group that underwent MIS (6.5%) and 3 in the benign patient group (9.7%). There were no deaths. The type of resections performed in the cancer group were: Low anterior resection/anterior resection, 12 patients, 34%; abdominperineal resection, 9 patients, 26%; sigmoid/rectosigmoidectomy, 5, 14%; right colectomy, 4, 11%; transverse colectomy, 3, 9%; and total proctocolectomy with ileal pouch, 2, 6%. The final cancer stage breakdown was Stage 0, 2 rectal cancer patients, 6% (T-0, N-0, pathologic complete response after neoadjuvant RT/chemotherapy), Stage 1, 10, 29%; Stage 2, 11, 31%; Stage 3, 12, 34%, and Stage 4, 0 (Table 1).

The indications for surgery in the benign disease group were diverticulitis, 18 patients, 58%; benign neoplasm, 10, 32%; ulcerative colitis, 2, 7%; constipation, 1 (3.2%). The operations performed were: sigmoid/rectosigmoid resection, 15 (48%); right colectomy, 6 (19%); lower anterior resection, 4 (13%); total colectomy/proctocolectomy, 3 (10%); Hartmann takedown with resection, 2 (7%); and transverse colectomy, 1 (Table 1). As regards the cancer group, in 23 patients the JP drain was placed in the pelvis whereas in 12 it was placed in the subcutaneous space beneath the main incision; 3 patients had both types of drains. In the benign pathology group the JP drains were placed in the pelvis in 8 patients and in the subcutaneous position in 23; 3 patients had both pelvic and subcutaneous drains. The greater number of pelvic JP drains in the cancer group reflects the fact that over 50 percent of the cancer cases were rectal cancer resections.

Plasma protein levels

The median plasma level at each postop time point was compared to the median preop level for each of the proteins. The total number of preop *vs* postop protein comparisons for each group (cancer and benign) was 32 (8 proteins × 4 time points). As regards the cancer group, significant elevations from baseline were noted postop at all of the time points while for the benign group significant elevations were noted at 29 of the 32 time points (90%). The 3 non-significant elevations concerned the POD 14-20 time point where the "n" for the benign group was 4 which made statistical analysis difficult. The extent of the increases over baseline varied from protein to protein and from time point to time point (Figures 1-8 and supplementary Tables 1 and 2). The range of the percent change from baseline values for each of the proteins over the 4 postop time points for the cancer group was comparable with the same data for the benign group (Table 2).

Wound fluid results

Study patients had either a pelvic or a subcutaneous JP drain except for 3 in each

Table 1 Demographic and clinical characteristics of the plasma and wound fluid study population (benign and cancer groups), *n* (%)

	Benign (*n* = 31)	Cancer (*n* = 35)
Age, yr (mean ± SD)	57.3± 14.1	63.6± 11.3
Sex (*n*):		
Male	11 (35.0)	21 (60)
Female	20 (65.0)	14 (40)
Incision length, cm (mean ± SD)	8.3 ± 5.3	9.8 ± 6.0
Operative time, min (mean ± SD)	339.1 ± 116.1	430.1 ± 121.0
Length of stay, d (mean ± SD)	5.6 ± 2.3	7.7 ± 6.6
Type of resection:		
Right	6 (19.0)	4 (11.0)
Transverse	1 (3.0)	3 (9.0)
Sigmoid/rectosigmoid	15 (48.0)	5 (14.0)
LAR/AR	4 (13.0)	12 (34.0)
APR	0 (0.0)	9 (26.0)
Hartman takedown with resection	2 (7.0)	0(0.0)
Total colectomy/proctocolectomy	3 (10.0)	2 (6.0)
Surgical method:		
Laparoscopic-assisted	21 (68.0)	22 (63.0)
Hand-assisted/hybrid Laparoscopic	8 (26.0)	9 (26.0)
Open	2 (6)	4 (11)

LAR: Lower anterior resection; AR: Anterior reselection APR: Abdominoperineal resection.

group that had two drains. Because, many drains were removed prior to hospital discharge and post discharge samples were obtained only once in most patients between POD 7-21, the "n"s of the pelvic and subcutaneous fluid subgroups were low for the POD 7-13 (cancer pelvic, 15; cancer subcutaneous, 7; benign pelvic, 6; benign subcutaneous, 10) and the POD 14-21 time points (cancer pelvic, 7; cancer subcutaneous, 2; benign pelvic, 2; benign subcutaneous, 2). The results of the protein assays performed on the WFL samples were first considered as to their origin (the pelvis or subcutaneous space) and the results compared; for all 8 proteins, at the great majority of time points, there was no statistical difference in protein levels between the pelvic and subcutaneous WFL (Supplementary Table 3). Therefore, in order to simplify the analysis and to increase the WFL "n" for the later time points, the pelvic and subcutaneous subgroups were combined to form a single larger WFL group the levels of which were compared to the plasma protein concentrations at each time point; the results of that comparison follow.

There were a total of 32 postop data points (8 proteins × 4 postop time points) to consider for both the cancer and benign groups. The median WFL levels at all time points were significantly higher than the corresponding median plasma level for all 8 proteins for both groups. The highest WFL levels were noted at the POD 7-13 or 14-20 time points for 6 of the 8 proteins in both the cancer and benign pathology groups. The magnitude of the difference between wound and plasma levels varied considerably from protein to protein and between the postop time points. What follows is a list of fold changes (multiples of the mean plasma level at each time point) followed by the percent of postop time points (n = 32) whose mean WFL value was equal to or greater than the stated fold change for the cancer groups data: ≥ 2 × mean plasma level, 97%; ≥ 3 ×, 91%; ≥ 5 ×, 69%; ≥ 10 ×, 47%; ≥ 30 ×, 29% and ≥ 40 ×, 22%. The comparable results for the benign group are as follows; ≥ 2 ×, 90%; ≥ 3 ×, 87%; ≥ 5 ×, 68%; ≥ 10 ×, 52%; ≥ 30 ×, 29%; and ≥ 40 ×, 26% (Table 3).

When the results for the individual proteins are considered the proteins can be divided into 3 categories. The greatest WFL elevations (*vs* plasma levels) were noted for VEGF, PLGF, and MCP-1 (mean wound value ≥ 30 × plasma levels at 9/12 cancer and 8/12 benign time points). The lowest elevations were noted for MMP-2, MMP-3, Ang-2, and CHI3L1 (cancer and benign groups, < 10 fold change *vs* plasma, 15/16 time points). The OPN results fall between the high and low groups (cancer and benign groups, 10-24 fold elevations at 75% of time points) (Table 3).

Pelvic vs subcutaneous wound fluid analysis

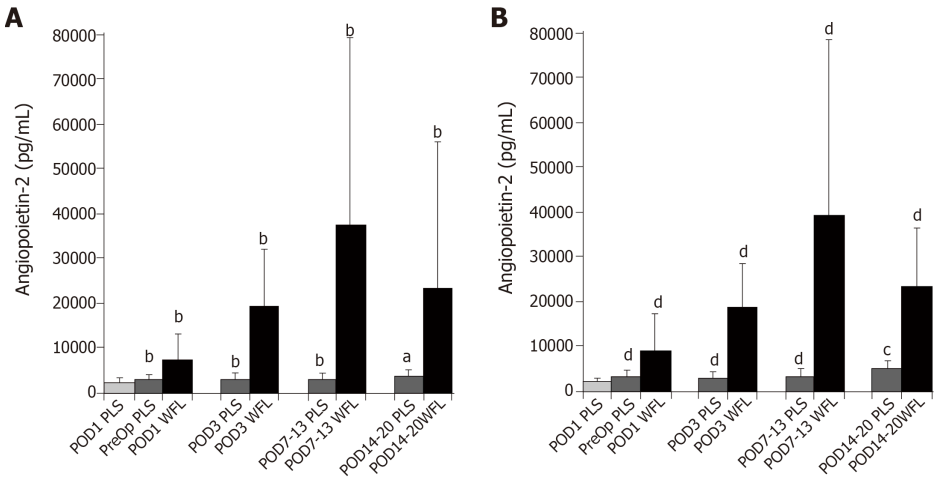

Figure 1 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative angiopoietin 2 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 26), $^{b}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 23), $^{b}P < 0.01$; Preoperative *vs* Postoperative day 7-13 (n = 16), $^{b}P < 0.01$; Preoperative *vs* Postoperative day 14-20 (n = 6), $^{a}P < 0.05$. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 26) *vs* Postoperative day 1 (wound fluid, n = 30), $^{b}P < 0.01$; Postoperative day 3 (plasma, n = 23) *vs* Postoperative day 3 (wound fluid, n = 28), $^{b}P < 0.01$; Postoperative day 7-13 (plasma, n = 16) *vs* Postoperative day 7-13 (wound fluid, n = 14), $^{b}P < 0.01$; Postoperative day 14-20 (plasma, n = 6) *vs* Postoperative day 14-20 (wound fluid, n = 4); $^{b}P < 0.01$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.01$); B: Colorectal surgery malignant patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 35), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 28), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 7-13 (n = 17), $^{d}P < 0.01$; postoperative *vs* Postoperative day 14-20 (n = 7), $^{c}P < 0.05$. Plasma *vs* wound fluid; Postoperative day 1 (plasma, n = 35) *vs* Postoperative day 1 (wound fluid, n = 33), $^{d}P < 0.01$; Postoperative day 3 (plasma, n = 28) *vs* Postoperative day 3 (wound fluid, n = 29), $^{d}P < 0.01$; Postoperative day 7-13 (plasma, n = 17) *vs* Postoperative day 7-13 (wound fluid, n = 20), $^{d}P < 0.01$; Postoperative day 14-20 (plasma, n = 7) *vs* Postoperative day 14-20 (wound fluid, n = 8), $^{d}P < 0.01$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as $^{c}P < 0.05$, $^{d}P < 0.01$. Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid.

The pelvic and subcutaneous WFL results at each time point were compared for both the cancer and benign pathology groups. Of note, as regards the POD 14-20 data, the n for subcutaneous WFL was only 2 for the cancer and benign groups and was also 2 for the benign pelvic subgroup; therefore, valid statistical analysis was not possible at that time point. As regards the 24 evaluable data points (8 proteins × 3 postop time points) no statistically significant differences were found between the subcutaneous and the pelvic WFL protein levels at 19 time points (79%) in the cancer group and at 20 time points (83%) in the benign group (Supplementary Table 3). In the cancer group subcutaneous protein levels were significantly greater than the pelvic results at 4 time points whereas in the benign disease group the pelvic fluid levels were greater than the subcutaneous results at 3 time points. Based on these results, for the comparison of the plasma and WFL protein levels at each time point the subcutaneous and pelvic WFL results were pooled.

Of note, for both the cancer and benign pathology groups, when the pelvic and subcutaneous WFL results were separately compared to the plasma protein results at each of the evaluable time points (POD 1, 3, 7-13), the WFL levels were significantly higher than the corresponding plasma levels at 92 % of time points (Supplementary Tables 4 and 5). Regardless of the source, mean WFL levels were greater than the corresponding mean plasma levels at all time points for all 8 proteins for both groups.

DISCUSSION

This study accomplishes 2 goals. First, it confirms that plasma levels of these 8 proangiogenic proteins are elevated from baseline for at least 3 wk after colorectal resection. Secondly, it demonstrates that the levels of these proteins in WFL samples taken simultaneously from the pelvic and abdominal wall wounds of CRC patients are significantly higher than the corresponding plasma levels at all time points. Notably, wound levels were 3 times higher than plasma levels at 87%-91% of time points and 10 times higher at 47%-52% of time points. Similar results were noted for both the cancer and benign pathology patient subgroups which suggests that it is the tissue trauma and/or subsequent healing rather than the indication for surgery that is the source of these elevations. Although the acute inflammatory response may contribute to the protein elevations during the first 4-7 d, in the authors' opinion, the most likely source of the added protein in the bloodstream for most of the postop

Table 2 Range of mean % of increase of plasma proteins during post-operative period from pre-operative mean value

	Benign	Malignant
ANG-2	49-101	38-70
VEGF	64-169	104-202
MMP-2	19-39	16-37
PLGF	36-55	29-53
MMP-3	60-162	43-143
OPN	85-187	108-146
MCP-1	28-344	44-63
CHI3L1	45-946	196-1006

ANG2: Angiopoetin-2; VEGF: Vascular endothelial growth factor; MMP-2: Matrix metalloproteinase-2; PLGF: Placental growth factor; MMP3: Matrix metalloproteinase-3; OPN: Osteopontin; MCP-1: Monocyte chemotactic protein-1; CHI3L1: Chitinase 3 like protein-1.

period are the healing surgical wounds. Since angiogenesis is a critical component of wound healing it is not surprising that the levels of these proteins would be increased in the wound where considerable neovascularization is occurring. Numerous previous investigators have documented the proangiogenic properties of WFL[43-47]. It is speculated that the proteins follow the concentration gradient from the wounds (3 to 106 × higher) to the bloodstream.

Also, the persistent and concomitant elevation of both wound and plasma protein levels for 2 wk or more suggests an association between the two sites. Further, blood levels have been shown to be increased for a month or longer[48-51], which is the time frame within which the lion's share of wound healing occurs. If the added proteins were generated elsewhere in the body after surgery and then were transported, *via* the bloodstream, to the wound, they would be doing so against the concentration gradient which seems highly unlikely. Also, since the primary tumor has been resected it cannot be the source of the protein increases noted post-surgery.

As mentioned, it has previously been shown that postop plasma stimulates proangiogenic EC behavior *in vitro*[12,13,22]. These proangiogenic plasma changes may stimulate tumor angiogenesis during the first month after surgery in patients with residual tumor deposits post resection of the primary lesion. Support for this hypothesis can be found in a clinical study that demonstrated that colorectal resection was associated with an increase in the size and intra-tumoral and peri-tumoral vascular density of pre-existing liver metastases 6-12 wk after resection of the primary colorectal tumor[52].

As mentioned in the introduction, each of the 8 proteins included in this study have been noted to have proangiogenic effects. It is important to also note that practically all of these proteins are overexpressed in a large variety of cancers and that, for some of the proteins, elevated serum or plasma levels have also been noted. Further, in many cases increased tumor expression or elevated blood levels have been associated with worse cancer outcomes. VEGF, the best studied and well known of the group, is absolutely critical to the process of neovascularization and is overexpressed by many cancers. PLGF may facilitate metastasis by increasing the motility and invasion of malignant cells; also, tumor overexpression of PLGF and VEGF together is associated with increased tumor angiogenesis and cancer growth[28]. MCP-1, in addition to promoting EC migration, has been shown to be overexpressed in multiple human cancers and is associated with tumor grade in ovarian cancer patients[53,54]. In regards to Chi3l1, in the murine setting, Chi3l1-overexpressing cancer cell lines exhibited 4.0-8.0 fold greater tumor growth and 1.8-2.0 fold greater vasculature density than controls[55]. Also, elevated blood levels of Chi3l1 have been noted in a large variety of cancer patients[56-61] and are associated with a poor prognosis in many[56,59,60]. OPN has been shown in some studies to enhance tumor progression and angiogenesis in association with VEGF[34,35]. Overexpression of OPN has been noted in breast, lung, liver and CRC patients and is associated with worse prognosis and early recurrence in patients with hepatocellular cancer[62]. MMP2 plays a unique role in tissue remodeling as regards angiogenesis and is associated with tumor progression and metastasis. Elevated MMP-2 activity has been linked to a poor prognosis in lung[63], breast[64], prostate[65] and CRC[66]. As mentioned, MMP-3 has been shown to play a role in the process of EMT which is an important component of wound healing and angiogenesis. MMP-3 has also been shown to play an important role in the growth

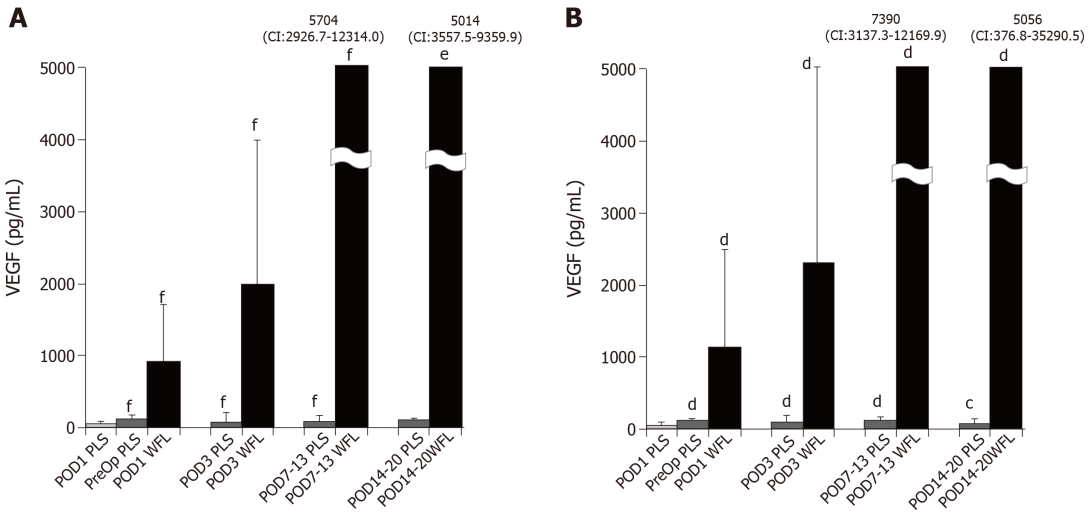

Figure 2 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative vascular endothelial growth factor levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative *vs* Postoperative plasma: Preoperative vs. Postoperative day 1 (n = 26), $^{f}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 23), $^{f}P < 0.01$ Preoperative *vs* Postoperative day 7-13 (n = 16), $^{f}P < 0.01$; Preoperative *vs* Postoperative day 14-20 (n = 6), ns. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 26) *vs* Postoperative day 1 (wound fluid, n = 30), $^{f}P < 0.01$; Postoperative day 3 (plasma, n = 23) *vs* Postoperative day 3 (wound fluid, n = 28) , $^{f}P < 0.01$; Postoperative day 7-13 (plasma, n = 16) *vs* Postoperative day 7-13 (wound fluid, n = 14), $^{f}P < 0.01$; Postoperative day 14-20 (plasma, n = 6) *vs* Postoperative day 14-20 (wound fluid, n = 4), $^{e}P < 0.05$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as $^{e}P < 0.05$, $^{f}P < 0.01$); B: Colorectal surgery malignant patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 35), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 28), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 7-13(n =17), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 14-20 (n = 7), $^{c}P < 0.05$. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 35) *vs* Postoperative day 1 (wound fluid, n = 33), $^{d}P < 0.01$; Postoperative day 3 (plasma, n = 28) *vs* Postoperative day 3 (wound fluid, n = 29), $^{d}P < 0.01$; Postoperative day 7-13 (plasma, n = 17) *vs* Postoperative day 7-13 (wound fluid, n = 20), $^{d}P < 0.01$; Postoperative day 14-20 (plasma, n = 7) *vs* Postoperative day 14-20 (wound fluid, n = 8), $^{d}P < 0.01$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. (Statistical significance is expressed as $^{c}P < 0.05$, $^{d}P < 0.01$ and $^{e}P < 0.05$). Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; VEGF: Vascular endothelial growth factor.

and/or metastatic transformation of cancers including breast cancer and hepatocellular carcinoma[67-74] and is overexpressed in some gastric and liver cancers.

Unfortunately, this study was quite small and, thus, it is not reasonable to draw any firm conclusions. It must be acknowledged, therefore, that there is no definitive evidence directly linking these plasma compositional changes to early recurrence or accelerated tumor growth after surgery. As mentioned, there is substantial clinical data supporting the concept that major surgery is associated with rapid cancer growth[3-5,75]. In 4 studies regarding patients with synchronous colon and liver lesions, rapid growth (within 2-3 mo) of the pre-existing liver metastases was noted after resection of the primary CRC as measured by serial computed tomography[4,5] and positron emission tomography scans[6,7]. Clearly, further studies are needed to determine the clinical ramifications of the persistent progangiogenic plasma compositional changes that have been noted.

If a clear link between surgery and accelerated tumor growth early postoperatively can be established then it would be logical to look for anti-cancer treatments that could safely be used during the first month after surgery which may be a particularly dangerous period for cancer patients with residual lesions. This is a time period that presently, with few exceptions, is not utilized for anti-cancer treatment; standard adjuvant chemotherapy is usually started 4 to 8 wk after surgery. Immunomodulating agents, tumor vaccines, anti-oxidants, and perhaps select monoclonal antibodies may be candidates for use in the early postop period. It is critical that any anti-cancer agent used in this time period not interfere with the healing process since that would likely lead to increased rates of anastomotic leaks and wound complications. As an example, anti-angiogenic therapy with agents such as bevacizumab is not a viable option because it would likely strongly interfere with wound healing.

This study assessed WFL from the pelvis and subcutaneous space within the main abdominal incision. Since prior evaluations of WFL's from different sources had not been performed, it was not known if the makeup of the 2 types of WFL would be similar. The results suggest that there are no significant differences in the levels of the 8 proteins in the 2 types of fluid at the great majority of time points, however, the study is underpowered in this regard and was not designed to answer this question. The dissimilar numbers of pelvic and subcutaneous samples in each group also makes comparison difficult. To definitively determine if the WFL source impacts WFL

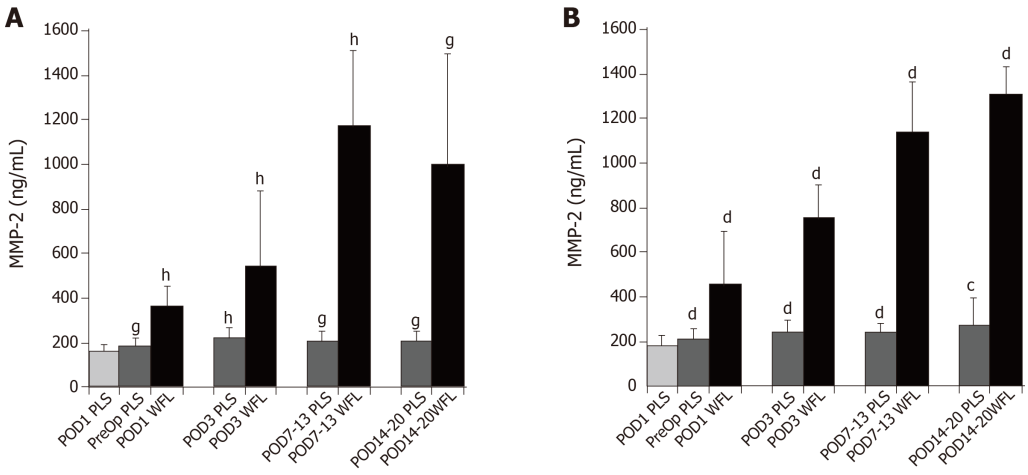

Figure 3 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative matrix metalloproteinase-2 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 26), $^{g}P < 0.05$; Preoperative *vs* Postoperative day 3 (n = 23), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 7-13 (n = 16), $^{g}P < 0.05$; Preoperative *vs* Postoperative day 14 -20 (n = 6), $^{g}P < 0.05$. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 26) *vs* Postoperative day 1 (wound fluid, n = 30), $^{h}P < 0.01$; Postoperative day 3 (plasma, n = 23) *vs* Postoperative day 3 (wound fluid, n = 28), $^{h}P < 0.01$; Postoperative day 7-13 (plasma, n = 16) *vs* Postoperative day 7-13 (wound fluid, n = 14), $^{h}P < 0.01$; Postoperative day 14-20 (plasma, n = 6) *vs* Postoperative day 14-20 (wound fluid, n = 4), $^{g}P < 0.05$; Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as $^{g}P < 0.05$, $^{h}P < 0.01$); B: Colorectal surgery malignant patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 35), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 28), $^{d}P<0.01$; Preoperative vs. Postoperative day 7-13 (n = 17), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 14-20 (n = 7), $^{c}P < 0.05$. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 35) *vs* Postoperative day 1 (wound fluid, n = 33), $^{d}P < 0.01$; Postoperative day 3 (plasma, n = 28) *vs* Postoperative day 3 (wound fluid, n = 29), $^{d}P < 0.01$; Postoperative day 7-13 (plasma, n = 17) *vs* Postoperative day 7-13 (wound fluid, n =20), $^{d}P < 0.01$; Postoperative day 14-20 (plasma, n = 7) *vs* Postoperative day 14-20 (wound fluid, n = 8), $^{d}P < 0.01$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as $^{c}P < 0.05$, $^{d}P < 0.01$. Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; MMP-2: Matrix metalloproteinase-2.

protein levels a substantially larger study would be needed. Despite these limitations it can be confidently stated that wound levels of these proteins are notably higher than corresponding plasma levels.

As mentioned, when the plasma protein results were considered alone, postop plasma protein levels were shown to be significantly elevated from their preop baseline levels for the 8 proteins at all time points in the cancer group and at 93% of time points in the benign pathology group. Of note, this is the first study to determine the late postop plasma levels of these 8 proteins simultaneously in a given population of patients. As mentioned, prior studies that looked at 1-3 proteins per study noted similar persistent late elevations in the plasma levels of the 8 proteins assessed in this study plus an additional 3 proangiogenic proteins (IL-8, progranulin, and keratinocyte growth factor) after MICR[12,16-21]. Of note, the current study did not assess plasma after the 3rd week after surgery since all wound drains had been removed by that point, however, prior studies have shown that the plasma elevations persist for 4-5 wk for some proteins.

Clear drawbacks to this study are the small "n"s for the post discharge time points (especially POD13-20) and the fact that the postop day on which the late sample(s) were obtained varied widely. As mentioned, once patients were discharged it was not possible to coordinate office visits so as to get study samples on a specific postop day. Therefore, late samples, by necessity, were "bundled". Also, because many of the drains had been removed in hospital, it was not possible to obtain late specimens from a good proportion of the patients. In addition, as mentioned, there were dissimilar numbers of pelvic and subcutaneous drains used in each group because the drains were not uniformly utilized (placed at the discretion of the surgeon) and because of the high proportion of rectal resections in the cancer group (more pelvic drains). A larger study would increase the "n"s but would likely still require bundling.

A comment must be made regarding the inclusion of open surgery patients in this study. Short lived increases in the extent and degree of the acute inflammatory response as judged by blood levels have been demonstrated in past studies for some cytokines in open (*vs* MIS) colorectal resection patients[76]. Further, no late postop plasma protein data was available for open CRC patients prior to this study. However, based on fact that the open colorectal resection *in vitro* EC culture results for the second and third postop weeks were similar to the MIS patients results, as mentioned earlier, the authors speculated that the proangiogenic cytokine response in

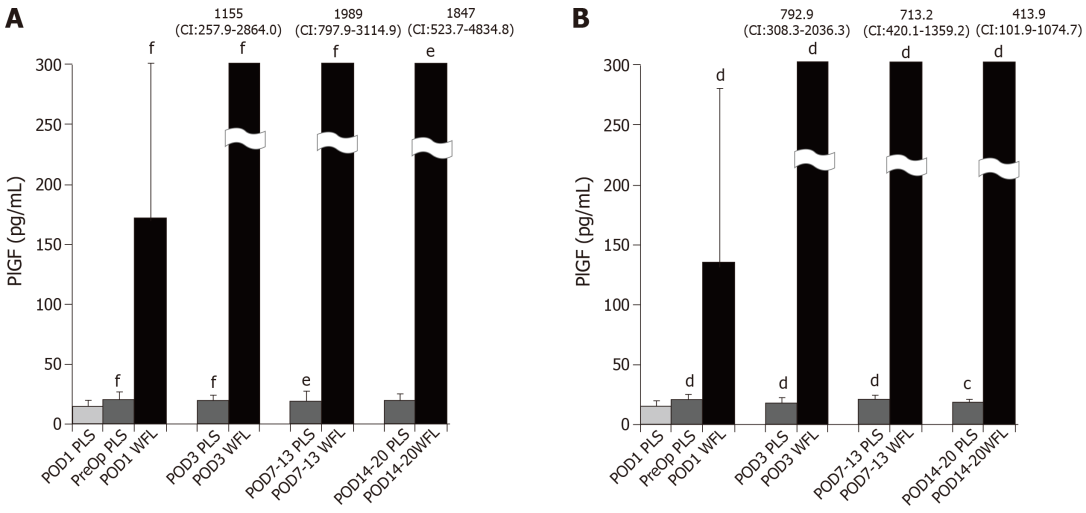

Figure 4 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative placental growth factor levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 26), ^{f}P < 0.01; Preoperative *vs* Postoperative day 3 (n = 23), ^{f}P < 0.01; Preoperative *vs* Postoperative day 7-13 (n = 16), ^{e}P < 0.05; Preoperative *vs* Postoperative day 14-20 (n = 6), ns. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 26) *vs* Postoperative day 1 (wound fluid, n = 30), ^{f}P <0.01; Postoperative day 3 (plasma, n = 23) *vs* Postoperative day 3 (wound fluid, n = 28), ^{f}P <0.01; Postoperative day 7-13 (plasma, n = 16) *vs* Postoperative day 7-13 (wound fluid, n = 14), ^{f}P < 0.01; POD14-20 (plasma, n = 6) *vs* POD14-20 (wound fluid, n = 4), ^{e}P < 0.05. Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as ^{e}P < 0.05, ^{f}P < 0.0.1); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 35), ^{d}P <0.01; Preoperative *vs* Postoperative day 3 (n = 28), ^{d}P < 0.01; Preoperative *vs* Postoperative day 7-13 (n = 17), ^{d}P < 0.01; Preoperative *vs* Postoperative day 14-20 (n = 7), ^{c}P < 0.05. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 35) *vs* Postoperative day 1 (wound fluid, n = 33), ^{d}P < 0.01; Postoperative day 3 (plasma, n = 28) *vs* Postoperative day 3 (wound fluid, n = 29), ^{d}P < 0.01; Postoperative day 7-13 (plasma, n = 17) *vs* Postoperative day 7-13 (wound fluid, n = 20), ^{d}P < 0.01; Postoperative day 14-20 (plasma, n = 7) *vs* Postoperative day 14-20 (wound fluid, n = 8), ^{d}P < 0.01. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as ^{c}P < 0.05, ^{d}P < 0.01). Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; PLGF: Placental growth factor.

the wounds and plasma would be similar. Of note, the small number of open patients in the present study (cancer, 4; benign, 2) precludes meaningful comparison between the open and MIS patients at most time points, however, clearly, wound levels are substantially increased for both methods. Also, when the wound and plasma results of the MIS patients (laparoscopic-assisted and hand-assisted laparoscopic) are assessed alone, significant differences persist for both the wound *vs* plasma and the preop *vs* postop plasma at all time points (data not shown).

The fact that these 8 proteins, shown to be increased late after surgery, all influence neovascularization suggests that there is considerable angiogenic activity in the wound late in the first postop month well after initial wound healing has occurred. Given these results, one would think that the other cytokines that play prominent roles in the wound healing process would also be persistently elevated after surgery. Interestingly, similar studies that measured postop plasma levels of FGF, TGF, HGF and EGF[77,78], however, have not demonstrated late elevations. However, further studies of other growth factors in this time window are warranted.

In summary, this study has demonstrated that plasma levels of the 8 proangiogenic proteins in question are significantly elevated over preop levels for 3 wk after colorectal resection and that protein levels in WFL samples taken at the same time points are many fold higher than the comparable plasma levels. Although not proven, the healing wounds appear to be a source of the added protein that raises plasma levels postoperatively, especially during weeks 2 and 3 after surgery. The indication for surgery (benign *vs* malignant) does not appear to impact these surgery-related changes (Supplementary Table 6). These proangiogenic plasma changes may stimulate tumor angiogenesis during the first month after surgery in patients with residual tumor deposits post resection of the primary lesion. Further study is needed to determine if the persistent proangiogenic plasma compositional changes are clinically relevant in cancer patients and, if so, then anti-cancer therapies that can be safely used in the perioperative time window need to be developed.

Table 3 The fall increased in wound fluid per corresponding mean plasma levels

Analyzed protein	The fall increased in wound fluid per corresponding mean plasma levels								Increased range	
	benign POD1	cancer POD1	benign POD3	cancer POD3	benign POD7-13	cancer POD7-13	benign POD14-20	cancer POD14-20	benign	cancer
ANG-2	3.3	3.0	5.5	6.0	12.8	12.0	8.0	3.5	3-13	3-12
VEGF	15.5	19.0	22.9	36.0	59.0	61.0	60.5	106.0	16-61	19-106
MMP-2	0.96	2.0	1.8	2.0	4.5	4.0	4.5	3.3	1-5	2-4
PLGF	40.8	16.0	88.2	61.0	100.3	46.0	106.1	27.2	41-106	16-61
MMP-3	2.6	5.2	4.6	5.0	6.5	3.2	8.8	5.4	3-9	3.2-5.3
OPN	4.9	3.1	14.0	12.0	24.2	14.0	15.5	9.3	5-23	3.1-14
MCP-1	39.7	40.0	74.7	37.0	85.2	59.0	16.0	57.1	16-86	37-59
CHI3L1	0.6	0.7	6.9	3.0	5.7	9.0	3.6	5.0	1-7	3-9

ANG2: Angiopoetin-2; VEGF: Vascular endothelial growth factor; MMP2: Matrix metalloproteinase-2; PLGF: Placental growth factor; MMP3: Matrix metalloproteinase-3; OPN: Osteopontin; MCP-1: Monocyte chemotactic protein-1; CHI3L1: Chitinase 3 like protein-1; POD: Post-operative day.

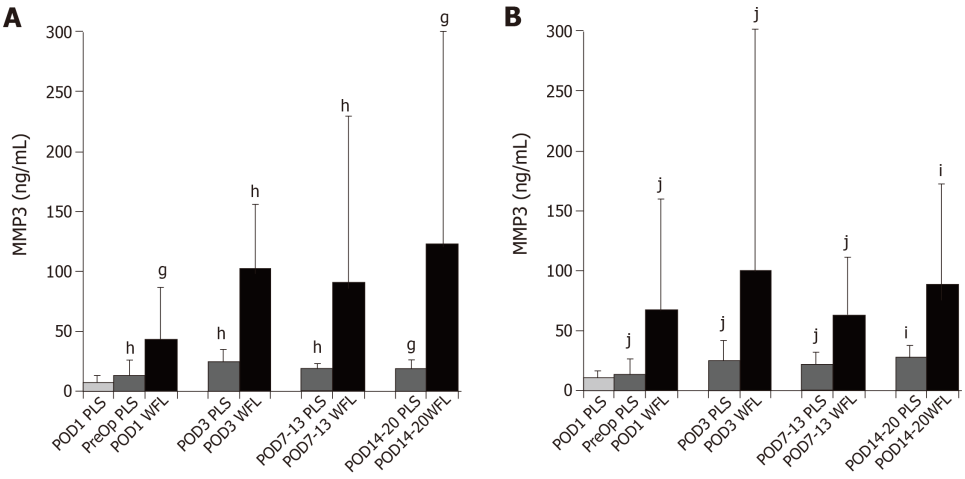

Figure 5 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative matrix metalloproteinase-3 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 26), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 23), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 7-13 (n = 16), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 14 -20 (n = 6), $^{g}P < 0.05$; Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 26) *vs* Postoperative day 1 (wound fluid, n = 30), $^{g}P < 0.05$; Postoperative day 3 (plasma, n = 23) *vs* Postoperative day 3 (wound fluid, n = 28), $^{h}P < 0.01$; Postoperative day 7-13 (plasma, n = 16) *vs* Postoperative day 7-13 (wound fluid, n = 14), $^{h}P < 0.01$; Postoperative day 14-20 (plasma, n = 6) *vs* Postoperative day 14-20 (wound fluid, n = 4), $^{g}P < 0.05$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. (Statistical significance is expressed as $^{g}P < 0.05$, $^{h}P < 0.01$); B: Colorectal surgery malignant patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 35), $^{i}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 28), $^{i}P < 0.01$; Preoperative *vs* Postoperative day 7-13 (n = 17), $^{i}P < 0.01$; Preoperative *vs* Postoperative day 14-20 (n = 7), $^{i}P < 0.05$. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 35) *vs* Postoperative day 1 (wound fluid, n = 33), $^{i}P < 0.01$; Postoperative day 3 (plasma, n = 28) *vs* Postoperative day 3 (wound fluid, n = 29), $^{i}P < 0.01$; POD7-13 (plasma, n = 17) *vs* POD7-13 (wound fluid, n = 20), $^{i}P < 0.01$; Postoperative day 14-20 (plasma, n = 7) *vs* Postoperative day 14-20 (wound fluid, n = 8), $^{i}P < 0.05$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as $^{i}P < 0.05$, $^{j}P < 0.01$. Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; MMP3: Matrix metalloproteinase-3.

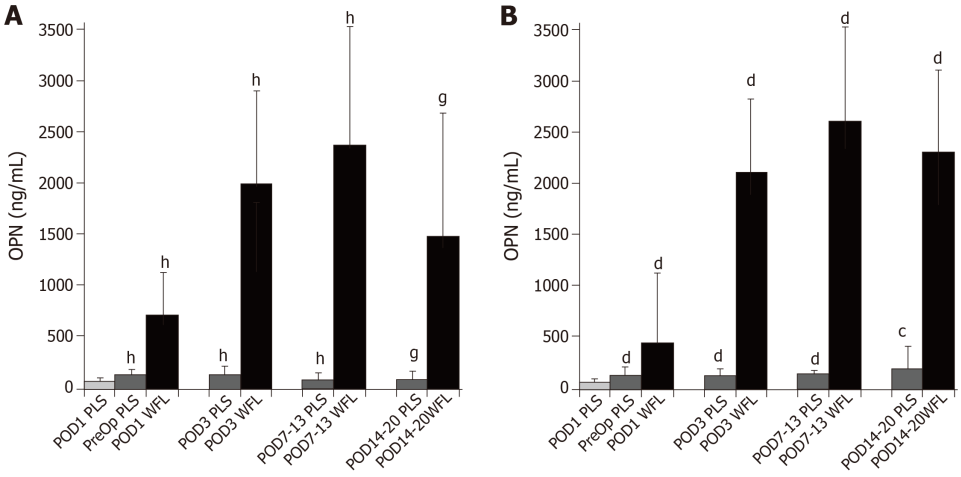

Figure 6 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative osteopontin levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 26), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 23), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 7-13 (n = 16), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 14-20 (n = 6), $^{g}P < 0.05$. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 26) *vs* Postoperative day 1 (wound fluid, n = 30), $^{h}P < 0.01$; Postoperative day 3 (plasma, n = 23) *vs* Postoperative day 3 (wound fluid, n = 28), $^{h}P < 0.01$; Postoperative day 7-13 (plasma, n = 16) *vs* Postoperative day 7-13 (wound fluid, n = 14), $^{h}P < 0.01$; Postoperative day 14-20 (plasma, n = 6) *vs* Postoperative day 14-20 (wound fluid, n = 4), $^{g}P < 0.05$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as $^{h}P < 0.05$, $^{g}P < 0.01$); B: Colorectal surgery malignant patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 35), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 28), $^{d}P < 0.01$; Preoperative *vs* Postoperative day 7-13 (n = 17), $^{d}P < 0.01$ Preoperative *vs* Postoperative day 14-20 (n = 7), $^{c}P < 0.05$. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 35) *vs* Postoperative day 1 (wound fluid, n = 33), $^{d}P < 0.01$; Postoperative day 3 (plasma, n = 28) *vs* Postoperative day 3 (wound fluid, n = 29), $^{d}P < 0.01$; Postoperative day 7-13 (plasma, n = 17) *vs* Postoperative day 7-13 (wound fluid, n = 20), $^{d}P < 0.01$; Postoperative day 14-20 (plasma, n = 7) *vs* Postoperative day 14-20 (wound fluid, n = 8), $^{d}P < 0.01$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as $^{c}P < 0.05$, $^{d}P < 0.01$. Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; OPN: Osteopontin.

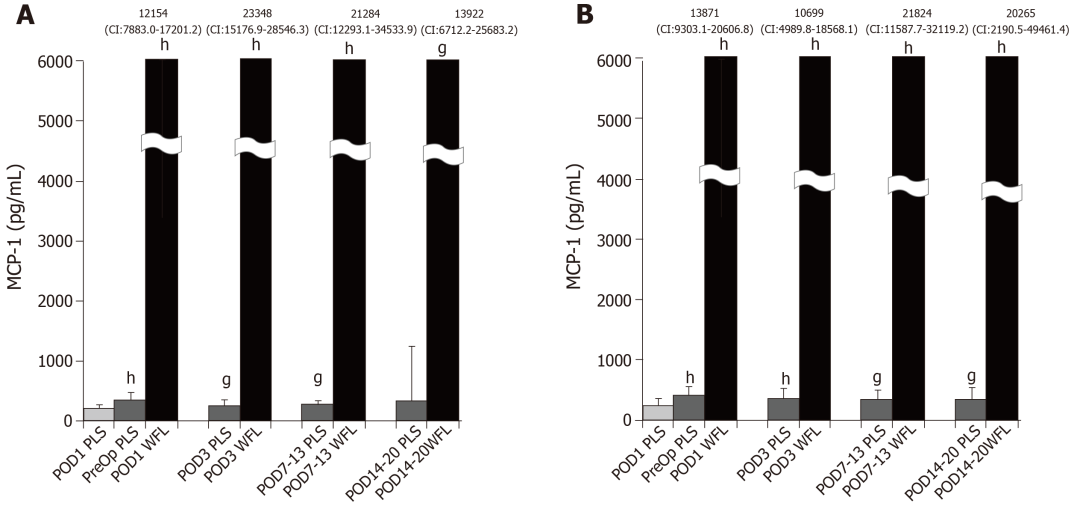

Figure 7 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative monocyte chemotactic protein-1 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 26), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 23), $^{g}P < 0.05$; Preoperative *vs* Postoperative day 7-13 (n = 16), $^{g}P < 0.05$; Preoperative *vs* Postoperative day 14-20 (n = 6), ns. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 26) *vs* Postoperative day 1 (wound fluid, n = 30), $^{h}P < 0.01$; Postoperative day 3 (plasma, n = 23) *vs* Postoperative day 3 (wound fluid, n = 28), $^{h}P < 0.01$; Postoperative day 7-13 (plasma, n = 16) *vs* Postoperative day 7-13 (wound fluid, n = 14), $^{h}P < 0.01$; Postoperative day 14-20 (plasma, n = 6) *vs* Postoperative day 14-20 (wound fluid, n = 4), $^{g}P < 0.05$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as $^{g}P < 0.05$, $^{h}P < 0.01$); B: Colorectal surgery malignant patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 35), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 3 (n = 28), $^{h}P < 0.01$; Preoperative *vs* Postoperative day 7-13 (n = 17), $^{g}P < 0.05$; Preoperative *vs* Postoperative day 14-20 (n = 7), $^{g}P < 0.05$. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 35) *vs* Postoperative day 1 (wound fluid, n = 33, $^{h}P < 0.01$; Postoperative day 3 (plasma, n = 28) *vs* Postoperative day 3 (wound fluid, n = 29), $^{h}P < 0.01$; Postoperative day 7-13 (plasma, n = 17) *vs* POD7-13 (wound fluid, n = 20), $^{h}P < 0.01$; Postoperative day 14-20 (plasma, n = 7) *vs* Postoperative day 14-20 (wound fluid, n = 8), $^{h}P < 0.01$. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as $^{g}P < 0.05$, $^{h}P < 0.01$. Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; MCP-1: Monocyte chemotactic protein-1.

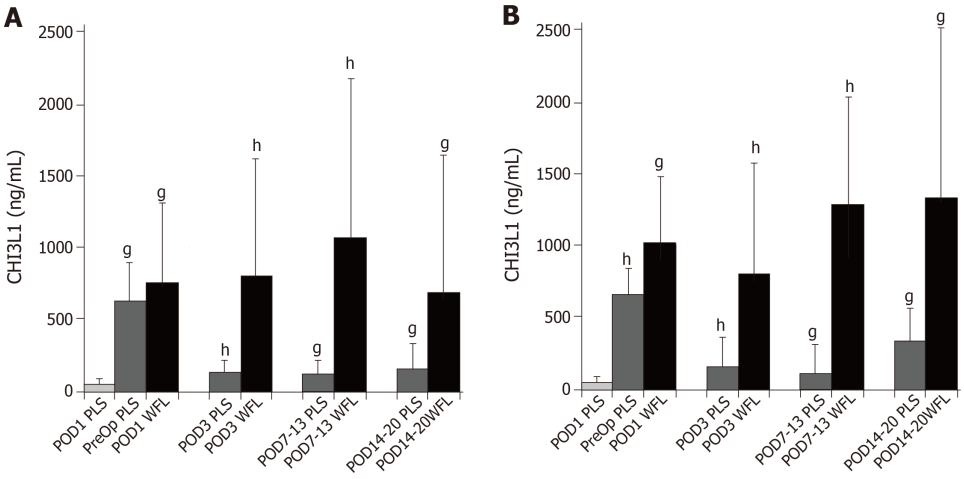

Figure 8 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative chitinase 3 like protein-1 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 26), ^{g}P< 0.05; Preoperative *vs* Postoperative day 3 (n = 23), ^{h}P < 0.01; Preoperative *vs* Postoperative day 7-13 (n = 16), ^{g}P < 0.05; Preoperative *vs* Postoperative day 14-20 (n = 6), ^{g}P < 0.05. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 26) *vs* Postoperative day 1 (wound fluid, n = 30), ^{g}P < 0.05; Postoperative day 3 (plasma, n = 23) *vs* Postoperative day 3 (wound fluid, n = 28), ^{h}P < 0.01; Postoperative day 7-13 (plasma, n = 16) *vs* Postoperative day 7-13 (wound fluid, n = 14), ^{h}P < 0.01; Postoperative day 14-20 (plasma, n = 6) *vs* POD14-20 (wound fluid, n = 4), ^{g}P < 0.05. Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as ^{g}P < 0.05, ^{h}P < 0.01); B: Colorectal surgery malignant patients. Preoperative *vs* Postoperative plasma: Preoperative *vs* Postoperative day 1 (n = 35), ^{h}P < 0.01; Preoperative *vs* Postoperative day 3 (n = 28), ^{h}P < 0.01; Preoperative *vs* Postoperative day 7-13 (n = 17), ^{g}P < 0.05; Preoperative *vs* Postoperative day 14-20 (n = 7), ^{g}P < 0.05. Plasma *vs* wound fluid: Postoperative day 1 (plasma, n = 35) *vs* Postoperative day 1 (wound fluid, n = 33), ^{g}P < 0.05; Postoperative day 3 (plasma, n = 28) *vs* Postoperative day 3 (wound fluid, n = 29), ^{h}P < 0.01; Postoperative day 7-13 (plasma, n = 17) *vs* Postoperative day 7-13 (wound fluid, n = 20), ^{h}P < 0.01; Postoperative day 14-20 (plasma, n = 7) *vs* Postoperative day 14-20 (wound fluid, n = 8), ^{g}P < 0.05. Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as ^{g}P < 0.05, ^{h}P < 0.0. Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; CHI3L1: Chitinase 3 like protein-1.

ARTICLE HIGHLIGHTS

Research background

Colorectal resection (CRR) has been previously shown to be associated with elevations in plasma levels of 11 proteins with proangiogenic effects [including vascular endothelial growth factor (VEGF)] that persist for 3 to 5 wk. Further, plasma from the second and third postop weeks has also been shown to promote endothelial cell proliferation, migration, and invasion which are critical to neovascularization. The noted persistent proangiogenic blood protein alterations might stimulate the growth of residual metastases that remain after resection of the primary tumor. The etiology of these elevations is unknown. The time course of the healing process and the fact that angiogenesis plays a critical role in wound healing makes the surgical wounds a possible source of the added protein in the blood. This study was done to simultaneously measure the levels of 8 proteins (VEGF, placental growth factor, angiopoetin-2, monocyte chemotactic protein-1, chitinase 3 like 1 protein, osteopontin, matrix metallo-proteinase-2 and matrix metallo-proteinase-3), all previously shown to be persistently elevated after CRR, in both the blood and fluid from the surgical wounds at multiple postop time points. The significance of this study is that it might demonstrate potentially important, heretofore unknown, systemic manifestations of wound healing.

Research motivation

The main topics of this study were: (1) Determination of the impact of CRR on blood levels of 8 proteins during the first 2 to 3 wks; and (2) To measure, at the same time points, the levels of the same 8 proteins in fluid from either pelvic or abdominal wall wounds. This is the first study to determine the perioperative levels of 8 proteins in the same population of patients and also the first to assess a population of benign pathology (cancer free) patients in addition to a group of cancer patients. If similar blood protein elevations were noted in the benign and cancer groups then it would be clear that the noted blood compositional changes were not related to the cancer diagnosis. A key motivation for this study was to determine the wound fluid (WFL) levels of 8 proangiogenic proteins as this would provide insight into wound healing, in general, and also might reveal a source of the plasma protein increases. Another motivation for this study was the desire to determine if the makeup of WFL from the pelvis in patients who had rectal resections would be similar to that obtained from the abdominal wall wounds. Therefore, this data should provide insight into wound healing in 2 different locations. Determining that wound levels of the proteins in question were notably higher than blood levels (which are also elevated from their baseline) at the same time points would establish that the healing wounds transiently but significantly alter the blood composition. This information would make clear the importance of minimizing the overall surgical trauma incurred in cancer patients. Also, this knowledge, by confirming the proangiogenic nature of the blood for 1 mo post-surgery, may compel doctors to

look for anti-cancer agents that could be given during the early postop period in an effort to negate these potentially tumor stimulatory conditions.

Research objectives

The main objectives of this study were the determination of plasma and WFL levels of the 8 proteins in question, simultaneously, at multiple postop time points. The hypothesis was that WFL levels of these proteins would be greater than blood levels because of the angiogenesis occurring in the healing wounds. Another objective was to confirm that after CRR the blood levels of the 8 proteins were persistently elevated for the first 3 wk. Yet other objectives were to determine if similar postop plasma increases were noted in cancer and benign colon disease patients and to ascertain if the protein concentrations in fluid from pelvic and abdominal wounds were similar or different. As mentioned, demonstrating that blood levels of these proangiogenic proteins remain elevated for 3 wk after surgery would confirm that surgery has long lasting systemic manifestations that have the potential to impact growth in residual cancer postop. If true, these results may motivate researchers to look for new anti-cancer agents that could be used early after surgery. Establishing that wound levels are higher than the corresponding blood levels would show that there is a concentration gradient between the healing wounds and the circulation; this would also suggest that the wounds may be a source of the additional protein in the blood. Determination of the similarity or difference between pelvic and abdominal wall WFL will provide insight into wound healing and will also guide future studies.

Research methods

This study concerned patients who underwent CRR for cancer or for benign colorectal pathology. This study was carried out under the auspices of two separate IRB protocols, one that called for obtaining multiple perioperative blood samples and clinical data for research purposes and the second that concerns harvesting of WFL samples from patients in whom Jackson Pratt drains were placed in either the pelvis or the main abdominal incision (consent obtained post-surgery). Preop blood samples were obtained before surgery from all patients. Blood and WFL samples were simultaneously obtained by research personnel on postop day (POD) 1, 3, and at least 1 late post-discharge time point provided the wound drain remained in place. The late samples, by necessity, were bundled into 2 "time points" (POD 7-13, POD 14-20). Post discharge late samples were obtained in only a fraction of the overall populations due to drain removal and the timing of the first office visit. WFL and blood samples were processed and aliquots of plasma and WFL frozen in a timely fashion. This is one of a small number of studies to collect fluid samples from both abdominal wall and pelvic wounds. WFL and plasma protein levels were determined in duplicate *via* highly specific commercially available Enzyme-Linked ImmunoSorbent Assays. This is the first study to assess perioperative blood levels of 8 proteins at multiple postop time points and the first, to our knowledge, to assess WFL levels for this number of proteins. Demographic, clinical, perioperative, and pathology data were obtained prospectively and entered into the IRB approved above mentioned data bank. The Wilcoxon signed-rank match paired test was used for the pre *vs* postop plasma comparison while the Mann-Whitney test was utilized for the plasma *vs* WFL comparisons.

Research results

A total of 35 cancer and 31 benign disease patients were studied. The vast majority underwent minimally invasive procedures; 11% of the cancer group and 6% of the benign disease group had open procedures. The majority of the cancer cases were rectal resections (60%) whereas the majority of the benign patients had either a sigmoid or right colectomy (67%). As regards the location of the Jackson Pratt drains, in the cancer group there were 23 pelvic and 12 subcutaneous abdominal wound drains whereas in the benign group there were 8 pelvic and 23 subcutaneous drains. As regards the preop *vs* postop plasma comparisons, there were a total of 32 points of comparison (8 proteins × 4 postop sampling points). The postop median plasma levels were significantly elevated from preop baseline at all 32 cancer time points and at 29 of 32 of the benign group time points. Of note, the range of the percent change from baseline values for the cancer and benign pathology groups were similar. This assessment of 8 proteins in the two populations verifies and substantiates the results of previous studies that each concerned 1 or, at most, 2 proteins. The results demonstrate that CRR is associated with plasma elevations that persist for at least 3 wk post-surgery. Further, these results prove that the elevations are related to the surgical procedure itself and not the indication for surgery (cancer *vs* benign pathology). These results also make clear the need to determine the oncologic consequences of the 3 to 5 wk long period when the blood is decidedly proangiogenic. New anti-cancer treatments that can be given during the first post month should be considered. Of note, when the pelvic and subcutaneous WFL results were compared, for all 8 proteins, at the great majority of time points, there was no statistical difference in protein levels between the 2 locations, thus, for the following analysis the WFL results from the 2 drain locations were combined. As regards the WFL *vs* plasma level comparisons for the 8 proteins, the median WFL levels were significantly greater than the corresponding plasma level at all 32 time points in both groups. The WFL median level was at least 3 × higher than plasma levels in 90%-91%, 5 × higher (or greater) in 68%-69%, and 30 × greater in 29% of patients in both groups. Of note, the highest WFL levels were noted at the POD 7-13 or 14-20 time points for 6 of the 8 proteins in both groups. These results prove that median wound levels of these proangiogenic proteins are notably greater than the corresponding plasma levels and that there is a substantial gradient between the wounds and circulation. Also, these results strongly support the hypothesis that the healing wounds are the source of the added protein in the blood. Similar studies that assessed different groups of proteins or different operations (gastrectomy, hepatectomy, pneumonectomy, *etc*.,) would

increase our understanding of surgery's systemic impact and perhaps lead to attempts to block, in some way, the deleterious systemic manifestations of major surgical trauma. Larger studies of this type would also allow a more detailed comparison between WFL from the pelvic and subcutaneous locations.

Research conclusions

There are 4 new findings of this study. The first is that WFL levels of the 8 proteins assessed are notably higher than the corresponding plasma levels which, in turn, are elevated from their preop baselines. These results support the hypothesis that the wounds are a major source of the added protein in the blood. These results also suggest that angiogenesis plays a prominent role in wound healing during the first month after surgery. The second new finding is the demonstration in this population of CRR patients that the plasma levels of all 8 proteins were significantly elevated for at least 3 wk after surgery (prior studies considered only 1-2 proteins per population). The third new finding is that plasma protein elevations similar to those found in cancer populations are found following surgery for benign pathology; thus the changes are related to the surgery and not the indication. The fourth new finding is that the make-up of WFL from 2 different locations are similar, as regards the levels of the proteins in question. This aspect needs further study and verification since the numbers of samples from each location limited the ability to detect differences at the later time points. The plasma results, by proving that long duration proangiogenic protein increases are present, raises the fear that these changes may promote tumor growth postoperatively in patients with residual disease. This realization should logically prompt studies to verify this hypothesis as well as to search for ways to limit these deleterious oncologic effects. The plasma and WFL results regarding 8 proteins in both cancer and benign pathology patients makes clear the fact that major surgery results in systemic blood compositional changes that last far longer than previously imagined; further, there is the potential that these changes may negatively impact cancer patients with residual disease. The new methods and study approaches put forth in this study are the simultaneous obtaining of blood and WFL samples at multiple time points during the first 3 wk after surgery and the assessment of 8 different proteins in a single population. As stated above, these results support the main hypothesis that the surgical wounds are the source of the added protein in the blood which significantly elevates plasma levels for weeks after surgery. The results also verify that long lasting plasma protein changes occur after surgery done for benign indications (as is the case for cancer populations).

Research perspectives

The results of this study add further evidence and support for the concept that CRR (and likely major surgery, in general) results in significant changes in the plasma levels of a substantial number of proteins that persist for at least 3 wk after surgery. Prior studies regarding the 8 proteins assessed in the present study have demonstrated that the full duration of the significant elevations is 3 to 5 wks. Documenting that all 8 proteins are persistently increased after surgery in patients with cancer or benign problems proves that these effects are related to the surgical procedure and not the presence of a cancer. The finding of much higher levels of these proteins in WFL than in the blood makes clear that wound healing is an involved and lengthy process in which that angiogenesis plays a central role. It also strongly suggests that the wounds are the source of the added protein. The fact that major surgery (tissue trauma) and the process of healing that follows alters the blood composition so that it is decidedly proangiogenic for a month's time has important implications. These systemic changes may accelerate the growth of residual tumor metastases by stimulating tumor angiogenesis. Future studies are needed to determine the clinical ramifications of the demonstrated plasma changes. Also, perioperative levels of other proteins that may influence tumor growth and establishment are indicated to better define surgery's effects. A better understanding of surgery's effects may lead to modifications in technique that will ameliorate the deleterious transient effects. Finally, the authors believe that anti-cancer drugs that can be used early after surgery should be sought so as to provide protection against accelerated tumor growth during the early postop period.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical efficacy of gemcitabine and cisplatin-based transcatheter arterial chemoembolization combined with radiotherapy in hilar cholangiocarcinoma

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Abstract

BACKGROUND

Radical surgical resection is regarded as the best treatment for hepatic hilar cholangiocarcinoma. However, 60%-70% of patients have lost the chance of surgery at the time of diagnosis. Simple biliary stent or drainage tube placement may fail in a short time due to tumor invasion or overgrowth, bile accumulation, or biofilm formation. Effective palliative treatments to extend the effective drainage time are of great significance for improving the quality of life of patients and changing the prognosis of patients.

AIM

To investigate the clinical efficacy of gemcitabine and cisplatin-based transcatheter arterial chemoembolization (TACE) combined with radiotherapy in hilar cholangiocarcinoma.

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METHODS

A retrospective analysis was conducted on patients clinically diagnosed with hilar cholangiocarcinoma from June 2014 to January 2017 at the Liaoning Provincial Cancer Hospital. Patients were evaluated by specialists, and those who were not suitable for surgery or unwilling to undergo surgery and met the inclusion criteria were included in the study. There were a total of 72 patients (34 males and 38 females) with an average age of 59.9 years (range, 40-72 years). According to percutaneous transhepatic biliary angiography and the patients' wishes, stent implantation or biliary drainage tube implantation was used to relieve biliary obstruction. The patients were divided into either a control group or a combined treatment group according to their follow-up treatment. The control group consisted of a total of 35 patients who received simple biliary drainage tube placement and biliary stent implantation (7 patients with bilateral stents and 6 with a unilateral stent) and 22 patients receiving biliary drainage tube placement alone. The combined treatment group received TACE and extracorporeal radiotherapy after biliary drainage or biliary stent implantation and consisted of a total of 37 patients, including 21 patients receiving combined treatment after biliary stent placement (14 patients with bilateral stents and 7 with a unilateral stent) and 16 undergoing combined therapy after implanting the biliary drainage tube. In the combination treatment group, the TACE chemotherapy regimen employed gemcitabine and cisplatin, and the embolic agent was iodized oil. A particular dose was determined according to the patient's body surface area and the tumor staining indicated by DSA. *In vitro* radiotherapy was performed with intensity-modulated radiotherapy or three-dimensional conformal radiotherapy at an average dose of 48.3 Gy. Both groups were followed from stent implantation or drainage tube implantation until the patient quitted or died. The median length of follow-up observation was 13 mo. The differences in overall survival time and the effect of different jaundice reducing methods (single stent, double stent, or biliary drainage) on the patency time and survival time of biliary stents were compared between the two groups; the related factors affecting overall survival time were analyzed.

RESULTS

The median survival time of the control group was 10.5 mo; the median survival time of patients with biliary stent implantation and those with percutaneous biliary drainage was 9.6 mo and 11.4 mo, respectively, and there was no statistically significant difference between them. The median survival time of the combined treatment group was 20.0 mo, which was significantly higher than that of the control group ($P < 0.05$). Among patients in the combined treatment group, the median survival time of patients who underwent biliary stent implantation and those who accepted percutaneous biliary drainage before the combination therapy was 19.5 mo and 20.1 mo, respectively, and there was no significant difference between them. In the combination treatment group, the mean time of median stent patency was 15.6 mo, which was significantly higher than that of the control group (7.0 mo; $P < 0.05$). The independent factors affecting survival time included age, whether to receive combination therapy, percutaneous biliary drainage tube implantation, and Bismuth-Corlette classification as type IV.

CONCLUSION

Gemcitabine and cisplatin-based TACE combined with radiotherapy can prolong the survival of patients with hilar cholangiocarcinoma. Independent predictors of survival include selection of combination therapy, Bismuth-Corlette classification as type IV, selection of percutaneous biliary drainage tube implantation, and age.

Key words: Hilar cholangiocarcinoma; Biliary stent; Percutaneous biliary drainage; Gemcitabine; Cisplatin; Radiotherapy; Transcatheter arterial chemoembolization

Core tip: In this study, hilar cholangiocarcinoma patients with obstructive jaundice were observed by different methods of reducing jaundice. The effectiveness of transcatheter arterial chemoembolization combined with radiotherapy was observed in extending the effective drainage time of the stent or drainage tube, improving the quality of life, and changing the prognosis of patients. The independent factors affecting survival were

analyzed. The results may be helpful to improve the systematic palliative treatment of hilar cholangiocarcinoma.

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INTRODUCTION

Hepatic hilar cholangiocarcinoma originates from the biliary mucosal epithelium and affects the left and right hepatic ducts at or near the junction of the biliary tract. Hilar cholangiocarcinoma is one of the most common malignant tumors of the biliary system, accounting for more than 70% of biliary tract tumors[1]. Radical surgical resection is the best treatment for long-term survival or cure in patients with hilar cholangiocarcinoma[2-5]. However, the incidence of hilar cholangiocarcinoma is a concealed, invasive growth, lacking specific symptoms in the early stage of the disease, and approximately 60%-70% of patients have lost the chance of surgery at the time of diagnosis.

The purpose of palliative biliary drainage[8-11] is to relieve bilirubinemia and cholangitis, provide conditions for surgery or other adjuvant treatments and has many advantages, such as reducing jaundice, being a relatively simple operation, and having a low cost. However, if the external drainage bile duct is carried for a long time, the quality of life may be seriously degraded due to inflammation of the puncture site, intercostal pain, and inconvenience in managing the tube[12-14]. Drainage may be reduced due to tube detachment or tumor bile duct growth. Percutaneous biliary stent implantation is considered a preferred solution for relieving high malignant biliary obstruction and achieving intrahepatic drainage. However, simple biliary stent placement may result in blockage due to tumor invasion or overgrowth, bile accumulation, or biofilm formation[15-18]. Extending the effective drainage time of the stent or drainage tube is of great significance for improving the quality of life of patients and the prognosis of patients.

A large number of studies have confirmed that gemcitabine combined with cisplatin chemotherapy[19,20], arterial chemoembolization[21-24], and radiation therapy[25,26] are safe and effective in the palliative treatment of cholangiocarcinoma. Therefore, this study retrospectively studied 72 patients with hilar cholangiocarcinoma with obstructive jaundice and explored the efficacy and prognosis of transcatheter arterial chemoembolization (TACE) combined with radiotherapy after percutaneous biliary drainage or stent implantation.

MATERIALS AND METHODS

Clinical data

A total of 72 patients with hilar cholangiocarcinoma complicated with obstructive jaundice were enrolled in this study from June 2014 to January 2017 at the Liaoning Provincial Cancer Hospital (Table 1), including 34 males and 38 females, with an average age of 59.9 years (range, 40-72 years old). All patients underwent contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MR) scanning and magnetic resonance cholangiopancreatography (MRCP). The inclusion criteria were: (1) Informed consent obtained from the patient; (2) Clinical or pathological diagnosis of hilar cholangiocarcinoma with obstructive jaundice and undergoing biliary stenting; (3) Imaging evaluations of tumors confined to the liver before initial treatment; and (4) Estimated survival periods > 3 mo. The exclusion criteria were: (1) Severe liver function disorder, kidney function disorder, or severe coagulopathy; (2) Extrahepatic metastases or multiple intrahepatic lesions; (3) General condition failure; and (4) History of hepatitis and cirrhosis.

The basic information of the two groups of patients (Table 1), including age, sex, Bismuth-Corlette classification, and jaundice reduction, were not statistically significant ($P > 0.05$).

Table 1 Basic information of stent implantation group and combined treatment group

Group	*n*	Age (yr)	Sex		Bismuth classification (cases)			Implantation method	
			M	F	II	III	IV	Unilateral	Bilateral
Control (35 cases)		61.6 ± 7.1	15	20	2	18	15	4	31
Stent	13		5	8	2	7	4	4	9
Drainage tube	22		10	12	0	11	11	0	22
Combined (37 cases)		58.2 ± 7.7	19	18	4	18	15	7	30
Stent	21		14	7	4	12	5	7	14
Drainage tube	16		5	11	0	6	10	0	16
P-value		0.91	0.49		0.74			0.88	

Percutaneous biliary puncture drainage tube and biliary stent implantation

Percutaneous biliary puncture drainage tube placement and stent implantation were performed under X-ray and ultrasound guidance. The drainage tubes (Cook, USA) with a diameter of 7F or 8.5F were used. The stent was a self-expanding bare metal stent (Wall stent, Boston Scientific Corp., USA), model 8 mm (diameter) x 60 mm (length) and 8 mm (diameter) x 40 mm (length). First, ultrasound-guided percutaneous transhepatic cholangiography was performed. The puncture device was a 22G micropuncture needle kit (NPAS-100-RH-NT, Cook). According to the angiographic results and preoperative imaging analysis, bile duct involvement was determined, and whether to use bilateral or unilateral drainage, as well as the diameter, length, and implanting methods of the biliary stent were also determined, as shown in Figures 1 and 2.

Patients needed to have tubes changed every 3-6 mo. In patients where the tube could not be recanalized or detached, a drainage tube was percutaneously implanted.

TACE

The chemotherapy drugs for TACE were gemcitabine and cisplatin, and the doses used were 1/2 of the systemic doses, *i.e.*, gemcitabine 500 mg/m^2 and cisplatin 35 mg/m^2, and the embolization agent was made with iodized oil. After treatment of the definite blood supply artery, selective embolization was performed (Figure 1). If the blood supply to the artery was not clear, arterial infusion was used. Rehydration and hydration were performed before and after treatment.

Intensity-modulated radiotherapy/three-dimensional conformal radiotherapy

The radiation therapy plan was developed on the ADAC Pinnacle^3 Treatment Planning System workstation (Philips, Best, The Netherlands). The gross tumor volume (GTV) included tumor and regional metastatic lymph nodes in the imaging findings. GTV was expanded outward by 0.5 cm as the clinical tumor volume (CTV), and CTV was expanded outward by 1 cm (for the cephalo-caudal direction) and 0.5 cm (for other directions), together with the tumor movement range, to form the planning tumor volume (PTV). The treatments were delivered with a VARIAN 6-MV X-ray linear accelerator (Varian Medical Systems, California, USA) using the following parameters: 3 to 7 coplanar or noncoplanar illumination fields, 90% isodose curve wrapped around PTV, 2.0 Gy each time, 5 times/wk, and total 50 Gy/25F.

Follow-up

The follow-up period began after stent or drainage tube implantation until the patient quitted study or died. Laboratory tests (routine blood and urine tests, indexes of hepatic and renal function, and tumor markers) and abdominal ultrasound were reviewed once a month; liver contrast-enhanced CT or MRI scans were reviewed every 3 mo. According to the imaging results and blood bilirubin levels, it was determined whether there was clogging in the biliary stent. For cases of obstructive jaundice, infection and other complications caused by biliary stent occlusion, biliary puncture drainage, anti-infection, and supportive treatments were given.

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 statistical software. Data that were normally distributed are expressed as the mean ± SD, while those with a non-normal distribution are expressed as the median, and independent sample *t*-test

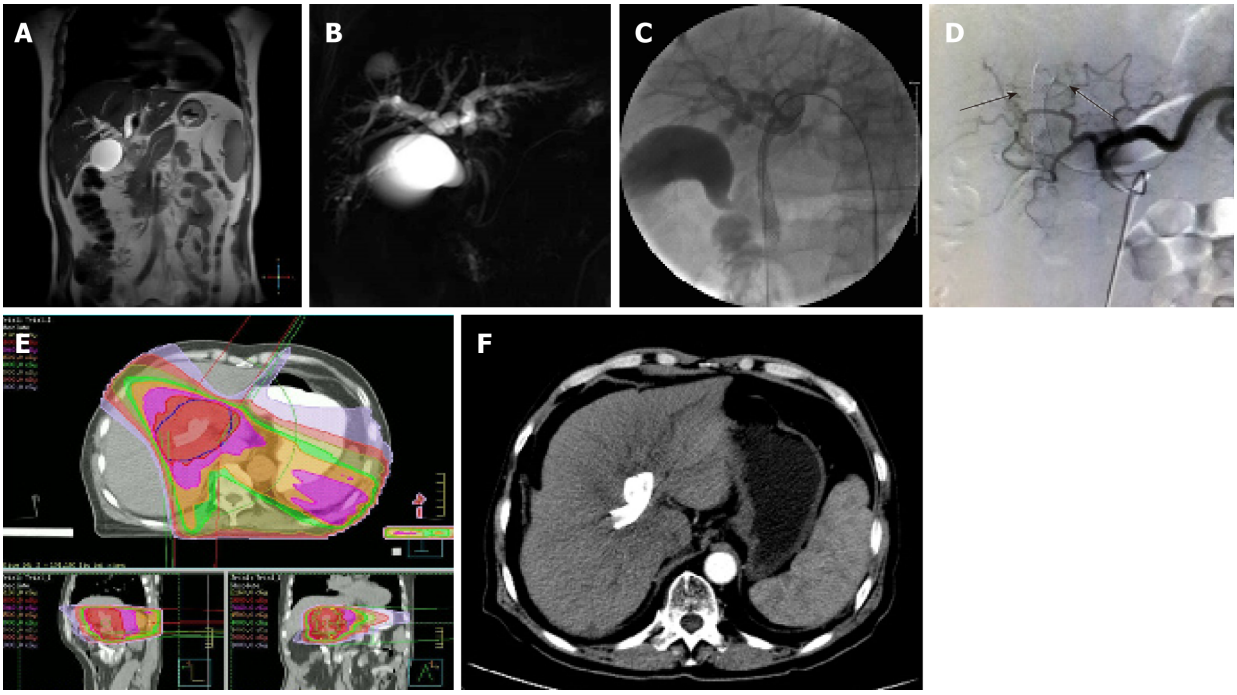

Figure 1 A 65-year-old man diagnosed with hilar cholangiocarcinoma. A and B: Magnetic resonance imaging (A) and magnetic resonance cholangiopancreatography (B) showed left hepatic duct and right hepatic duct branch involvement (Bismuth-Corlette type IIIa); C: Patients underwent percutaneous double stent placement; D: During transcatheter arterial chemoembolization, the branches of the hepatic artery were responsible for the blood supply of the lesion area in the hepatic angiography (arrows); E: Intensity-modulated radiotherapy plan is shown; F: Liver cirrhosis was observed at 11 mo after receiving treatment.

was performed. The Kaplan-Meier method was used to compare the stent patency time and survival time. Cox proportional hazard regression analysis was used to analyze the risk factors affecting the prognosis of patients. $P < 0.05$ was considered statistically significant.

RESULTS

Observations after treatment and follow-up results

The total bilirubin values of the control group and the combined treatment group were both less than 80 μmol/L 5-6 wk after receiving biliary drainage or stent implantation. Postoperative complications included biliary hemorrhage and cholangitis. No operation-related deaths were observed within 30 d of the operation.

Twelve patients received intensity-modulated radiotherapy, and 25 patients received three-dimensional conformal radiotherapy. There were 10 (27.0%) patients with grade II-III adverse reactions 2 wk after radiotherapy, and 3 (8.1%) of these patients could not complete the treatment course due to upper abdominal pain, nausea, and vomiting. A total of 102 TACE treatments were given in 37 cases. Twenty-one (29.2%) patients developed grade II adverse events of neutropenia after the first or subsequent TACE treatments.

The main causes of death in the control group and the combination treatment group included multiple organ failure caused by extensive metastasis in 12 patients, biliary infection and upper gastrointestinal bleeding after biliary recanalization in 21, liver failure after biliary recanalization in 35, unexplained death in 2, and loss to follow-up in 2.

Comparison of stent patency time between the two groups

The median patency time of the biliary stent was 15.6 mo in the combined treatment group and 7.0 mo in the control group, and there was a significant difference between them ($P < 0.05$) (Figure 3).

Comparison of survival between the two groups

The median survival time of the control group was 10.5 mo; the median survival time of patients with biliary stent implantation and those with percutaneous biliary drainage was 9.6 mo and 11.4 mo, respectively, and there was no statistically significant difference between them. The median survival time of the combined

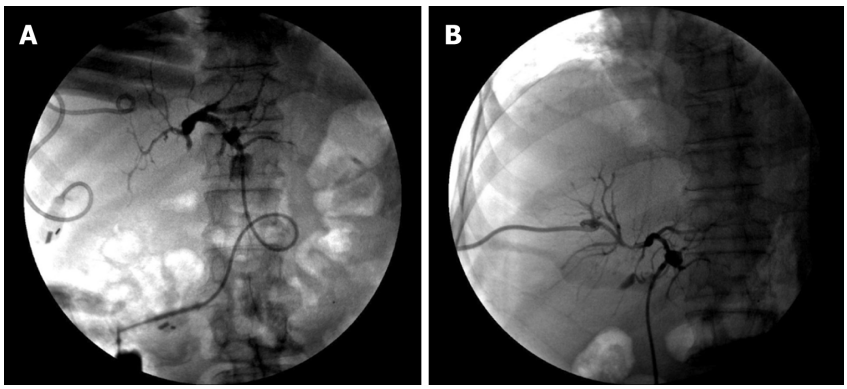

Figure 2 A patient with hilar cholangiocarcinoma who underwent biliary drainage. A: Before transcatheter arterial chemoembolization combined with radiotherapy, the obstruction of the junction between the left and right hepatic ducts was shown during cholangiography; B: The obstruction was reduced and local stenosis was observed after treatment.

treatment group was 20.0 mo and was significantly higher than that of the control group (10.5 mo; $P < 0.05$). In the combined treatment group, the median survival time of the patients who underwent biliary stent implantation those who underwent percutaneous biliary drainage before combined therapy was 19.5 mo and 20.1 mo, respectively, and there was no significant difference between them (Figure 4).

Analysis of independent predictors of survival

Multivariate analysis was performed on the sex, age, Bismuth-Corlette classification, jaundice reduction, combination therapy or not, and baseline value of CA19-9 (reviewed 3 wk after jaundice) in the study cases, and the results are shown in Table 2.

DISCUSSION

Obstructive jaundice is a common clinical symptom of hilar cholangiocarcinoma. Persistent biliary obstruction can lead to liver failure, secondary renal failure[27,28], severe hypoproteinemia, electrolyte imbalance, septic shock, *etc*., affecting the quality of life and survival of patients[16,29,30]. Clinically, percutaneous drainage tube or biliary stent implantation in various ways is an effective treatment for obstructive jaundice[17,31-39].

Most of hilar cholangiocarcinoma cases progress along the bile duct and invade the surrounding tissue, but distant metastasis rate is relatively low[40]. After the metal stent is placed, tumor invasion or overgrowth, bile accumulation, or biofilm formation may cause clogging in the stent. In this study, the median patency time of a simple metal stent was 7 mo in patients with high biliary obstruction, which is similar to that of other previous studies[16,17,29,30](7-9 mo).

The Asia-Pacific Consensus for Hepatic Portal Cholangiocarcinoma[31] indicates that percutaneous stent drainage is superior to endoscopic palliative care in advanced cases of type III or IV, and the purpose of palliative stent implantation is to ensure adequate liver drainage (>50%). Studies have shown that[17] effective liver drainage is important for patient survival, and the study by Vienne *et al*[33] showed that effective liver drainage (HR = 4.158, $P = 0.040$) was an independent predictor of survival. The most common classification of hilar cholangiocarcinoma is the Bismuth-Corlette classification, which evaluates local tumor spread but does not provide information on vascular invasion or metastatic disease and may be limited in assessment of prognostic value[34,40,41]. However, the Bismuth-Corlette classification determines the involvement of the biliary tract branch to a certain extent, which indicates the feasibility of achieving the maximum effective drainage of the liver and the stent or drainage tube implantation method; low classification (I or II) is easier to achieve the maximum effective drainage of the liver than high classification (III or IV). Type III or IV often requires multiple stents or drainage tubes. Our study showed that Bismuth-Corlette type IV was an independent predictor of survival and was inversely related to survival, and these results are similar to a previous study[33]. The control group showed a median survival time that was not statistically different from that in patients who underwent stent implantation or drainage tube implantation.

Drainage tubes were routinely required to undergo cholangiography and replaced

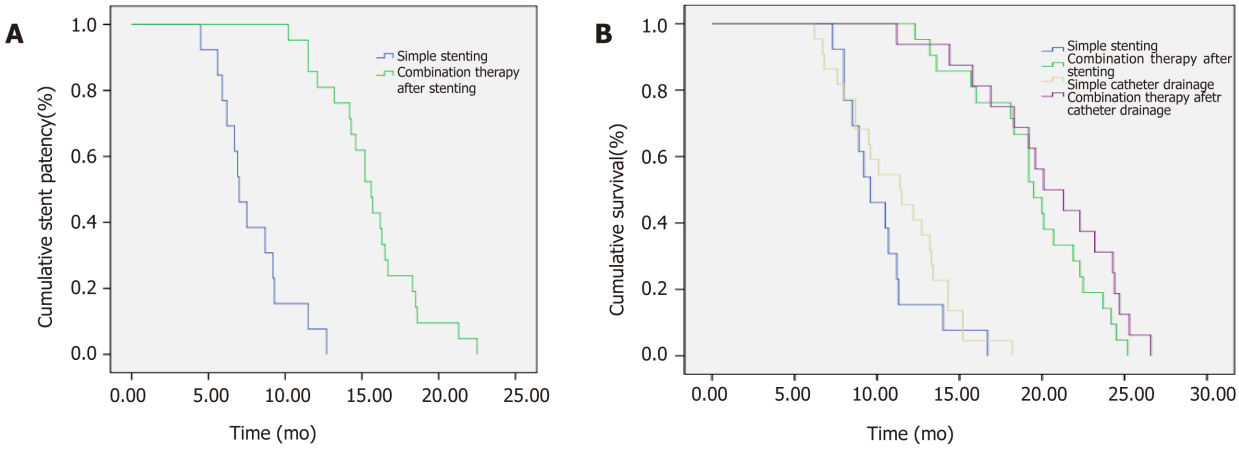

Figure 3 Comparison of stent patency time and survival between control group and combined treatment group. A: Comparison of stent patency time; B: Comparison of survival.

every 3-6 mo; however, there were still 10 (26.3%) patients with 13 tube shedding events. In addition, 2 (5.3%) patients with implanted metastases at puncture sites of the right intercostal space were observed in the study. Studies have shown that compared with biliary stent implantation, the choice of biliary drainage tube implantation is an independent predictor of survival that is positively correlated with survival and the reimplantation and replacement of the drainage tube may lead to prolonged effective drainage time.

This study showed that CA19-9 level cannot be used as a predictor of survival. In addition, serum CA19-9 concentration was determined by the Lewis blood group antigen phenotype of red blood cells[42], and approximately 5%-14% of the population had the Lewis α-β-type, which cannot produce CA19-9. In the CA19-9 test of this study, we did not take these factors into consideration, which may also result in biased results.

Studies have shown that gemcitabine plus cisplatin chemotherapy can prolong the survival of patients with cholangiocarcinoma in palliative care. A meta-analysis of 1368 patients with locally advanced cholangiocarcinoma by Eckel *et al*[19] showed that gemcitabine and platinum combination therapy resulted in higher response rates and tumor control rates. The ABC-02 trial by Valle *et al*[20] explored the addition of cisplatin plus gemcitabine to unresectable and metastatic cholangiocarcinoma and suggested that cisplatin plus gemcitabine resulted in a significant increase in progression-free survival (PFS) (8.0 mo *vs* 5.0 mo, $P < 0.001$) and overall survival (OS) (11.7 mo *vs* 8.1 mo, $P < 0.001$) compared to gemcitabine alone. Some research on the treatment of cholangiocarcinoma with arterial therapy has also achieved some results. The results of the study by Gusani *et al*[23] indicate that gemcitabine-based TACE was well-tolerated in patients with unresectable cholangiocarcinoma and that combination therapy (cisplatin or oxaliplatin) prolonged patient survival (OS 11.7 mo) compared to controls. Park *et al*[24] reported that conventional lipiodol-based chemoembolization (C-TACE) increased survival from 3.3 mo to 12.2 mo. Mahadevan *et al*[25] recently reported the results of 32 patients with major unresectable hepatocellular carcinoma treated with stereotactic body radiotherapy (SBRT), with local control and OS rates of 88% and 58%, respectively, and a median survival of 17 mo. Adverse events included duodenal ulcer in 2 patients, liver abscess in 1, and cholangitis in 1. Kopek *et al*[26] reported 27 patients with hepatic hilar cholangiocarcinoma treated with SBRT, with median PFS and OS of 6.7 and 10.6 mo, respectively, of which 6 patients developed gastroduodenal ulcers.

In patients with type III or IV cholangiocarcinoma in the combination treatment group, complete drainage was not achieved even by bilateral stent placement, and liver damage caused by obstructive jaundice may exist throughout the course of the disease. Therefore, combined treatment requires more attention to the patient's liver function status. In our institution, patients with hilar cholangiocarcinoma were strictly evaluated for liver function before receiving chemotherapy, TACE, and radiotherapy. Patients with a total bilirubin index less than 51.3 μmol/L (3 mg/dL) and Child-Pugh grade B or better might be more tolerant to treatment-induced liver damage. A liver protectant was given during the treatment, and the biochemical indicators of liver function were closely monitored during TACE and radiotherapy in our study. In radiation therapy, the total dose was controlled at approximately 48.3 Gy. There were

Table 2 Cox regression analysis of risk factors affecting patient prognosis

Influencing factor		Proportional risk factor	95%CI	*P*-value
Gender	M	1		
	F	1.312	0.771-2.223	0.316
Age	≤60 yr	1		
	>60 yr	1.819	1.032-3.207	0.038
Bismuth-Corlett classification	II	1		
	III	2.633	0.992-6.984	0.052
	IV	6.102	2.040-18.251	0.001
Ways to reduce jaundice	Stenting	1		
	Drainage	0.263	0.134-0.516	0.000
Comprehensive treatment	No	1		
	Yes	0.039	0.016-0.091	0.000
CA19-9	-	1.002	0.999-1.004	0.147

no patients with peptic ulcer, hemorrhage, or hepatic abscess in this study; 10 (27.0%) patients had grade II-III adverse reactions 2 wk after radiotherapy, 3 (8.1%) of whom were unable to complete the treatment course due to upper abdominal pain, nausea, and vomiting. The indications for TACE treatment during follow-up after radiotherapy were as follows: (1) The CA 19-9 index continued to increase for 3 mo during follow-up; (2) Imaging evaluation could measure lesion enlargement; (3) The total bilirubin index less than 3 mg/dL and Child-Pugh grade B or better; and (4) From the last treatment interval of more than 6 mo. During the operation, selective arterial administration was strictly required, and lesions with insignificant arterial blood supply were carefully embolized. In most patients, decreased appetite and fatigue occurred after treatment. Twenty-one (29.2%) patients developed grade II adverse reactions and neutropenia after the first or subsequent TACE treatment. No other serious complications were found to result in the inability to accept TACE. However, it was observed that 5 patients in the combination treatment group had irreversible cirrhosis and portal hypertension within 7-12 mo after treatment, which may be related to liver damage caused by TACE and radiotherapy.

This study showed that gemcitabine plus cisplatin-based TACE combined with radiotherapy extended the median survival time of patients with hilar cholangiocarcinoma after receiving stent or drainage tube implantation (20.0 mo *vs* 10.5 mo, $P < 0.05$). Accepting combination therapy, Bismuth-Corlette type IV, and accepting biliary drainage tube implantation were the independent predictors of survival of patients with hilar cholangiocarcinoma. Limitations of the study include that this study was a retrospective study conducted in only one center, and cases of hepatitis, cirrhosis, and extrahepatic metastasis were excluded from the case selection. These factors may affect the patient's survival and the observation of other research indicators.

ARTICLE HIGHLIGHTS

Research background

Because of the occultation of hepatic hilar cholangiocarcinoma, most patients have lost the opportunity for surgical radical treatment at the time of diagnosis. Palliative treatment is important for patients with hepatic hilar cholangiocarcinoma.

Research motivation

Simple reduction of jaundice may fail due to tumor progression, and effective and combined palliative treatment may prolong the effective drainage time of the biliary stent or drainage tube, thereby prolonging patient survival. However, relevant research is rare at present.

Research objectives

This study mainly investigated the effects of transcatheter arterial chemoembolization (TACE) combined with radiotherapy on the survival of patients with hilar cholangiocarcinoma after biliary stent or drainage tube implantation, and analyzed the influencing factors.

Research methods

This study used a retrospective cohort analysis to determine the significance of TACE combined with radiotherapy by comparing the differences between the two groups and comparing the

different methods of reducing jaundice (stent or drainage tube placement) within the group. Regression analysis of the overall data helps to identify the independent factors influencing survival.

Research results

There was no significant difference in survival between the control group and the combination treatment group, which were treated by stent or tube implantation for the treatment of reducing jaundice, while the survival of patients receiving TACE combined with radiotherapy was significantly longer than that of patients receiving simple reduction of jaundice.

Research conclusions

The results showed that TACE combined with radiotherapy can significantly extend the effective drainage time of stent or tube and prolong the survival of patients. Co-treatment, Bismuth-Corlett type IV, percutaneous biliary drainage, and age were independent predictors of survival.

Research perspectives

Accepting a reasonable and standardized palliative treatment will prolong the survival of patients with unresectable hilar cholangiocarcinoma and improve their living conditions. With the development of immunotherapy and targeted therapy, relevant research may be carried out in the future.

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ORIGINAL ARTICLE

Retrospective Study

Relationship between celiac artery variation and number of lymph nodes dissection in gastric cancer surgery

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Author contributions: Huang Y designed this study; Liu ZM, Wu XH, Qin XG, Chen ZB, and Zeng YJ conducted the study; Huang Y, Mu GC, and Chen ZB collected the data; Mu GC and Chen ZB interpreted the results; Mu GC and Huang Y drafted the article; Liu ZM revised the paper; All authors are in agreement with the content of the manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University.

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

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Abstract

BACKGROUND

Radical D2 lymphadenectomy for advanced gastric cancer as a standard procedure has gained global consensus. Mounting studies have shown that the number of lymph nodes dissection directly affects the prognosis and recurrence of gastric cancer. Our previous study showed that there was no obvious lymph node around the abnormal hepatic artery derived from the superior mesenteric artery.

AIM

To investigate the relationship between celiac artery variation and the number of lymph nodes dissection in gastric cancer surgery.

METHODS

The clinicopathological data of 421 patients treated with radical D2 lymphadenectomy were analyzed retrospectively. The difference of the number of lymph nodes dissection between the celiac artery variation group and the normal vessels group and the relationship with prognosis were analyzed.

RESULTS

Celiac artery variation was found in 110 patients, with a variation rate of 26.13%. Celiac artery variation, tumor staging, and Borrmann typing were factors that affected lymph node clearance in gastric cancer, and the number of lymph nodes

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dissection in patients with celiac artery variation was significantly less than that of non-variant groups ($P < 0.05$). Univariate analysis showed that there was no significant difference in survival time between the two groups ($P > 0.05$). Univariate and multiple Cox regression analysis showed that celiac artery variation was not a prognostic factor for gastric cancer ($P > 0.05$). Tumor staging, intraoperative bleeding, and positive lymph node ratio were prognostic factors for gastric cancer patients (all $P < 0.05$).

CONCLUSION
The number of lymph nodes dissection in patients with celiac artery variation was reduced, but there was no obvious effect on prognosis. Therefore, lymph nodes around the abnormal hepatic artery may not need to be dissected in radical D2 lymphadenectomy.

Key words: Gastric cancer; Celiac artery variation; Lymphadenectomy; Number of lymph nodes; Prognosis

Core tip: Celiac artery variation has been given great importance by surgeons. However, the distribution of the lymph nodes around the variant celiac artery and its effect on prognosis has rarely been examined. This study shows that variation of the celiac artery is an important factor affecting the lymph node clearance of gastric cancer, and the decrease in the number of lymph nodes dissection does not affect the prognosis. Therefore, lymph nodes dissection around abnormal hepatic artery, especial for the abnormal hepatic artery derived from the superior mesenteric artery, is not recommended.

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INTRODUCTION

Gastric cancer is the fifth most common malignant tumor, and its mortality rate ranks second in the world. In 2015 alone, 679,000 new cases were estimated in China, and about 498,000 patients died of gastric cancer[1]. Radical D2 lymphadenectomy as a standard procedure has gained global consensus. The current seventh edition of the International Union against Cancer/American Joint Committee on Cancer (UICC/AJCC) tumor, node, and metastases (TNM) staging for gastric cancer recommended that at least 16 or more lymph nodes should be dissected for satisfactory histological examination[2]. A German multicenter study showed that clearance of more than 25 lymph nodes was an independent prognostic factor for all types of pathological staging[3]. Studies showed that the number of lymph nodes dissection directly affected the prognosis and recurrence of gastric cancer[4].

According to our previous results[5], there was no obvious lymph node around the abnormal hepatic artery derived from the superior mesenteric artery, and the relationship was unclear between the total number of lymph nodes dissection and the impact on the prognosis in celiac artery variation patients. In addition, Arifuzzaman*et al*[6] found that the celiac artery variation rate was 30.9%, suggesting that it may be important clinically to investigate the difference in the number of lymph nodes dissection in patients with celiac artery variation and those with normal vessels. These findings could provide clinical basis for precise individualized lymph nodes dissection of gastric cancer.

MATERIALS AND METHODS

General information

Four hundred and fifty-two gastric cancer patients who underwent D2 lymphadenectomy that was performed by the same surgical team at the Department of Gastrointestinal Surgery of the First Affiliated Hospital of Guangxi Medical University from January 2009 to March 2017 were included in this study and screened according to the following criteria: (1) Preoperative gastroscopic pathology was gastric carcinoma; (2) Patient underwent upper abdominal multi-slice spiral computed tomography angiography(MSCTA) examination; (3) Preoperative comprehensive evaluations indicated D2 lymphadenectomy; (4) Patient was adopted for D2 lymphadenectomy. For the patients with vascular variations, we carefully dissected and cleaned the surrounding lymph nodes during the operation; (5) Complete clinical and pathological data were recorded; (6) No neoadjuvant therapy was accepted; and (7) Excluding other malignant tumors. Finally, 421 cases satisfied the conditions mentioned above. Three hundred and six cases (72.7%) were men, and the median age was 56.1 years old (19-86 years old). After the surgery, all the patients were staged according to the 7th AJCC TNM staging standard: IA stage-49 cases, IB stage-41 cases, IIA stage-47 cases, IIB stage-62 cases, IIIA stage-52 cases, IIIB stage-56 cases, and 114 in stage IIIC. This study was approved by ethical review committee at 27 August 2018 [Approval number: 2018(KY-E-056)].

Information acquisition of celiac artery

Inspection equipment (LightspeedVCT) was provided by the American GE company. The preparation, scanning parameters, and data and image processing of the CT scan have been detailed in the literature[7]. After image reconstruction, two senior radiologists analyzed the reconstructed three-dimensional vascular images and observed whether celiac artery variation was present.

Surgery

The 421 gastric cancer patients underwent standard radical gastrectomy by the same experienced surgical team. Lymph node clearance was carried out according to the requirements of the Japanese Gastric Cancer Association protocol. The range of lymph nodes dissection and gastrectomy were performed in accordance with the requirements of the Japanese Gastric Cancer Association guidelines[8]. The lymph nodes in each group were selected by the senior resident who participated in the operation, in accordance with the regulations of the Japanese gastric cancer protocol at the end of the operation. After the operation, additional sorting was carried out with touch method, and the lymph nodes extracted in the operation were sent for pathological examination.

Follow up

The standard follow-up protocol for patients with gastric cancer was every 3 mo for at least 2 years, every 6 mo for the next 3 years, and every 12 mo after five years for life.The follow-up items included physical examination, tumor markers, computed tomographic scan, and gastroscopy. Follow-up deadline was to 31 May 2018, and the survival time was calculated from operation time to death or follow-up deadline. Sixteen cases were lost midway during follow-up, and the loss rate was 3.8%. The patients were followed up for 12.0-112.0 mo, and the median follow-up period was 42.6 mo.

Statistical analysis

SPSS 16 statistical software (Chicago, IL, United States) was used to analyze the data. The count data was compared by χ^2 test. Two independent samples t test or one-way analysis of variance were used to analyze normal distribution data. Survival analysis was performed by Kaplan-Meier method, and survival rate was compared by Log-rank. Univariate and multivariate Cox regression analysis was used to analyze the survival of gastric cancer. The difference was statistically significant if $P < 0.05$.

RESULTS

The variation of celiac artery

The preoperative MSCTA images showed 311 cases of normal celiac artery, 110 cases of variant celiac artery, and the variation rate was 26.13%. Celiac artery types in all 421 cases detected by preoperative MSCTA were conformed intraoperatively. Ninety-seven cases had an abnormal hepatic artery and were classified according to Hiatt's standard[9] (Figure 1). Among them, abnormal hepatic artery derived from superior mesenteric artery was seen in 48 cases, the hepatic artery ran in front of the pancreas in two cases(Figure 2) and behind the pancreas in 46 cases. In the post-pancreas type, the hepatic artery arising from the superior mesenteric artery ran behind the

pancreatic neck and the initial segment of the portal vein. Then, it ran behind the right hepatic duct and entered the liver ligament.

The left gastric artery derived from the abdominal aorta in eight cases, the splenic artery derived from the superior mesenteric artery in two cases, and in three cases, the celiac trunk and the superior mesenteric artery with a common trunk derived from the abdominal aorta directly.

The relationship between the number of lymph nodes dissection and the clinicopathological features of radical D2 lymphadenectomy for gastric cancer

In total, 2243 lymph nodes were dissected in 110 cases of celiac artery variation, with an average of 20.4 (4-50)/case, 671 positive lymph nodes, and 6.1 average lymph node metastases. The total number of lymph nodes detection in 311 cases without vascular variation was 7373, with an average of 23.7 (3-70/case), 1663 positive lymph nodes, and 5.3 average lymph node metastases. In general, the number of lymph nodes dissection in patients with celiac artery variation was significantly less than that in patients without celiac artery variation (P = 0.000). In stage I and II, there was significant difference between the two groups of lymph node clearance (P = 0.000),but there was no significant difference in stage III (P = 0.229). There was no significant difference in age, sex, tumor location, tumor stage, pathological type, Borrmann typing, or adjuvant chemotherapy between the two groups (P > 0.05) (Table 1).

The number of lymph nodes dissection in radical D2 lymphadenectomy for gastric cancer was not only related to the variation of celiac artery but also affected by late tumor stage and high Borrmann typing (P < 0.05)and was not affected by sex, age, tumor location, and pathological type (all P > 0.05) (Table 2).

Survival analysis

The survival rate at 1, 3, and 5 years in the celiac artery variation group was 84.5%, 57.6%, and 47.6%, respectively. The survival rate at 1, 3, and 5 years in the non-variation group was 85.2%, 56.8%, and 45.2%, respectively. There was no statistical difference in the survival time between the two groups (χ^2 = 0.056, P = 0.813) (Figure 3).

The age, sex, tumor staging, tumor location, pathological type, Borrmann typing, number of lymph nodes, lymph node metastasis, positive lymph node ratio, celiac artery variation, operation time, and intraoperative bleeding amount and postoperative survival of gastric cancer patients were analyzed by univariate Cox regression analysis. The results showed that tumor staging, tumor location, Borrmann typing, operation time, intraoperative bleeding amount, number of lymph nodes dissection, number of lymph node metastases, and positive lymph nodes ratio were prognostic factors of gastric cancer(Table 3). Variables of statistical significance in univariate Cox regression analysis were introduced into the multivariate Cox regression analysis equation (Forward: LR method), and the selected standard was 0.05. The results showed that tumor stage, intraoperative bleeding amount, and positive lymph node ratio were independent risk factors for prognosis of gastric cancer, as shown in Table 4.

DISCUSSION

At present, D2 lymphadenectomy is widely accepted as the standard of surgery for advanced gastric cancer all around the world. The current 7th UICC/AJCC TNM staging of gastric cancer requires that lymph nodes dissection should include at least 16 or more lymph nodes for histological examination[2]. In recent years, many studies have shown that there is a correlation between the overall survival time and the number of lymph nodes dissection after gastric cancer surgery[10,11]. However, the scope of precise lymph nodes dissection for different stages and tumor locations remains controversial. Lu *et al*[12] found that radical distal gastrectomy with more than 16 lymph nodes dissection and radical total gastrectomy with more than 21 lymph nodes dissection was the standard and will be more conducive to the analysis and evaluation of the prognosis of the patients. A multicenter study in the United States has shown that clearance of more than 16 lymph nodes in patients with IA-III-A can significantly improve long-term survival[13]. According to the results of a multicenter study including 1654 cases in Germany, more than 25 lymph nodes should be removed for gastric cancer patients with stage II[3]. In a word, the number of lymph nodes dissection in gastric cancer is still controversial. In the era of precision surgery, many scholars suggest that a reasonable range of lymph nodes dissection should be selected according to the individual factors, such as tumor location, tumor staging, and human anatomy, in order to reduce postoperative complications and improve the

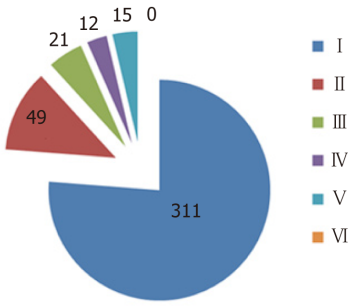

Figure 1 Abnormal types of hepatic artery.

long-term survival rate and postoperative living quality. The variation of the celiac artery is an important anatomical factor for gastric cancer patients. An earlier study found that the abnormal hepatic artery derived from the superior mesenteric artery was not obviously linked to lymph node distribution[5]. We speculate that the anatomic variation of the celiac artery may affect the number of lymph nodes dissection in gastric cancer.

The results of this study showed that the variation rate of the celiac artery was as high as 26.13%, which was similar to that reported in Ugurel *et al*[14]. Among them, hepatic artery system variation was the most common, and the abnormal hepatic artery derived from superior mesenteric artery accounted for 43.6% of the hepatic artery system variation, which was a little higher than our previous report(37.0%)[15]. The existence of celiac artery variation increases the difficulty of operation, prolongs the operation time, increases the amount of bleeding during the operation, and may increase the incidences of intraoperative and postoperative complications. Therefore, the surgeon attaches great importance to celiac artery variation[16-18]. However, the distribution of the lymph nodes around the variant celiac artery has rarely been concerned. The results of this study show that celiac artery variation, tumor stage, and Borrmann typing are factors that affect the lymph nodes dissection of gastric cancer. The higher tumor stage and Borrmann typing, the more lymph nodes could be observed. At present, lymph nodes were sorted with touch method after surgery, which may be associated with overlooking some hidden tiny lymph nodes. High tumor stage and Borrmann typing may increase the rate of lymph node enlargement around the stomach, thus potentially increasing the number of lymph nodes dissection.

The number of lymph nodes dissection in patients with celiac artery variation is significantly less than those without variation, which further validates the prediction of the results in our earlier study. We speculate that the reasons for the reduction of the number of lymph nodes may be as follows. Firstly, the lymphatic reflux system of the stomach is special and complex. Kajitani in Japan suggests that the lymph around the stomach flows retrograde along the artery, and the artery is more fixed. Finally, it was determined to use the trip of the arterial system and the branch of the artery as a fixed anatomical sign to describe the lymph circumfluence of the stomach[19]. Variation of the celiac artery may be accompanied by a change in the lymphatic reflux, leading to a reduction in the distribution of the perivascular lymph nodes. Secondly, the 14th version of the Japanese gastric cancer treatment protocol lists No.12a as a routine cleaning object and No.12p and No.12b as unconventional cleaning objects. The No.12a lymph node is distributed along the hepatic artery from the confluence part of the left and right hepatic duct to the superior border of the pancreas. Normally, the proper hepatic artery goes ahead of the left anterior of the hepatoduodenal ligament and anterior of the portal vein, but the abnormal right hepatic artery and common hepatic artery derived from the superior mesenteric artery are common in the rear of the portal vein and medial of the common bile duct[20].The lymph nodes around this part of the abnormal hepatic artery are easily ignored without dissection during the operation because they are mistaken for No.12b or No.12p, eventually leading to a reduction in the number of lymph nodes dissection. Thirdly, the abnormal arteries, especiallythe abnormal hepatic artery derived from the superior mesenteric artery, are mostly of the post-pancreas type, and it is difficult to dissect the peripheral lymph nodes and adipose tissue around the root of the abnormal hepatic artery and the posterior part of the pancreas.

The number of lymph nodes dissection in the patients with celiac artery variation was not more than those of normal blood vessels, but there was no difference in the

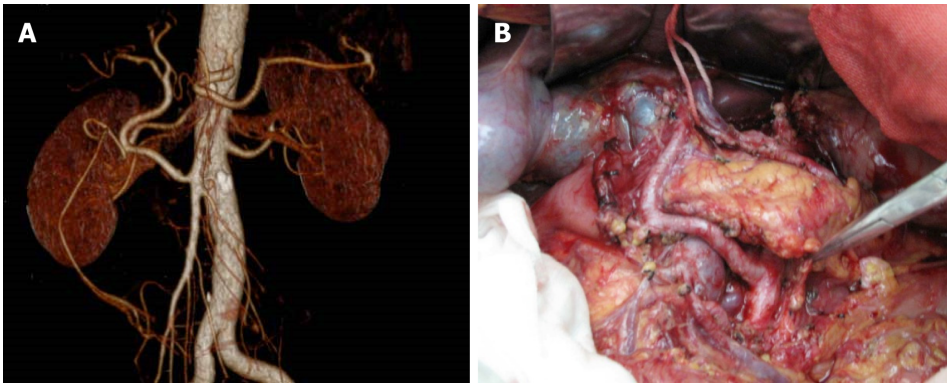

Figure 2 The common hepatic artery derived from the superior mesenteric artery (anterior-pancreas type).

prognosis of the two groups. The univariate and multivariate Cox regression analysis showed that the variation of celiac artery was not an independent risk factor for the prognosis of gastric cancer. In view of this, we do not recommend routine cleaning the lymph nodes around the variant celiac artery, especially the abnormal hepatic artery derived from the superior mesenteric artery. The reasons are three points: (1) No lymph nodes are found around the abnormal hepatic artery of the post-pancreas and pre-pancreas type arising from the superior mesenteric artery during the D2 radical lymphadenectomy. Also, the tissues around the abnormal vessels were dissected for routine HE staining and CK20, CEA immunization, and no metastasis was found[5]; (2) The majority of abnormal hepatic arteries derived from the superior mesenteric artery belonged to the post-pancreas type, greatly increasing the difficulty of lymph nodes dissection and the risk of damaging the abnormal hepatic artery and pancreas, which may lead to increased risk of intraoperative bleeding, postoperative liver function damage, and pancreatic fistula and an increase in operation time; and (3) The results of this study showed that the number of lymph nodes dissection was reduced in celiac artery variation patients. However, prognosis was not affected, and the variation of the celiac artery was not an independent risk factor for the prognosis of gastric cancer.

In summary, variation of the celiac artery is an important factor affecting the lymph node clearance of gastric cancer, and the decrease in the number of lymph nodes dissection does not affect the prognosis. We do not recommend routine cleaning for the abnormal hepatic artery, especially the abnormal hepatic artery derived from the superior mesenteric artery. As this study was retrospective, the next step is to perform a prospective control study on the distribution difference of the peripheral lymph nodes based on the detailed vascular variation types, which would yield a more reliable basis for the development of a precise and individualized treatment plan for patients with gastric cancer.

Table 1 Comparison of clinical data between two groups of patients with celiac artery variation and without vascular variation

Clinicopathological features	Abnormal vessel group, *n* = 110	Normal vessel group, *n* = 311	χ^2	*P* value
Gender				
male	74	232	2.196	0.138
female	36	79		
Agein yr				
≤45	23	54	0.881	0.644
46-60	46	143		
>60	41	114		
Tumor stage				
I	24	66	2.848	0.241
II	22	87		
III	64	158		
Tumor location				
Proximal stomach	13	38	0.977	0.802
Gastric body	14	45		
Gastric antrum	73	208		
Whole stomach	10	20		
Pathology classification				
Adenocarcinoma	99	270	2.489	0.477
Mucous carcinoma	5	10		
Signet ring cell carcinoma	2	13		
Undifferentiated carcinoma	4	18		
Borrmann classification				
I	8	11	3.830	0.280
II	31	75		
III	53	164		
IV	18	61		
Adjuvant chemotherapy				
Yes	79	217	0.163	0.687
No	31	94		
Complication				
Yes	18	29	4.059	0.044
No	92	282		
Lymph node clearance	20.391 ± 0.693	23.707 ± 0.587	3.651	0.000
Lymph node clearance in different stages				
I	13.917 ± 0.558	20.470 ± 1.010	3.822	0.000
II	17.863 ± 0.728	23.402 ± 1.095	4.231	0.000
III	23.688 ± 0.932	25.228 ± 0.871	1.207	0.229

Table 2 The relationship between the number of lymph nodes dissection and the clinicopathological features of radical D2 lymphadenectomy for gastric cancer

Influence factors	*n*	mean ± S	SS	MS	*t* or *F* value	*P* value
Gender						
Male	306	22.869 ± 0.573	-	-	0.097	0.922
Female	115	22.765 ± 0.839				
Agein yr						
≤ 45	77	23.091 ± 1.313	15.536	7.768	0.081	0.922
46-60	189	22.937 ± 0.625				
> 60	155	22.600 ± 0.817				
Variation of celiac artery						
Yes	110	20.391 ± 0.693	-	-	3.651	0.000
No	311	23.707 ± 0.587				
Tumor stage						
I	90	18.722 ± 0.814	-	-	13.365	0.000
II	109	22.284 ± 0.910				
III	222	24.784 ± 0.676				
Tumor location						
Proximal stomach	51	21.588 ± 1.273	510.539	170.180	1.801	0.146
Gastric body	59	21.509 ± 1.199				
Gastric antrum	281	23.000 ± 0.576				
Whole stomach	30	26.100 ± 2.207				
Pathology classification						
Adenocarcinoma	369	22.846 ± 0.511	54.376	18.125	0.190	0.903
Mucous carcinoma	15	24.333 ± 2.656				
Signet ring cell carcinoma	15	21.733 ± 1.963				
Undifferentiated carcinoma	22	22.500 ± 2.123				
Borrmann classification						
I	19	17.053 ± 1.733	2492.710	830.903	9.261	0.000
II	105	20.171 ± 0.842				
III	218	23.303 ± 0.655				
IV	79	26.506 ± 1.165				

MS: Mean square;SS: Standard deviation square.

Table 3 The results of univariate Cox regression analysis in gastric cancer

Clinicopathological features	B	SE	Wald	OR (95%CI)	*P* value
Age	0.204	0.104	3.820	1.227(0.999-1.505)	0.051
Gender	0.104	0.159	0.429	1.110(0.812-1.517)	0.512
Tumor stage	1.004	0.124	65.481	2.728(2.140-3.479)	0.000
Tumor location	-0.265	0.088	8.958	0.767 (0.645-0.913)	0.003
Pathology classification	0.020	0.089	0.050	1.020 (0.857-1.214)	0.823
Borrmann classification	0.605	0.098	37.822	1.832 (1.511-2.222)	0.000
Celiac artery variation	0.038	0.163	0.055	1.039 (0.755-1.430)	0.815
Operation time	0.002	0.001	8.270	1.002 (1.001-1.003)	0.004
Intraoperative bleeding	0.001	0.000	21.216	1.001 (1.000-1.001)	0.000
No. of lymph nodes metastases	0.071	0.008	90.075	1.074 (1.058-1.090)	0.000
No. of lymph nodes	0.018	0.007	6.083	1.018 (1.004-1.032)	0.014
Positive lymph node ratio	2.491	0.242	106.057	12.072 (7.514-19.393)	0.000

Table 4 The results of multivariate Cox regression analysis

Variables	B	SE	Wald	OR (95%CI)	*P* value
Comprehensive staging	0.626	0.145	18.494	1.870 (1.406-2.486)	0.000
Intraoperative bleeding	0.001	0.000	10.575	1.001 (1.000-1.001)	0.001
Positive lymph node ratio	1.466	0.318	21.206	4.330 (2.320-8.079)	0.000

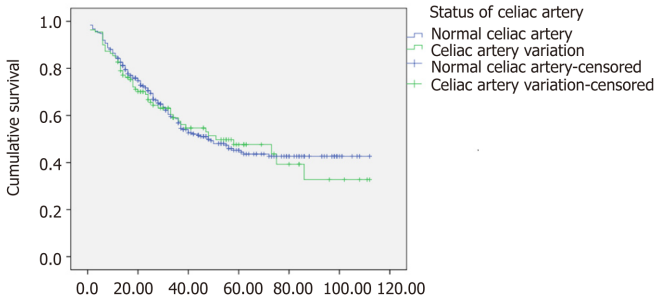

Figure 3 Comparison of survival curves of the celiac artery variation group and the normal vessels group.

ARTICLE HIGHLIGHTS

Research background

The number of lymph nodes dissection directly affects the prognosis and recurrence of gastric cancer. In addition, celiac artery variation is quite common clinically. However, there are few studies that discuss the relationship between celiac artery variation and the number of lymph nodes dissection in gastric cancer surgery.

Research motivation

According to our previous study, the number of lymph nodes dissection in gastric cancer surgery might be different between variant celiac artery patients and normal celiac artery patients. Therefore, we conducted this study to investigate the relationship between celiac artery variation and the number of lymph nodes dissection in gastric cancer surgery.

Research objectives

To investigate the relationship between celiac artery variation and the number of lymph nodes dissection in radical D2 lymphadenectomy of gastric cancer and the effect on prognosis.

Research methods

The clinicopathological data of 421 patients treated with radical D2 lymphadenectomy were analyzed retrospectively. The difference in the number of lymph nodes dissection between celiac artery variation group and normal vessels group and the relationship with prognosis were analyzed.

Research results

The number of lymph nodes dissection in patients with celiac artery variation was significantly less than that of non-variant groups, but there was no significant difference in survival time between the two groups. Univariate and multiple Cox regression analysis showed that celiac artery variation was not a prognostic factor for gastric cancer.

Research conclusions

Celiac artery variation is an important factor affecting lymph node clearance in patients with gastric cancer. The number of lymph nodes dissection in patients with celiac artery variation is reduced, but there is no obvious effect on the prognosis. Therefore, lymph nodes around the abnormal artery, especially for the abnormal hepatic artery derived from superior mesenteric artery, may not need to be dissected in radical D2 lymphadenectomy.

Research prospective

As this was a small-scale study, we propose future studies with a larger sample sizes. At the same time, the relationship between celiac artery variation and the number of lymph nodes dissection in different celiac artery variation types should be evaluated. We propose that lymph nodes around the abnormal artery, especially for the abnormal hepatic artery derived from superior mesenteric artery, do not need to dissected in radical D2 lymphadenectomy. However, further prospective and controlled studies are required to verify this theory.

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