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CASE REPORT

ABOUT COVER

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REVIEW

Behind the curtain of non-coding RNAs; long non-coding RNAs regulating hepatocarcinogenesis

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common and aggressive cancers worldwide. HCC is the fifth common malignancy in the world and the second leading cause of cancer death in Asia. Long non-coding RNAs (lncRNAs) are RNAs with a length greater than 200 nucleotides that do not encode proteins. lncRNAs can regulate gene expression and protein synthesis in several ways by interacting with DNA, RNA and proteins in a sequence specific manner. They could regulate cellular and developmental processes through either gene inhibition or gene activation. Many studies have shown that dysregulation of lncRNAs is related to many human diseases such as cardiovascular diseases, genetic disorders, neurological diseases, immune mediated disorders and cancers. However, the study of lncRNAs is challenging as they are poorly conserved between species, their expression levels aren't as high as that of mRNAs and have great interpatient variations. The study of lncRNAs expression in cancers have been a breakthrough as it unveils potential biomarkers and drug targets for cancer therapy and helps understand the mechanism of pathogenesis. This review discusses many long non-coding RNAs and their contribution in HCC, their role in development, metastasis, and prognosis of HCC and how to regulate and target these lncRNAs as a therapeutic tool in HCC treatment in the future.

Key words: Tumor suppressor genes; Oncogenes; Long non-coding RNAs; Proliferation; Hepatocellular carcinoma; Metastasis

Core tip: Recent researches are focusing on targeting non-coding RNAs in an attempt to find therapeutic means for many health problems. Here, we are shedding the lights on the regulation of several proteins in hepatocellular carcinoma (HCC) by long non-coding RNAs (lncRNAs). lncRNAs try desperately to halt the aberrantly expressed oncogenic network by the fact that single lncRNA can have multiple downstream targets in one or more signaling pathway. This is an approach in an attempt to find an efficient radical cure for HCC.

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INTRODUCTION

Epigenetic regulations

If we look up the word epigenetic in the dictionary, the result will be: the process by which the expression of genetic information is modified on a molecular level without alteration to the DNA sequence. The word epigenetic was also defined by previous researchers as "in addition to changes in genetic sequence", "to act "on top of" or "in addition" to genetics" and "heritable changes in gene activity and expression that occur without alteration in DNA sequence"[1-4]. Various kinds of epigenetic processes have been discovered during the years, which include methylation, acetylation, phosphorylation, ubiquitylation and non-coding RNA. Such alterations can be transmitted to daughter cells or, as suggested in recent experiments, can be reversed. Epigenetic processes are significant to normal organism functions, however, if they develop incorrectly, severe unwanted health and mental effects could arise[4].

The most studied epigenetic process is DNA methylation. It's the covalent addition or a methyl group (CH3) removal to the fifth position of the cytosine base within CpG dinucleotides. This modification is catalyzed by DNA methyltransferases (DNMTs), as DNMT1, DNMT3a and DNMT3b. DNMT3a and DNMT3b are considered de novo methyltransferases, initiating methylation to unmethylated CpGs during embryonic development or in cancer cells[5-7]. On the contrary, DNMT1 functions as the maintenance methyltransferase by methylating hemimethylated CpGs after mitosis, hence transmitting the methylation patterns to daughter strands, along with contributing to the de novo methylation process[8,9]. Both classes are said to function co-operatively to methylate DNA usually in regions known as CpG islands where the occurrence of CpG dinucleotides is high. DNA methylation causes gene silencing through two mechanisms; firstly, by decreasing the affinity of transcription factors to gene promoters through steric hindrance and the second mechanism is by the direct binding of methyl CpG binding domain (MBD)-containing proteins to the methylated DNA, leading to transcription repression through chromatin condensation[10]. This gene silencing could be reversed by active DNA demethylation which mainly happens by the removal of the methyl group from 5-methylcytosine *via* Methyl-CpG binding domain proteins[11].

Another epigenetic regulation is histone modification which encounters any post translational modification regulating chromatin structure and function. Chromatin consists of DNA and proteins bundled together in a compact way to fit inside the nucleus[4]. These complexes can be modified mainly through acetylation and methylation of the histone lysine residues. The resultant effect differs according to the type of modification and its location on the histone. The lysine residues at the histone terminals are subject to acetylation or deacetylation by histone acetyltransferases or histone deacetylases. Acetylation decreases the positive charges of lysine residues and decreases the affinity between histones and DNA which results in decondensation of the chromatin hence, disrupting the chromatin structure. Moreover, acetylated residues act as binding sites for histone modifying enzymes or chromatin remodeling factors that facilitate gene expression[12,13]. Methylation of histone occurs on different lysine residues with different degree of methylation thus giving a wide variety of results either repressive or activating depending on the combination of factors[14]. Methylation of the lysine at the fourth residue of histone H3 (H3K4Me) promotes a transcriptionally active conformation, as H3K9Me which promotes a transcriptionally repressive conformation. H3K36Me can be activating or repressive, depending upon proximity to a gene promoter region[15].

Non-coding RNAs

RNA species beyond mRNA which lack clear potential to encode proteins or peptides, and they include intronic RNAs, microRNAs (miRNAs), circular RNAs (circRNAs), extracellular RNAs and long non-coding RNAs (lncRNAs), that will be our main focus in this review[16].

lncRNAs

LncRNAs are a diverse group of transcripts whose natural functions and potential as drug targets remain largely undefined. These RNA species are greater than 200 nucleotides in length and do not encode protein. lncRNAs are thought to involve nearly 30000 different transcripts in humans, lncRNA transcripts account for the major part of the non-coding transcriptome. LncRNA

discovery remains at a primary stage.

LncRNAs biogenesis

Several long non-coding RNAs (lncRNAs) or classes of lncRNAs are differentially regulated at different levels of their biogenesis, maturation and degradation. At the chromatin level state, lncRNAs and mRNAs exhibit similar properties, For example, an enrichment of H3K4me3 at promoters; however, lncRNA genes have a higher enrichment of H3K27ac and are definitely more repressed by certain chromatin remodelling complexes, such as Swr1, Isw2, Rsc and Ino80. Transcription starts from divergent promoters differs for the sense (mRNA) and the antisense (lncRNA) directions; divergent antisense transcription is enriched for H3K56ac and phosphorylation of RNA polymerase II (Pol II) Tyr1. Transcription in the divergent direction is additionally increased by the SWI/SNF proteins and repressed by CAF-1. Transcriptional elongation is more strongly regulated by DICER1 and MYC for lncRNAs than for mRNAs. The occurrence of U1 and polyadenylation signals differs on either side of bidirectional promoters (along the U1–PAS axis), favoring the splicing of mRNAs in the sense direction and the cleavage and polyadenylation in the divergent, antisense direction. Despite the the fact that mRNAs localize very specifically to ribosomes in the cytoplasm, lncRNA localization is substantially more differed, as certain lncRNAs can occupy the chromatin, subnuclear domains, the nucleoplasm or the cytoplasm. Finally, whereas mRNAs are primarily degraded in the cytoplasm by decapping and 5′-to-3′ exonuclease digestion, numerous unstable lncRNA transcripts are liable to the nuclear exosome or to cytosolic nonsense-mediated decay (NMD)[17].

LncRNAs functions and mechanism of action

LncRNAs can regulate gene expression and the synthesis of protein in various ways. Some lncRNAs are relatively highly expressed, and functions as scaffolds for particular subnuclear domains. Carrying on, lncRNA have secondary structures which facilitate their interactions with DNA, RNA and proteins. Also, its binding to DNA or RNA is sequence-specific. Gene regulation may take place in cis (in close proximity to the transcribed lncRNA) or in trans (at a distance from the transcription site). In the function of chromatin modulation, the effect of lncRNA is usually gene-specific, exerted at a local level (in cis) but the regulation of chromatin can also take place in trans[18].

Some lncRNAs already had their functions experimentally elucidated and have proved to be involved in gene regulation processes including: Chromatin modification and structure, direct transcriptional regulation, regulation of RNA processing such as splicing, editing, localization, translation and degradation, post-translational regulation of protein activity and localization, facilitation of ribonucleoprotein (RNP) complex formation, modulation of microRNA regulation, in addition to gene silencing through endogenous siRNA (endo-siRNA) and regulation of genomic imprinting.

Recently, lncRNA functions have been classified into main different molecular mechanisms. One lncRNA may be described as one or more of the following five models: First, the Signal model that functions as a molecular signal or indicator of transcriptional activity. The Decoy model in which it binds and titrates away other regulatory RNAs (*e.g.*, microRNAs) or proteins (*e.g.*, transcription factors). Third type is the Guide model in which it directs the localization of ribonucleoprotein complexes to specific targets (*e.g.*, chromatin modification enzymes are recruited to DNA). Last but not least the Scaffold model that has a structural role as platform upon which relevant molecular components (proteins and or RNA) can be assembled into a complex or spatial proximity. And the last type is the Enhancer model which controls higher order chromosomal looping in an enhancer-like model[19].

Epigenetic regulation and lncRNAs in hepatocellular carcinoma

The lncRNA X-inactive specific transcript (XIST) recruits EZH2 which is a component of polycomb repressive complex "PRC2", to the X chromosome, leading to histone methylation and silencing of one X chromosome[20]. Also ANRIL, a lncRNA which recruits polycomb repressive complexes "PRC1 and PRC2" to the locus, causing the reduction transcription of ARF, CDKN2B, and CDKN2A[21,22]. Another lncRNA " TUG1" can regulate KLF2 expression in the epigenetic level by binding to PRC2. And it was found that those lncRNAs are deregulated in hepatocellular carcinoma (HCC) beside other lncRNAs that will be mentioned below.

LncRNAs in cancer

Numerous previous studies concluded that long non-coding RNAs play an important role in regulating many biological processes including gene expression, cell cycle regulation and cellular differentiation[23]. Hence, deregulated lncRNA expression may contribute to disease pathogenesis and pathophysiology such as cancers, cardiovascular disorders and metabolic disorders. This relation unveils the role lncRNAs could have as therapeutic targets or biomarkers for diseases[24]. LncRNAs are usually observed in cancer cases due to the expression difference between cancerous cells and normal cells[25]. Moreover, they fall under two categories; either oncogenic, promoting cancer or tumor suppressor, inhibiting cancer progression and both have equal importance in cancer therapeutic treatment. Long non-coding RNAs exert their effect through various mechanisms for example, modulating epigenetic regulations including imprinting as X-inactivation in females, controlling cell apoptosis signaling as well as

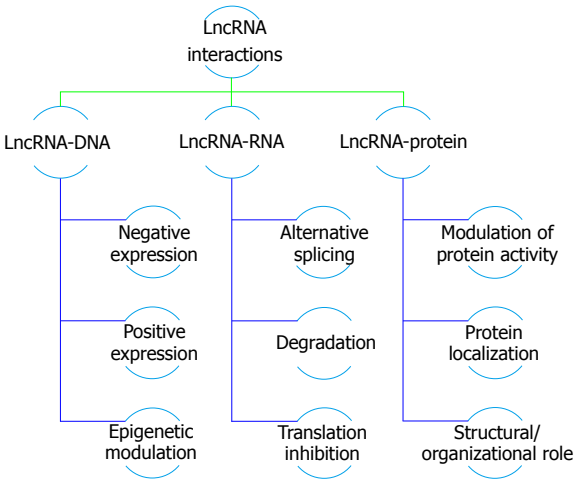

Figure 1 Long non-coding RNAs actions and interaction in cancer development; a schematic representation to the role of deregulated long non-coding RNAs in various types of cancers. lncRNAs: Long non-coding RNAs.

cellular proliferation pathways. Sometimes, they interact with RNA-binding proteins mediating transcriptional processes, bind to their intracellular steroid receptors activating or inhibiting downstream targets or transcription factors. Furthermore, lncRNAs could induce chromatin modification through recruiting chromatin remodeling complex to specific loci and regulate gene expression at the post transcriptional level as well (as shown in Figure 1)[26].

As an example to the function of deregulated lncRNAs in various types of cancers, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is reported overexpressed in breast cancer, prostate cancer, uterine cancer, colon cancer, pancreatic cancer, osteosarcoma, NSCLC and other cancers[26-28]. Additionally, HOX transcript antisense RNA (HOTAIR) is found upregulated in breast cancer and prostate cancer aiding in tumor invasion and metastasis[29,30]. Furthermore, H19 present on chromosome 11, is reported crucial in bladder, lung, breast, cervix, prostate and colorectal cancers. Another famous lncRNA is urothelial carcinoma-associated 1 (UCA1) which serves as an oncogene in colon, cervix, lung, bladder, breast and stomach carcinomas through enhancing cellular proliferation and migration[27]. ANRIL, one of the antisense lncRNAs is found in high levels in prostate cancers and leukemia[26,27]. BC200, an intergenic gene on chromosome 2 modulates translational processes in breast, cervix, esophagus, lung, ovary and tongue cancers[26]. On the contrary, MEG3 has proved to be one of the tumor suppressor genes in cancers like pituitary adenomas as it inhibits cell proliferation and modulates angiogenesis through regulating p53[31]. Another tumor suppressor gene is growth arrest-specific transcript 5 (GAS5) that induces apoptosis in breast and prostate cancers by inhibiting several responsive genes leading to altering various cellular processes[26].

LncRNA IN HCC

Tumor suppressor lncRNA

lncRNA SVUGP2 is downregulated in HCC cells and its overexpression inhibits cellular proliferation and invasion through the inhibition of several mRNA and proteins of growth markers and invasion markers; hence acting as a tumor suppressor[32]. AF113014 acts as a tumor suppressor by regulating the expression of the tumor suppressor gene, Egr2 through interaction with miR-20a causing a decrease in cellular proliferation[33]. Uc.134 is reported to be significantly downregulated in HCC cells; its overexpression suppresses HCC by inhibiting cellular invasion and proliferation *via* repression of CUL4-mediated ubiquitination of LATS1 and activation of YAPS127 phosphorylation[34]. SchLAH is a lncRNA that is downregulated in HCC *via* possible histone deacetylation; its overexpression inhibits cell migration and metastasis through interaction with FUS protein and modulation of RhoA and Rac1 downstream targets[35]. SRA1 is one of the downregulated RNAs in HCC cells that serves as a tumor suppressive through its regulation of tumor size and serum glucose levels[36]. DGCR5 is one of the downregulated lncRNAs that can acts as a tumor suppressor in HCC patients however its molecular mechanism is yet to be studied[37]. Also, the lncRNA ZNF674-AS1 is found to be downregulated in HCC patients with significant relation to poor prognosis in patients; however, no significant molecular mechanism is established yet[38]. STARD13 is a tumor suppressor as its expression levels in HCC is low and its upregulation enhances cellular apoptosis by acting as a ceRNA for Fas and increases tumor sensitivity to chemotherapy[39].

Linc-cdh4-2 is downregulated in HCC and its overexpression causes a significant decrease in migration and invasion with no effect on cell viability and proliferation. The decrease in migration could be through an increase in the protein levels of R-cadherin and decrease in the protein levels of small GTPase RAC1, as an increase in RAC1 protein levels enhances cell invasion abilities[40]. Low levels of LINC RP1130-1 are significant in HCC patients compared to normal patients. This decrease is associated with number of tumors, portal vein tumor thrombus, microvascular invasion and most importantly a shorter recurrence-free survival hence, classifying it as a tumor suppressor lncRNA[41]. ZNFX1-AS1 is significantly downregulated in HCC and its overexpression suppressed cell proliferation, colony formation and promoted cell apoptosis. miR-9 exhibits a positive correlation with ZNFX1-AS1 hence an upregulation of miR-9 and a downregulation of its promoter methylation is observed with ZNFX1-AS1 overexpression[42]. LINC00052 is one of the tumor suppressor lncRNAs as its upregulation inhibit cell

invasion and proliferation abilities of HCC cells which is achieved through regulating NTRK3 expression by establishing complementary base pairing with miR-128 ad miR-485-3p. Downregulation of NTRK3 promotes cellular invasion and proliferation abilities just like the low levels of LINC00052[43]. PRAL is a tumor suppressor lncRNA present on chromosome 17p13.1 usually in low levels in HCC patients and its overexpression inhibits tumor growth. This occurs due to the binding of PRAL to HSP90 which enhances its binding to p53, opposes MDM2 induced p53 degradation hence promoting HCC cell apoptosis[44]. Moreover, studying several deregulated genes in HCC revealed the downregulation of AF070632 associated with various cellular processes such as oxidation-reduction, cofactor binding in catabolic processes[45]. The expression of the lnc-Dreh was evaluated and found to be downregulated in HCC tissues proving to be a tumor suppressor as it binds to vimentin, decreasing its expression and disrupting the normal cytoskeleton structure required for growth and invasion hence inhibiting metastasis in HCC or HBV related HCC patients[46]. LET is considered as one of the lncRNAs that function as a tumor suppressor as it inhibits HCC metastasis and invasion when overexpressed on chromosome 15. This occur through a complex positive feedback loop of HIF-1α/HDAC3/lncRNA-LET/NF90 that modulates HIF-1α response and gives the tumor suppressive effect[47].

An interesting mechanism of tumor suppression is encountered with PTENP1, a pseudogene of PTEN, where it induces autophagy of HCC cell inhibiting its survival. This is achieved as PTENP1 acts as an endogenous sponge for miR-17, miR-19b and miR-20a abolishing their inhibitory ability of PTEN hence inhibiting the PIK3T/AKT pathway and inducing pro-death autophagy[48]. CPS1-IT1 acts as tumor suppressor in HCC by inactivation of HIF-1α which results in decreased the expression of epithelia-mesenchymal transition (EMT) related proteins[49]. TUSC7 acts as tumor suppressor through the TUSC7-miR-10a-EphA4 axis leading to EMT suppression[50]. WT1-AS may function as a tumor suppressor in HCC by reversing the oncogenic effects of WT1. WT1-AS downregulates WT1 expression in HCC tumors and promotes apoptosis by binding to the promoter region of WT1[51]. AOC4P lncRNA also plays role as an HCC tumor suppressor by promoting vimentin degradation and suppressing the EMT[52]. SRHC is noncoding tumor suppressor that can inhibit HCC cell proliferation and promote its differentiation[53]. CASC2 inhibits cell proliferation, migration and invasion and promotes apoptosis by inactivation of the MAPK pathway this indicates that CASC2 may act as a tumour suppressor in HCC[54]. lncRNA-AK058003 can inhibit HCC proliferation and metastasis acting as a tumor suppressor by suppressing the SNCG in a HuR-dependent manner[55]. Suppression of treRNA by miR-190a significantly inhibited migration of HCC cells. This lead to reduced expression of Vimentin and SNAI1 (mesenchymal markers), as well as induced expression of E-cadherin and Claudin-1 (epithelial markers)[56].

Oncogenic lncRNA

HCG11 acts as an oncogenic lncRNA as it induces cell proliferation and invasion of HCC tumor cells, suppresses apoptotic indicators, such as: p21 and caspase-3, promotes anti-apoptotic factors including ERK, JNK and p38 *via* the regulation of IGF2BP1 protein and activation of MAPK pathway which consequently promoted HCC progression[57]. LOC90784 long non-coding RNA functions as an oncogene as its knockdown promotes Bax and represses CDK4 and Cyclin D protein expression which induces apoptosis and suppresses proliferation. Moreover, its downregulation inhibits cellular invasion and migration by repressing MMP2 and MMP9 expression in HCC cells[58]. Another lncRNA that is found to be upregulated in HCC cells is UBE2CP3; it induces the process of cell invasion and migration in HCC by increasing the expression of Snail-1 and N-cadherin while decreasing the expression of E-cadherin hence, promoting the EMT leading to tumor metastasis[59]. Linc00462 is reported to be upregulated in HCC tumors promoting tumor progression through the regulation of PI3K/AKT pathway, inducing cellular proliferation and migration[60]. TINCR lncRNA is found to be upregulated in HCC cells acting as an oncogene regulating differentiation, invasion and metastasis of tumor cells[61]. AB019562 is reported to be upregulated in HCC cells; its knockdown inhibits cell proliferation, arrests cell cycle at G0/G1 phase, suppresses cellular metastasis, induces apoptosis and activates caspase-3 activity[62]. LncARSR is a long non-coding RNA that regulates the sensitivity of the tumor cells to doxorubicin. Upregulation of lncARSR in HCC cells induces its resistance to doxorubicin, indicating poor prognosis through PTEN mRNA depletion and PI3k/Akt activation[63]. AWPPH serves as an oncogene as its overexpression in HCC promotes cell proliferation, migration and metastasis. This is achieved through YBX1 interaction and its mediated activation of downstream transcription factors such as SNAIL1 and PIK3CA and their pathways[64].

Prostate cancer-associated transcript (PCAT)-14 is overexpressed in HCC cells; it induces proliferation, migration and cell cycle arrest. Moreover, it regulates the expression of ATAD2 and Hedgehog pathway *via* its inhibitory effect on miR-372 that's achieved by methylation of miR-372's promoter region. Conversely, miR-372 eliminates PCAT-14's effects on proliferation and invasion of HCC cells[65]. Another crucial lncRNA is P73 antisense 1 T that is upregulated in HCC cell lines; its knockdown resulted in reduced cellular proliferation, invasion and downregulated HMGB1/RAGE signaling pathway that also correlates with dysregulated cell death and survival. miR-200a has a negative correlation with TP73-AS1 and HMGB1 expressions and studies revealed that both compete

for the same binding site on miR-200a concluding that TP73-AS1 could be oncogenic through miR-200a dependent HMGB1/RAGE regulation[66]. Linnc00441 is an oncogene that's upregulated in HCC promoting cell proliferation, tumor growth and downregulating RB1 tumor suppressor gene. Transcription factor (TCF)-4 and H3K27 acetylation were found to contribute to Linc00441 upregulation which in turn downregulated RB1 expression through possible enhancement of CpG islands methylation in its promoter region *via* DNMT3A Linc00441-dependent recruitment[67]. HOST2 is reported to be upregulated in various HCC cell lines and its silencing results in decreased cell proliferation, blocked cell cycle at G0/Gi phase to S phase and reduced cell migration and invasion. Therefore, its marked as an oncogene that supports cellular proliferation, metastasis and inhibits apoptosis in human HCC cells[68]. UC001kfo is an overexpressed lncRNA in HCC cells that positively regulates cellular proliferation and migration through the modulation of its target gene, α-SMA, in a positive manner. α-SMA also serves as a crucial marker for EMT which in turn regulates cellular migration and invasion *via* its effect on UC001kfo function[69]. LncBRM is an upregulated RNA in HCC and liver CSCs; it may contribute to the recurrence and self-renewal of the tumor cells. LncBRM modulates BRG1/BRM switch that in turn activates YAP1 signaling pathway *via* a KLF4-dependent manner giving a result that's positively correlated with tumor severity and self-renewal ability of the cells[70]. HOX Antisense lincRNA HOXA-AS2 is an oncogene that's overexpressed in HCC cells and its upregulation correlates with poor prognosis, increased proliferation and metastasis. The knockdown of HOXA-AS2 significantly suppressed proliferation, migration and promoted apoptosis[71]. CRNDE is reported to be overexpressed in HCC cells inducing cellular proliferation, migration and invasion. It is found that CRNDE inhibits the expression of miR-384 that's usually downregulated in HCC while enhancing the expression of NF-κB and p-AKT, suggesting that CRNDE-miR-384 axis is promising for treatment of HCC[72].

One of the antisense lncRNAs investigated is ZEB2-AS1; it is reported to be overexpressed in HCC cells especially Huh7. The study confirmed the that changes in ZEB2-AS1 levels fluctuates EMT-induced markers and the silencing of the RNA decreases cellular proliferation, viability and invasion[73]. Unigene56159 is an HBV related lncRNA that could be induced by HBV infection aiding in HCC development, migration and EMT. This happens because Unigene56159 directly binds to miR-140-5p acting as a competing endogenous RNA (ceRNA) for the miRNA, abolishing its inhibitory effect on its other target Slug hence, promoting EMT in HCC cells[74]. CCHE1 is an oncogene that's noted to be upregulated in HCC and its knockdown enhanced growth arrest and cell apoptosis. The knockdown also reduced tumorigenicity and inhibited ERK/MAPK pathway, with a function in cellular proliferation, differentiation and survival, hence the decreased cellular viability[75]. GPC3-AS1 is suggested to be upregulated in HCC due to possible increase in histone acetylation in the promoter region. GPC-AS1 upregulation recruits PCAF to the GPC3 body upregulating its transcription and hence promoting cellular proliferation and migration[76]. GIHCG is upregulated in HCC cells and significantly promotes tumor growth and metastasis through association with EZH2. This upregulates H3K27me3 and DNA methylation levels by DNMT1 of miR-200b/a/429 promoter hence epigenetically silencing miR-200b/a/429 expression in HCC cells[77]. LncCAMTA1 is overexpressed in HCC and is associated with poor prognosis, increased HCC proliferation and tumorigenesis. LncCAMTA1 is noted to inhibit CAMTA1 transcription by promoting a repressive chromatin structure of the gene and this relation is essential for lncCAMTA1 proliferative effect on HCC cells[78]. LncSox4 is upregulated in HCC and tumor initiating cells (TIC) that has proved essential for liver TIC self-renewal and tumor initiation. LncSox4 interacts with and recruits Stat3 to the Sox4 promoter enhancing Sox4 expression which is also required for TIC self-renewal[79]. Small nucleolar rna host gene (SNHG)3 shows high expression levels in HCC patients and is significantly correlated with tumor size, overall survival, poor prognosis and malignant status[80].

SNHG12 is overexpressed in HCC tissues promoting cellular proliferation and inhibiting apoptosis *via* functioning as a sponge for miR-199a/b-5p reducing their inhibitory action on MLK3, thus enhancing MLK3 expression and its downstream targets in the NF-κB pathway[81]. Furthermore, SNHG15 is reported to be upregulated in HCC tissues compared to normal control and positively correlates with histological grade, vein invasion and poor prognosis hence, is a promising clinical biomarker for HCC[82]. BAIAP2-AS1 is overexpressed in HCC tissues with positive correlation to proliferation and invasion processes through the regulation of MAPKAP1, E2F3and RAF1 which are downstream targets of several miRNAs that BAIAP2-AS1 functions as a sponge for[83]. Additionally, one of the studies investigating deregulated lncRNAs in HCC concluded that LINC01419 and AK021443 are upregulated in early HCC stages hence concluding their involvement in cell cycle progression and tumor initiation[45]. Another interesting study investigated the levels of lnc-HEIH in HBV related HCC cases and reported to be upregulated and involved in cell cycle modulation through the association with EZH2 and suppression of p16, p27 and p21 which mainly regulate the cell cycle arrest. All these actions facilitate its oncogenic properties and promote cellular proliferation and invasion in HCC tissues[84]. A study was done revealing that hypoxic conditions in HCC tissues could modulate lncRNAs expression such as linc-ROR that's overexpressed in hypoxic HCC tissues. Linc-ROR itself regulates cellular responses during hypoxic conditions by altering the expression of HIF-1α

and its downstream target PDK1 in a positive manner through inhibiting the expression of miR-145[85]. On a further note, linc-ROR modulate chemotherapy induced apoptosis and cell survival through p53 dependent signaling[86]. Lnc-β-Catm associates with β-catenin and the methyltransferase EZH2, leading to activation of Wnt–β-catenin signaling, resulting in cancer severity in people with HCC[87].

UC338 enhances cell proliferation as it directly interacts with BMI1 and suppresses p21 expression in HCC[88]. LINC01225 in HCC, stimulates proliferation and invasion through binding to protein, leading to increase the level of epidermal growth factor receptor (EGFR) as a consequence, thus fine tuning the EGFR/MAPK signaling pathway[89]. PlncRNA-1 act as an oncogene in HCC progression, by induction of EMT signaling[90]. BANCR overexpression is associated with HCC carcinogenesis. Downregulation of BANCR would decrease cell proliferation, enhance cell apoptosis, and impair cell invasion and migration[91]. LncRNA CARLo-5 enhances proliferation, migration and invasion of HCC and its upregulation was associated with poor prognosis of HCC patients[92]. MVIH enhanced cell growth and inhibited cell apoptosis of HCC through inhibition of miR-199a expression; si-MVIH inhibited HCC cell viability and enhanced cell apoptosis, but this effect was reversed by miR-199a inhibitor as miR-199a had a direct binding ability to MVIH RNA[93]. Overexpression of ZEB1-AS1 reduces protein levels of epithelial markers and increases protein levels of mesenchymal markers, proving that ZEB1-AS1 promotes EMT and its role in tumor progression[94]. lncRNA Sox2ot may play a tumor oncogenic role in HCC as the up-regulation of lncRNA Sox2ot promotes the metastasis ability of HCC cells[95]. ZFAS1 acts as tumor promoter in HCC progression by binding miR-150 and abolishing its tumor-suppressive function. miR-150 repressed HCC cell invasion by inhibiting ZEB1 and the matrix metalloproteinases MMP14 and MMP16. Conversely, ZFAS1 activated ZEB1, MMP14, and MMP16 expression, inhibiting these effects of miR-150. This mean that upregulation of ZFAS1 in HCC enhances tumor metastasis in a miR-150–dependent manner[96]. The lincRNA-UFC1, a target of microRNA 34a, promotes proliferation and decreases apoptosis in HCC cells through interaction with the mRNA stabilizing protein HuR to regulate levels of β-catenin in HCC cells[97]. Also, URHC may function as an oncogene that regulates cell proliferation and apoptosis through down-regulating of ZAK expression, and inactivation of the ERK/MAPK pathway by URHC-ZAK regulation[98]. LncRNA-PE promotes HCC cell invasion by upregulating ZEB1, the master inducer of EMT *via* inhibiting transcription of miR-200a/b[99]. ZNF667 serves as oncogene, promotes the cell proliferation, invasion and migration *via* interference with the expression of Bcl-2 and BAX[100]. The upcoming section discusses individual lncRNA that were found to be extensively studied.

Highly up-regulated in liver cancer

Highly up-regulated in liver cancer (HULC), present on chromosome 6, could be identified as the most over-expressed lncRNA in HCC with various mechanisms in promoting tumorigenesis and carries a huge perspective in RNA-targeted therapy[101]. High levels of HULC in plasma of HCC patients could be exploited as a noninvasive biomarker for HCC diagnosis[102]. HULC is reported to have high plasma levels in HCC patients and its combination with Linc00152 and AFP would achieve better diagnostic accuracy[103]. Overexpression of HULC is reported with upregulation in HEIH which resulted in increased proliferation, invasion and snail protein expression in HepG2 cells[104]. HULC is overexpressed in HCC as it promotes growth in cancer cells through enhancing ubiquitin-specific peptidase 22 (USP22) which reduces ubiquitin-mediated degradation of COX-2 protein hence stabilizing and upregulating COX-2 protein[105]. A similar mechanism of HULC in HCC is reported where it promotes the deubiquitination of sirt1 protein through inducing USP22 which promotes the levels of sirt1 proteins necessary for autophagy induction in chemotherapy treatment. Another set of miRNAs miR-6825-5p, miR-6845-5p and miR-6886-3p work opposite to HULC and suppress USP22 and they were found to be inhibited by HULC expression which modulate the sensitivity of cancer cells to chemotherapy[106]. HULC promotes tumor progression through acting as a ceRNA for miR-200a-3p inhibiting its function hence upregulating ZEB1 expression and inducing EMT, tumor proliferation and metastasis[107]. HULC contributes to HCC angiogenesis through interaction and downregulation of miR-107 which upregulates E2F1 and finally activates SPHK1 which aids in tumor angiogenesis (as illustrated in Figure 2)[108].

HULC is reported to be regulated by the Specificity protein (Sp) transcription factors that significantly modulate HCC proliferation and migration. The anti-diabetic drug, metformin downregulates Sp transcription factors and by default Sp-regulated genes such as HULC, giving metformin a strong evidence to be used in combination treatments for HCC patients[109]. One of the microRNAs that interact with HULC is miR-203 which shows a negative relation on HULC as its upregulation inhibits cell proliferation and induces cell apoptosis through downregulation of HULC and another oncogene, ADAM9. Also, the overexpression of HULC diminishes the antitumor effect of miR-203[110]. HULC contributes to the deregulation of lipid metabolism in HCC due to the upregulation of transcription factor PPARA which activates ACSL1. Moreover, an interaction with miR-9 is present as it is downregulated by HULC through promoter methylation and miR-9 itself inhibits PPARA expression as well. ACSL1 is capable of upregulating HULC *via* RXRA activation in hepatoma cells thus

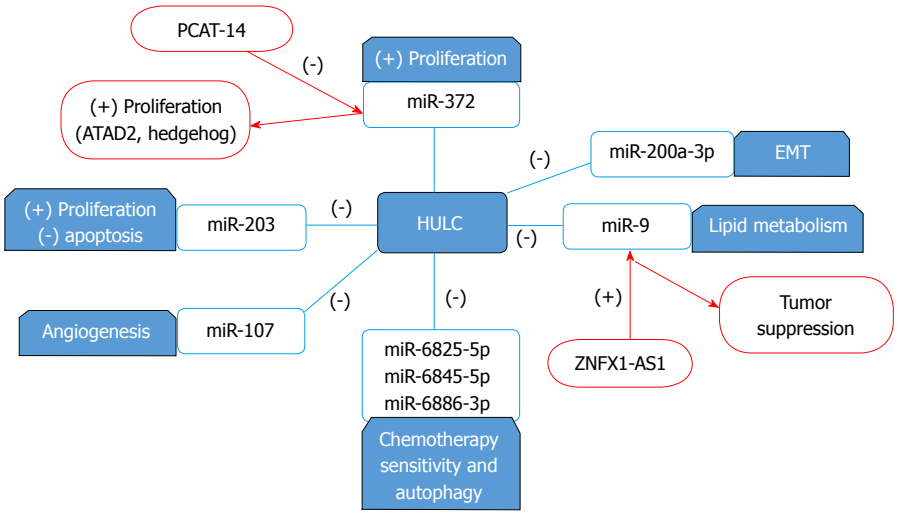

Figure 2 Highly up-regulated in liver cancer interactions with miRNA. Highly up-regulated in liver cancer (HULC) inhibits the actions of miR-6825-5p, miR 6845-5p and miR 6886-3p which modulates the cell sensitivity to chemotherapy and autophagy. Moreover, it inhibits the function of miR-200a-3p hence inducing EMT and metastasis. HULC downregulates miR-107 promoting angiogenesis and tumor proliferation. Overexpression of HULC diminishes miR-203 antitumor effect. While HULC contributes to lipid deregulation and tumor progression through the downregulation of miR-9, ZNFX1-AS1 upregulates miR-9 resulting in tumor suppression HULC and PCAT-14 promote proliferation through the inhibition of miR-372 however through different mechanisms. (-): Inhibits/Downregulates (+): Upregulates. EMT: Epithelia-mesenchymal transition; PCAT: Prostate cancer-associated transcript.

suggesting a positive feedback loop HULC/miR-9/PPARA/ACSL1/HULC is present in HCC cells[111]. HULC binds to YB-1 protein and promotes its phosphorylation through ERK and induces the translation of associated mRNAs hence increasing proliferation n HCC through promoting G1/S transition[112]. Another study suggests that HULC promotes hepatocarcinogenesis through the disruption of the circadian rhythm and upregulating circadian oscillator CLOCK in hepatoma cells[113]. Moreover, HULC is reported to be upregulated by HBx protein which results in increased cellular proliferation due to the inhibition of the tumor suppressor gene p18 on the mRNA and protein level[114]. An interesting study revealed the binding of CREB to the promoter region of HULC, activating its expression and function as an endogenous sponge to miR-372 which in turn results in further activation of HULC in an auto-regulatory loop of CREB/HULC/miR-372/PRKACB/CREB/HULC enhancing cellular proliferation (as illustrated in Figure 3)[115].

HOTAIR

HOTAIR is a lncRNA of 2158 bp length in chromosome 12 representing an antisense strand of homeobox C gene[116]. High levels of HOTAIR in HCC patients compared to normal control is significantly correlated with rapid proliferation, large tumor size and poor prognosis[117]. Another study reported that significantly high levels of HOTAIR is related with shorter recurrence-free survival period and its suppression reduced cell viability and induced apoptosis and chemotherapy sensitivity of HCC[118]. HOTAIR upregulation in HCC patients enhances tumor progression, migration and recurrence *via* the regulation of Wnt/β-catenin pathway[119]. Increased expression of HOTAIR in HCC patients show increased risk of recurrence and lymph node metastasis through the regulation of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor protein (VEGF)[120]. In a relevant study, HOTAIR overexpression in HCC patients is reported to upregulate autophagy related 3 (ATG3) and ATG7 expressions which induces cellular proliferation[121].

A study investigating the molecular mechanism of HOTAIR as an oncogene in HCC tissues revealed that it achieves its action through abolishing SETD2 expression and phosphorylation, inhibiting H3K36me3 binding to hMSH2 and SKP2 and modulating various epigenetic changes such as DNA damage repair, microsatellite instability and abnormal gene expression[122]. Interestingly, HOTAIR's overexpression in HCC patients unveiled several molecular relations with transcription factors and miRNAs, as it is partially regulated by the transcription factor FOXC1 which binds to HOTAIR promoter regions and activates its expression. Moreover, HOTAIR is found to downregulate miR-1 and vice versa due to the presence of miR-1 binding site on HOTAIR[123]. In an extensive study, HOTAIR was found to act as a binding site to both DDX5 and Mex3b in a competitive manner where the binding of Mex3b induces the ubiquitination of SUZ12 and derepression of PRC2 targets in G2 phase of the cell cycle promoting hepatocarcinogenesis in HBV patients[124]. A study on 52 paired HCC specimens proved HOTAIR as an oncogene in HCC as its knockdown activates P16(Ink4a) and P14(ARF) downstream signaling of miR-218, enhances miR-218 expression and suppresses Bmi-1 expression hence HOTAIR suppression inhibits cell viability and tumorigenesis[125]. Additionally, it was proposed that HOTAIR promotes HCC proliferation and migration

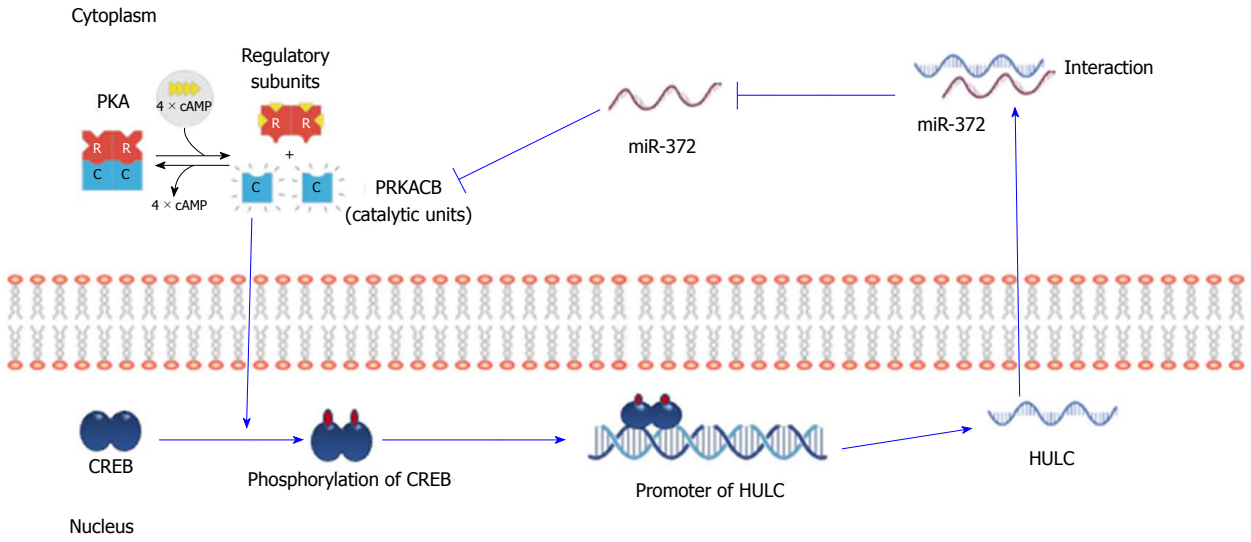

Figure 3 The auto-regulatory loop of highly up-regulated in liver cancer through inhibition miRNA-372 in hepatocellular carcinoma. miR-372 binding to HULC can repress the expression and activity of miR-372. Inhibition of miR-372 leads to a reduction in translational repression of its target gene PRKACB, which in turn induces phosphorylation of CREB, thereby increasing the amount of CREB that can bind to the proximal promoter of HULC to induce HULC expression. HULC: Highly up-regulated in liver cancer.

through the inhibition of RNA binding motif protein 38 (RBM38) which is crucial for biological processes and exhibits a negative relation with HOTAIR[126].

MALAT1

LncRNA MALAT1 is located on chromosome 11q13. It has been found to participate in the carcinogenesis of several types of tumors such as lung cancer, breast cancer, nasopharyngeal carcinoma, and so on[127-129]. MALAT1 acts as an oncogene since it suppressed miR-143-3p. MALAT1 functioned as a molecular sponge for miR-143-3p, and inhibited its function. This study results suggested that MALAT1 regulated ZEB1 expression and the proliferation and invasion through miR-143-3p in HCC cells[130]. Another study showed another mechanism for how MALAT-1 acts as oncogene. MALAT1 could promote tumor growth and metastasis by activating and upregulation of LTBP3 (latent transforming growth factor β-binding protein 3), which could also be up-regulated by hepatitis B virus X protein(HBx), which is known to be involved in HCC progression. These results introduce a vital mechanism of hepatocarcinogenesis through the signaling of HBx-MALAT1/LTBP3 axis[131]. In addition, lncRNA MALAT1 act as a molecular sponge of miR-146b-5p to down-regulate its expression in HCC. MiR-146b-5p acts as tumor suppressive by inhibiting the phosphorylation of Akt mediated by TRAF6. As they found that TNF receptor associated factor 6 (TRAF6) is a direct target of miR-146b-5p in HCC. This proves that miR-146b-5p inhibits tumor growth and metastasis of HCC by targeting TRAF6 mediated Akt phosphorylation[132].

lncRNA MALAT1 plays a crucial role in tumor progression as study showed that patients with high expression level of *malat-1* have high risk of tumor recurrence after liver transplant. Inhibition of MALAT1 would significantly decrease cell viability, motility, invasiveness, and increase the sensitivity to apoptosis[133]. MALAT1 is a proto-oncogene upregulated in HCC that acts by activation of Wnt pathway and promotion of the oncogenic splicing factor SRSF1. Induction of SRSF1 by MALAT1 modulates SRSF1 splicing targets, by modulating the alternative splicing of S6K1, promoting the production of antiapoptotic splicing isoforms and activating the mTOR pathway. Inhibition of SRSF1 expression or mTOR activity reduces the oncogenic properties of MALAT1, concluding that SRSF1 induction and mTOR activation may be vital for MALAT1-induced transformation[134]. Studies have indicated that the MALAT1 not only regulates tumorigenesis in HCC, but also controls cell cycle progression in hematopoietic cells. Researchers have found that MALAT1 plays different biological functions in hematopoietic cells, including the regulation of the cell cycle. MALAT1 was upregulated during liver regeneration. MALAT1 accelerated hepatocyte proliferation by enhancing cell cycle progression from the G1 to the S phase and inhibiting apoptosis *in vitro*. Moreover, MALAT1 was found to be regulated by p53 during liver regeneration, and hence, p53 may be a key regulator of MALAT1 activity. This study found that MALAT1 activated the Wnt/β-catenin pathway by inhibiting the expression of Axin1 and adenomatous polyposis coli (APC), causing expression promotion of cyclin D1. The results of this study suggest that MALAT1 is a crucial molecule for liver regeneration[135, 136]

MEG-3

Maternally expressed gene 3 (MEG3) is the first lncRNA to be found to have tumor suppressor function, which is expressed in many human normal tissues[137].

Frequently, the expression levels of miR-26a and MEG3 were found to be downregulated in HCC tissues compared to non-malignant tissues. Upregulation of miR-26a markedly decreased the proliferation, invasion and migration of HCC cells. They thought that DNA methyltransferase 3b (DNMT3B) was a direct target gene of miR-26a. Overexpressed miR-26a suppressed the expression level of DNMT3B. Inhibited expression of DNMT3B showed similar tumor suppressive effects induced by miR-26a upregulation, and resulted in the upregulation of MEG3. Moreover, the expression levels of DNMT3B were upregulated in the HCC tissues and it was inversely correlated with miR-26a and MEG3 in HCC tissues. These findings demonstrated a new mechanism miR-26a/DNMT3B/MEG3 axis to HCC development[138].

A study confirmed that the TF, NF-κB, could bind to the MEG3 promoter region and might affect the transcription of MEG3. MEG3 overexpression would competitively "sponge" miRNA-664, relieving the inhibition of miR-664 on the transcription and translation of alcohol dehydrogenase 4 (ADH4) playing a role in inhibiting HCC cell proliferation[139]. P53 is an important tumor suppressor which is incorporated in preventing cancer through taking place as a transcription factor to regulate its target genes. In this study, MEG3 acts as a tumor suppressor in hepatoma cells through interacting with p53 protein to activate p53-mediated transcriptional activity and affect the expression of partial p53 target genes[140-143]. MEG3 regulates HCC cell proliferation and apoptosis partially through p53 accumulation.

Down-regulation of UHRF1, a new identified oncogene which is required for DNA methylation, caused MEG3 overexpression in HCC cell lines, which could be reversed by the up-regulation of UHRF1. Furthermore, overexpression of MEG3 in HCC cells partially abolished the promotion of proliferation induced by UHRF1. These data suggest that MEG3 functioning as a potential biomarker in predicting the prognosis of HCC, was regulated by UHRF1 *via* recruiting DNMT1 and regulated p53 expression. This means that UHRF1/DNMT1/MEG3/p53 axis signaling might be involved in HCC development[144]. Another study showed that enforced expression of MEG3 in HCC cells significantly decreased cell growth, and induced apoptosis. This study found that miR-29, which can modulate DNMT 1 and 3, could regulate MEG3 expression. Overexpression of mir-29a increased expression of MEG3. These findings show that methylation-dependent tissue-specific regulation of the lncRNA MEG3 by miR-29a may contribute to HCC growth and highlight the inter-relationship between two classes of non-coding RNA, miRNAs and lncRNAs[145]. Due to resistance of HCC to chemotherapy, this gives novel cancer treatment methods an overwhelming significance. Epigenetic therapy in cancer is useful in reversing some of the cancer defects because of reversibility of the epigenetic alterations. A study found that the "Dendrosomal curcumin" DNC dependent overexpression of miR-29a and miR-185 can decrease the expression of DNMT1, 3A and 3B and hence increases the expression of MEG3. So, DNC may enhance DNA hypomethylation and re-expression of silenced tumor suppressor genes in HCC. These findings suggest that DNC could be an effective choice for epigenetic therapy of HCC[146].

Actin filament associated protein 1-antisense RNA 1

lncRNA actin filament associated protein 1-antisense RNA 1 (AFAP1-AS1) is significantly upregulated in HCC patients with association to poor prognosis. Its knockdown inhibits cellular proliferation, invasion and metastasis and increased apoptosis *in vivo*[147]. Moreover, AFAP1-AS1 reported to induce cellular proliferation and invasion and inhibit apoptosis by the upregulation of RhoA/Rac2 pathway in the cell cycle, MMP-9 and PCNA protein levels and apoptotic indexes cyclinD1 along with the downregulation of Bax[148].

ANRIL (CDKN2B-AS)

A study results showed that , lncRNA ANRIL could serve as a potential therapeutic target as lncRNA ANRIL expression is significantly increased in HCC tissues and strongly correlated with advanced clinical features, and the down-regulated expression of lncRNA ANRIL could inhibit HCC cells proliferation, migration and invasion[149]. Another study showed that its overexpression may be an important factor for HCC progression, through silencing of Kruppel-like factor 2 (KLF2) by binding with PRC2[150].

ATB

lncRNA-ATB enhanced the expression of ZEB1 and ZEB2 by competitive binding to the miR-200 family and hence, induced EMT and invasion. Furthermore, lncRNA-ATB stimulated organ colonization of disseminated tumor cells by binding IL-11 mRNA, autocrine induction of IL-11, and triggering STAT3 signalling. Generally, lncRNA-ATB promotes the invasion-metastases cascade[151].

Colon cancer associated transcript 1

Colon cancer associated transcript 1 (CCAT1) is found to be overexpressed in HCC patients and could promote cellular proliferation and migration in tumor cells *in vitro*[152]. Additionally, CCAT1's overexpression in HCC patients is regulated by c-myc expression that binds to the E-box of CCAT1 promoter region, increasing it expression and cellular proliferation and tumorigenesis effect[153]. In an interesting study, CCAT1 is found to exert its effect in HCC through acting as a molecular sponge for let-7, inhibiting its functions and restoring the levels of HMGA2 and c-myc hence enhancing cell proliferation and migration[154]. CCAT1 regulated CDK1 expression through functioning

as a ceRNA and inhibiting miR-490-3p in HCC hence promoting cellular proliferation and invasion[155].

CCAT2

CCAT2 is referred to as an oncogene as it is over-expressed in HCC, promoting cellular proliferation, invasion and metastasis while inhibiting apoptosis[156]. The upregulation of CCAT2 present in HCC patients induces tumor growth and invasion *via* enhancing EMT through regulating its factors, increasing vimentin and Snail2 expressions while decreasing E-cadherin expression hence accelerating HCC progression[157].

DANCR

DANCR is upregulated in HCC tumor tissue and plasma levels of patients with better diagnostic value and accuracy than alpha fetoprotein (AFP). High levels of DANCR promotes microvascular invasion and metastasis and its knockdown inhibits cellular proliferation and metastasis through inhibiting β-catenin signaling pathway[158]. DANCR is also found to increase the stem-like characters of HCC cells aiding tumorigenesis, proliferation and invasion *via* the interaction with CTNNB1 which reverses the inhibitory effect miR-214, miR-320a and miR-199a exerted on CTNNB1[159].

EGFR

It was found the EGFR-AS1 was up regulated in HCC, furthermore it was determined that it promotes HCC development by improving the ability of invasion and proliferation of HCC cell, Beside the fact that it affects the cell cycle. To sum up, EGFR-AS1 may act as a prognostic factor in HCC and , on the other hand, it was observed that the inhibition of EGFR-AS1 in HCC cells significantly impeded cells proliferation and invasion *in vivo* which might provide a potential possibility for targeted therapy for HCC[160]. Another study showed that lnc-epidermal growth factor receptor (EGFR) up regulation in tergs correlates positively with the tumor size and expression of EGFR/Foxp 3, but negatively with INF-y expression in patients and xenografted mouse models. Lnc-EGFR stimulates treg differentiation, suppression CTL activity and promotes HCC growth in an EGFR-dependent manner. Lnc-EGFR links an immunosuppressive state to cancer by promoting treg cell differentiation, thus offering a potential therapeutic target for HCC[161].

Five prime to XIST

Lnc-FTX (five prime to XIST) represses Wnt/β-catenin signaling activity by competitively sponging miR-374a and inhibits HCC cell EMT and invasion. Furthermore, lnc-FTX binds to the DNA replication licensing factor MCM2, impeding DNA replication and inhibiting proliferation in HCC cells. These findings suggest that lnc-FTX can function as a tumor suppressor in HCC by physically binding miR-374a and MCM2[162]. A study showed that the lncRNA Ftx exerted the oncogenic function *via* miR-545. As RIG-I is a downstream mediator of miR-545 function in HCC. Additionally, lncRNA Ftx or miR-545 caused upregulation of cell cycle regulator Cyclin D1 and downregulation of p27 by activation of PI3K/Akt. These findings suggest that lncRNA Ftx/miR-545 axis promotes HCC progression by activation of Akt signaling pathway by targeting RIG-I [163].

GAS5

A study showed that lncRNA GAS5 was down-regulated in the HCC, The aberrant expression of GAS5 was linked to a poor prognosis[164]. Another study showed that the expression level of GAS5 is significantly downregulated in HCC tissues compared to adjacent normal controls. GAS5 overexpression decreases hepatoma cell proliferation and invasion, promoted the apoptosis of hepatoma cells, downregulated the vimentin level and upregulated E-cadherin level in hepatoma cells[165]. Overexpression of GAS5 decreases the migration and invasion of HCC cells and high expression of miR-21 eliminates GAS5-mediated suppression of HCC cell migration and invasion.This mean that GAS5 acts as a tumor suppressor in HCCs through negative regulation of miR-21[166].

H19

lncRNA H19 is paternally imprinted and maternally expressed[167], and is located on chromosome 11p15.5, which is always linked to various diseases and higher incidence of tumorigenesis. The studies have proven that LncRNA H19 acts as an oncogene or a suppressor gene. lncRNA H19 promotes bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression[168]. In contrast, H19 acts as tumor suppressor, lncRNA H19 may suppress HCC metastasis through enhancing hnRNPU/PCAF/RNAPol II, and activating miR-200 family[169]. Moreover, another study showed that inhibition of lncRNA H19 and miR-675 may promote migration and invasion of HCC cells through activating the AKT/ GSK-3β/Cdc25A signaling pathway[170].

HOXA transcript at the distal TIP

The upregulation of HOXA transcript at the distal TIP (HOTTIP) in HCC could be a result of loss in miR-125b, upregulation in HOXA13, downregulation of miR-192 and miR-204 or several other interactions with ncRNAs[171]. HOTTIP is discovered to be significantly upregulated in HCC patients with positive relation to levels of HOXA13. This increased loop of HOTTIP/HOXA13 enhances cell proliferation and metastasis in HCC patients[172]. Furthermore, HOTTIP is shown to be the downstream target of miR-192 and miR-204 which in turn positively modulate glutaminase (GLS1). miR-192 and miR-204 negatively regulate HOTTIP hence inhibiting GLS1

mediated glutaminolysis which interrupts HCC proliferation and cell viability[173]. The knockdown of HOTTIP caused by overexpression of miR-125b inhibited HCC proliferation, migration and tumorigenicity along with some HOXA gene expression[174].

Nuclear enriched abundant transcript 1

Nuclear enriched abundant transcript 1 (NEAT1) was concluded to be upregulated in HCC patients accelerating cellular proliferation, metastasis and vaso-invasion. Additionally, the high levels were associated with the expression of MTDH, NM23 and MALAT1[175]. The expression of NEAT1 in HCC patients was significantly increased which promoted cell viability and inhibited apoptosis. The mechanism underlying this effect could be the inhibition of miR-129-5p by NEAT1 through regulating VCP/ $I_{\kappa}B$ which are the downstream targets of the miRNA[176]. The high levels of NEAT1 in HCC patients promote cellular proliferation and migration through binding to U2AF65 and regulating hnRNP A2 expression[177].

Long intergenic non-coding RNA 00152

Long intergenic non-coding RNA 00152 (linc00152) is significantly upregulated in HCC patients with fine diagnostic accuracy between HCC samples and control[103]. Linc00152 promotes cellular proliferation and tumor growth through the phosphorylation and activation of the mechanistic target of rapamycin(mTOR) signaling pathway by binding to EpCAM promoter and upregulating its expression[178].

P21 (CDK-interacting protein 1)

lincRNA-p21 is one of the lncRNAs, which contains longer than 200 nucleotides. It is initially identified as a direct transcriptional target of p53 and acts as a translational suppressor by direct binding to the target mRNA[179]. A study showed that overexpression of lincRNA-p21 led to downregulation of Notch signal-related proteins Hes-1 and NICD and the expression of EMT-related proteins E-cadherin and Claudin-1 was increased, and the expression of proteins N-cadherin and snail was reduced causing inhibition of invasion[180]. Another study showed that lincRNA-p21 negatively regulated miR-9 expression level, and miR-9 was upregulated in human HCC tissues and cells. The knock down of miR-9 suppressed HCC migration and invasion *in vitro*, since E-cadherin was a direct target of miR-9, the expression level of E-cadherin was found to be regulated by lincRNA-p21 and miR-9. The data suggested that lincRNA-p21 inhibits migration and invasion of HCC by regulating miR-9-mediated E-cadherin cascade signaling pathway[181].

PCAT-1

A study showed that overexpression of PCAT-1 led to increase cell proliferation and migration, and inhibit apoptosis. Findings suggest that PCAT-1 acts as an tumor promotor in HCC and silencing PCAT-1 may be a potential novel therapeutic strategy for HCC[182]. Another study suggests that the increased expression of PCAT-1 was correlated with advanced clinical parameters and poor overall survival of HCC patients, indicating that PCAT-1 up-regulation may serve as a novel biomarker of poor prognosis in HCC patients[183].

PVT1 (Pvt1 oncogene)

A study on serum lncRNA for diagnosis of HCC concluded that PVT1 and uc002mbe.2 in combination served as an accurate diagnostic biomarker that can be associated with tumor size and BCLC value with a predictive ability better than AFP[184]. Human PVT1 is upregulated in HCC tissues and is associated with poor prognosis, enhanced cellular proliferation and migration which is achieved through binding and stabilizing NOP2, a cell cycle gene, all under the control of TGF-β1[185]. As an oncogene, PVT1 enhances tumor growth and migration through functioning as an endogenous sponge for miR-186-5p abolishing its inhibitory action on YAP1, restoring its upregulation in HCC hence promoting tumorigenesis[186].

SNHG1

SNHG1 overexpression in HCC is correlated with large tumor size, poor differentiation and poor prognosis. SNHG1 promoted cell proliferation and inhibited cell apoptosis through the inhibitory action on p53 and p53 target genes[187]. SNHG1 is found to be significantly upregulated in HCC aiding in cellular proliferation, invasion a migration in HepG2 cells which is achieved through the possible inhibition of miR-195 proving to be one of its downstream targets[188].

SNHG6

SNHG6 is reported to be among the dysregulated genes in HCC and its expression is related to co-expressed genes on 8q involved in structural integrity of ribosome and translation[189]. Among various SNHG6 transcripts, SNHG6-003 only showed to be oncogenic enhancing cell proliferation and drug resistance. SNHG6-003 functions as a ceRNA, acting as a sponge for miR-26a and miR-26b inhibiting their functions hence, modulating the levels of transforming growth factor-β-activated kinase 1(TAK1). Additionally, SNHG6-0003 shows a significant co-expression pattern with TAK1 as both are upregulated in various kinds of cancers[190].

SNHG20

SNHG20 is significantly upregulated in HCC patients and positively correlated to tumor size, cellular proliferation, invasion and poor prognosis. Its knockdown results in remarkable inhibition of cellular proliferation and

invasion in SK-Hep-1 cells[191]. The consequence of high levels of SNHG20 on HCC cells occurs due to the binding of SNHG20 to the enhancer of zeste homolog 2 (EZH2) that regulates E-cadherin expression along with SNHG20's ability to modulate ZEB1, ZEB2, N-cadherin and vimentin in a positive relation[192].

Sprouty 4-intron transcript 1

Sprouty 4-intron transcript (SPRY4-IT) was significantly increased in HCC cells, and overexpression of SPRY4-IT1 can promote cell proliferation and increase the cell invasion ability *via* promoting EMT progression and interacting with Estrogen Related Receptor α (ERR-α)[193]. Moreover, it can suppress Twist1 and vimentin expression and increase E-cadherin expression[194].

The taurine upregulated gene 1

The taurine upregulated gene 1 (TUG1) many studies have found that TUG1 plays vital roles in many human cancers, such as HCC, osteosarcoma, glioma and bladder cancer[195-198]. A study showd that TUG1 was up-regulated in HCC tissues than that in corresponding non-tumor tissues. The findings suggest that TUG1 upregulation was induced by nuclear transcription factor SP1 and TUG1 can regulate KLF2 expression in the epigenetic level by binding to PRC2[195].

TCF7

LncRNA TCF7, as a T cell factor, is well known for its function in T lymphocyte development and multi-potential hematopoietic cell self-renewal[199]. A study found that IL-6 transcriptionally activated the expression of lncTCF7 in HCC cells by activating STAT3, a transcription activator which binds to promoter regions of lncTCF7. Moreover, STAT3 knock down reduced lncTCF7 expression. Importantly, RNA interference-based attenuation of lncTCF7 prevented IL-6-induced EMT and cell invasion. These findings highlights the existence of an aberrant IL-6/STAT3/lncTCF7 signaling axis that leads to HCC aggressiveness through EMT induction[200]. Another study showed that lncTCF7 can promote liver CSC self-renewal and tumor progression by activation of Wnt signaling through recruiting the SWI/SNF complex to the TCF7 promoter[201].

UCA1

A study indicated that UCA1 expression may potentially promote the EMT in HCC, by upregulating the expression of Snail2. MiR-203 was a target of UCA1 and UCA1 upregulated Snail2 by negatively regulating miR-203. This indicated a novel UCA1/miR203/Snail2 signaling pathway regulatory network in HCC[202]. A study discovered that UCA1, upregulated by HBx, displayed a crucial role in G1/S transition in both hepatic and hepatoma cells. Moreover, a positive correlation between the expression of UCA1 and HBx and a negative correlation between UCA1 and p27 were observed in HCC specimens, suggesting the significance of UCA1 in HBx-mediated hepatocarcinogenesis. Also, they demonstrated that UCA1 repressed p27 expression at least partly through associating with chromatin-modifying complexes PRC2 component EZH2 in HCC cells. So, they provided a significant mechanism of hepatocarcinogenesis through the signaling of HBx-UCA1/EZH2-p27Kip1 axis[203]. UCA1 acts as an oncogene by promoting malignant progression of human HCC, UCA1 acts as an endogenous sponge to reduce miR-216b expression, resulting in derepression of FGFR1 expression and activation of FGFR1/ERK signaling pathway in HCC, providing a novel signaling pathway UCA1-miR-216b-FGFR1-ERK in HCC[204].

XIST

During previous studies, it is concluded that XIST and its activator JPX are downregulated in HCC patients and their levels serve as a biomarker of poor prognosis[205]. XIST modulates the regulation of miR-181a on PTEN as siXIST enhances cellular proliferation and migration in HCC patients *via* binding to miR-181a at multiple binding sites[206]. XIST functions as a tumor suppressor through interacting with miR-92b and inhibiting its miR-92b/Smad7 axis hence inhibiting its oncogenic effect on HCC cells[207]. On the contrary to previous studies, XIST was reported upregulated in HCC patients associated with poor prognosis and enhanced tumorigenesis. This is achieved through activation of AKT signaling *via* modulation of miR-139-5p/PDK1 axis as XIST has a reciprocal repression with miR-139-5p[208].

INTERPLAY OF lncRNAs WITH miRNAs

Emerging evidences proved an intensive interaction between lncRNAs and microRNAs as summarized in Table 1.

Emerging studies have demonstrated the manipulation of lncRNAs expression in different models. Mice models as well as *in vivo* and *in vitro* models are summarized in Table 2.

CONCLUSION

In conclusion, despite the various unanswered questions about ncRNAs, specifically lncRNAs; their role in HCC has been exhaustively researched. This research reached to a conclusion that lncRNA act as an important regulator in various biological processes rendering its dysregulation a factor in various diseases, including HCC. HCC-related lncRNAs play a crucial role in tumor initiation, progression and treatment. Their functions could be exploited for diagnosis as well as treatment giving them an exceptional potential in targeted therapy of HCC. During the years, various lncRNAs have been studied and several molecular mechanisms have been understood, however, further

Table 1 Interplay between long non-coding RNAs and miRNAs

lncRNA	miRNA	Effect/ relationship	Pathway
HULC	miR-6825-5p, miR-6845-5p, miR-6886-3p	Inhibitory	Increasing chemotherapy sensitivity through USP22 and sirt1 levels controlling autophagy
	miR-200a-3p	Inhibitory	Increasing ZEB1 expression, EMT and metastasis
	miR-107	Inhibitory	Upregulates E2F1, activates SPHK1 and angiogenesis
	miR-203	Inhibitory	Inhibits cellular proliferation and induces apoptosis
	miR-9 (ZNFX1-AS1)	Inhibitory	Positive feedback loop of HULC/miR-9/PPARA/ACSL1/HULC for lipid metabolism regulation
	miR-372 (PCAT-14)	Inhibitory	Auto-regulatory loop of CREB/HULC/miR-372/PRKACB/CREB/HULC enhancing cellular proliferation
XIST	miR-181a	Inhibitory	Binds to several binding sites on miR-181a modulating its function
	miR-92b	Inhibitory	Suppresses miR-92b/Smad7 oncogenic axis
	miR-139-5p	Inhibitory	Modulate the miR-139-5p/PDK1/AKT axis
CCAT1	Let-7	Inhibitory	Restoring HMGA2 and c-myc levels and enhancing cell proliferation
	miR-490-3p	Inhibitory	Regulate CDK1 and HCC proliferation
AF113014	miR-20a	Interaction	Regulate Erg2 expression
CRNDE	miR-384	Inhibitory	Enhances expression of NF-κB and p-AKT
DANCR	miR-214, miR-320a, miR-199a	Cancels its effect	Cancels the inhibitory effect they exert on CTNNB1 hence enhancing tumorigenesis
FTX	miR-374a	inhibitory	represses Wnt/β-catenin signaling pathway
	miR-545	interaction	Tumor promotor through activation of PI3K/Akt
GAS5	miR-21	inhibitory	downregulated the vimentin level and the upregulated E-cadherin level
GIHCG	miR-200a/b/429	Inhibitory	Associates with EZH2 and silences miRNA
H19	miR-200	activation	tumor-suppressive by mediating hnRNPU/PCAF/RNAPol II
	miR-675	inhibitory	Activation of the AKT/ GSK-3β/Cdc25A signaling pathway
HOTAIR	miR-1	Inhibitory	
	miR-218	Inhibitory	Inhibits P16(lnk4a) and P14(ARF) expression, activates Bmi-1 and tumorigenesis
HOTTIP	miR-192, miR-204	Negative	Inhibit HOTTIP expression, GLS1 and HCC proliferation
	miR-125b	Negative	Inhibits HOTTIP expression and HOXA genes
Linc00052	miR-128, miR-485-3p	Complementary base pairing	Modulating NTRK3 expression
Linc-ROR	miR-145	Inhibitory	Enhances HIF-1α/PDK1 expression
MALAT-1	miR-143-3p	inhibitory	regulated ZEB1 expression
	miR-146b-5p	inhibitory	targeting TRAF6 mediated Akt phosphorylation
MEG-3	miR-29a	interaction	modulate DNMT 1 and 3
	miR-26a	Negative	Modulates miR-26a/DNMT3B/MEG3
NEAT1	miR-129-5p	Inhibitory	Inhibits the miRNA through regulating VCP/IκB, its downstream pathway
lincRNA-p21	miR-9	inhibitory	Tumor suppressive
PCAT-14	miR-372 (HULC)	Inhibitory	Regulates ATAD2 and hedgehog pathway
PTENP1	miR-17, miR-19b, miR-20a	Inhibitory	Inhibiting PIK3T/AKT pathway and inducing autophagy
PVT1	miR-186-5p	Inhibitory	Restores YAP1 expression levels
SNHG1	miR-195	Inhibitory	
SNHG6	miR-26a, miR-26b	Inhibitory	Modulate TAK1 expression
SNHG12	miR-199a/b-5p	Inhibitory	ceRNA, Enhancing MLK3 expression and NF-κB pathway
TP73-AS1	miR-200a	Inhibitory	miR-200a dependent HMGB1/RAGE regulation
UCA1	miR-203	interaction	UCA1/miR203/Snail2 signaling pathway regulatory network
	miR-216b	inhibitory	activation of FGFR1/ERK signaling pathway
Unigene56159	miR-140-5p	Inhibitory	ceRNA, Restoring slug expression and EMT
ZNFX1-AS1	miR-9	Positive	Tumor suppression

HOTTIP: HOXA transcript at the distal TIP; lncRNAs: Long non-coding RNAs; PCAT: Prostate cancer-associated transcript; HULC: Highly up-regulated in liver cancer; XIST: X-inactive specific transcript; TCF7: Transcription factor 4; TUG1: Taurine upregulated gene 1; EGFR: Epidermal growth factor receptor; CCAT1: Colon cancer associated transcript 1; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; UCA1: Urothelial carcinoma-associated 1; SPRY4-IT: Sprouty 4-intron transcript; SNHG: Small nucleolar rna host gene.

researches need to be done to enlighten more research calibers about lncRNAs giving it the distinction it deserves to intrigue the biopharmaceutical field. During this review, certain lncRNAs such as HULC, HOTAIR, MALAT1 and MEG3 showed extensive roles related to HCC tumorigenesis. Moreover, in this review, the focus was showing the interplay between lncRNAs and other ncRNAs contributing to HCC progression or treatment. Complex pathways have been discovered that included lncRNAs affecting miRNAs and proteins aiding or suppressing HCC. These pathways are good candidates for targeted therapy in HCC patients. Moreover, the serum and tissue levels of lncRNAs serve as an accurate diagnostic marker for HCC patients

Table 2 Different models used in studies

lncRNA	Study	Model	Ref.
HULC	Molecular mechanism of HEIH and HULC in the proliferation and invasion of hepatoma cells.	*In vitro*	[104]
	lncRNA HULC promotes the growth of hepatocellular carcinoma cells *via* stabilizing COX-2 protein	*In vitro*	[105]
	lncRNA HULC triggers autophagy *via* stabilizing Sirt1 and attenuates the chemosensitivity of HCC cells.	*In vitro* and *in vivo* (nude mice)	[106]
	lncRNA HULC enhances epithelial-mesenchymal transition to promote tumorigenesis and metastasis of hepatocellular carcinoma *via* the miR-200a-3p/ZEB1 signaling pathway.	*In vitro* and *in vivo* (nude mice)	[107]
	lncRNA HULC promotes tumor angiogenesis in liver cancer by up-regulating sphingosine kinase 1 (SPHK1)	*In vitro* and *in vivo* (nude mice)	[108]
	Specificity protein (Sp) transcription factors and metformin regulate expression of the long non-coding RNA HULC	*In vitro*	[109]
	miR-203 suppresses the proliferation and metastasis of hepatocellular carcinoma by targeting oncogene ADAM9 and oncogenic long non-coding RNA HULC.	*In vitro*	[110]
	Long noncoding RNA HULC modulates abnormal lipid metabolism in hepatoma cells through an miR-9-mediated RXRA signaling pathway.	*In vitro* and *in vivo* (nude mice)	[111]
	Long noncoding RNA HULC modulates the phosphorylation of YB-1 through serving as a scaffold of extracellular signal-regulated kinase and YB-1 to enhance hepatocarcinogenesis.	*In vitro*	[112]
HOTAIR	Clinical significance of the expression of long non-coding RNA HOTAIR in primary hepatocellular carcinoma.	*In vitro*	[117]
	Overexpression of long non-coding RNA HOTAIR predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation.	*In vitro*	[118]
	Long non-coding RNA HOTAIR is a marker for hepatocellular carcinoma progression and tumor recurrence.	*In vitro* and *in vivo* (nude mice)	[119]
	Large intervening non-coding RNA HOTAIR is associated with hepatocellular carcinoma progression	*In vitro*	[120]
	The long noncoding RNA HOTAIR activates autophagy by upregulating ATG3 and ATG7 in hepatocellular carcinoma	*In vitro*	[121]
	LncRNA HOTAIR promotes human liver cancer stem cell malignant growth through downregulation of SETD2	*In vitro* and *in vivo* (mice)	[122]
	HOTAIR, a long non-coding RNA driver of malignancy whose expression is activated by FOXC1, negatively regulates miRNA-1 in hepatocellular carcinoma.	*In vitro* and *in vivo* (mice)	[123]
	Hotair mediates hepatocarcinogenesis through suppressing miRNA-218 expression and activating P14 and P16 signaling.	*In vitro* and *in vivo* (mice)	[125]
	Long non-coding RNA HOTAIR promotes cell migration and invasion *via* down-regulation of RNA binding motif protein 38 in hepatocellular carcinoma cells.	*In vitro*	[126]
SNHG	SNHG3 correlates with malignant status and poor prognosis in hepatocellular carcinoma.	*In vitro*	[80]
	Long non-coding RNA small nucleolar RNA host gene 12 (SNHG12) promotes tumorigenesis and metastasis by targeting miR-199a/b-5p in hepatocellular carcinoma.	*In vitro*	[81]
	Long noncoding RNA SNHG15, a potential prognostic biomarker for hepatocellular carcinoma	*In vitro*	[82]
	Long noncoding RNA SNHG1 predicts a poor prognosis and promotes hepatocellular carcinoma tumorigenesis	*In vitro*	[187]
	Expression of Long Non-Coding RNA (lncRNA) Small Nucleolar RNA Host Gene 1 (SNHG1) Exacerbates Hepatocellular Carcinoma Through Suppressing miR-195	*In vitro*	[188]
	The long non-coding RNA, SNHG6-003, functions as a competing endogenous RNA to promote the progression of hepatocellular carcinoma.	*In vitro*	[190]
	Up-regulation of LncRNA SNHG20 Predicts Poor Prognosis in Hepatocellular Carcinoma.	*In vitro*	[191]
	Long non-coding RNA SNHG20 predicts a poor prognosis for HCC and promotes cell invasion by regulating the epithelial-to-mesenchymal transition.	*In vitro*	[192]
MALAT1	Long Non-Coding RNA MALAT1 Regulates ZEB1 Expression by Sponging miR-143-3p and Promotes Hepatocellular Carcinoma progression.	*In vitro*	[130]
	HBx-related long non-coding RNA MALAT1 promotes cell metastasis *via* up-regulating LTBP3 in hepatocellular carcinoma.	*In vitro* and *in vivo* (Male BALB/C nude mice)	[131]
	Down-regulation of miR-146b-5p by long noncoding RNA MALAT1 in hepatocellular carcinoma promotes cancer growth and metastasis.	*In vitro* and *in vivo* (nude mice)	[132]
	Long non-coding RNA MALAT-1 overexpression predicts tumor recurrence of hepatocellular carcinoma after liver transplantation.	*In vitro*	[133]
MEG3	Armored long non-coding RNA MEG3 targeting EGFR based on recombinant MS2 bacteriophage virus-like particles against hepatocellular carcinoma.	*In vitro* and *in vivo* (BALB/c nude mice)	[137]
	MicroRNA-26a inhibits proliferation and metastasis of human hepatocellular carcinoma by regulating DNMT3B-MEG3 axis.	*In vitro*	[138]

	Overexpression of Long Non-Coding RNA MEG3 Inhibits Proliferation of Hepatocellular Carcinoma Huh7 Cells *via* Negative Modulation of miRNA-664.	*In vitro*	[139]
	Long Noncoding RNA MEG3 Interacts with p53 Protein and Regulates Partial p53 Target Genes in Hepatoma Cells	*In vitro*	[141]
	The aberrant expression of MEG3 regulated by UHRF1 predicts the prognosis of hepatocellular carcinoma.	*In vitro*	[144]
	microRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer.	*In vitro*	[145]
AFAP1-AS1	Long noncoding RNA AFAP1-AS1 indicates a poor prognosis of hepatocellular carcinoma and promotes cell proliferation and invasion *via* upregulation of the RhoA/Rac2 signaling.	*In vitro* and *in vivo* (mice)	[148]
	Critical role for the long non-coding RNA AFAP1-AS1 in the proliferation and metastasis of hepatocellular carcinoma.	*In vitro* and *in vivo* (mice)	[147]
ANRIL	High expression of long non-coding RNA ANRIL is associated with poor prognosis in hepatocellular carcinoma.	*In vitro*	[149]
ATB	A long noncoding RNA activated by TGF-beta promotes the invasion metastasis cascade in hepatocellularcarcinoma.	*In vitro* and *in vivo* (nude mice)	[151]
CCAT1	Aberrant Expression of CCAT1 Regulated by c-Myc Predicts the Prognosis of Hepatocellular Carcinoma.	*In vitro*	[153]
	CCAT1 promotes hepatocellular carcinoma cell proliferation and invasion.	*In vitro*	[152]
	Long noncoding RNA CCAT1 promotes hepatocellular carcinoma progression by functioning as let-7 sponge.	*In vitro*	[154]
CCAT2	Long non-coding RNA CCAT2 is associated with poor prognosis in hepatocellular carcinoma and promotes tumor metastasis by regulating Snail2-mediated epithelial-mesenchymal transition.	*In vitro*	[157]
	Long non-coding RNA CCAT2 functions as an oncogene in hepatocellular carcinoma, regulating cellular proliferation, migration and apoptosis	*In vitro*	[156]
DANCR	Long noncoding RNA DANCR increases stemness features of hepatocellular carcinoma by derepression of CTNNB1.	*In vitro* and *in vivo* (mice)	[159]
	DANCR Acts as a Diagnostic Biomarker and Promotes Tumor Growth and Metastasis in Hepatocellular Carcinoma	*In vitro* and *in vivo* (mice)	[158]
EGFR	The long noncoding RNA, EGFR-AS1, a target of GHR, increases the expression of EGFR in hepatocellular carcinoma.	*In vitro* and *in vivo*	[160]
FTX	Ftx non coding RNA-derived miR-545 promotes cell proliferation by targeting RIG-I in hepatocellular carcinoma.	*In vitro* and *in vivo* (nude mice)	[163]
GAS-5	Decreased expression of long non-coding RNA GAS5 indicates a poor prognosis and promotes cell proliferation and invasion in hepatocellular carcinoma by regulating vimentin.	*In vitro*	[165]
	Down-regulation of long non-coding RNA GAS5 is associated with the prognosis of hepatocellular carcinoma.	*In vitro*	[164]
H19	Epigenetic activation of the MiR-200 family contributes to H19-mediated metastasis suppression in hepatocellular carcinoma.	*In vitro* and *in vivo* (Nude mice)	[169]
HOTTIP	Long non-coding RNA HOTTIP is frequently up-regulated in hepatocellular carcinoma and is targeted by tumour suppressive miR-125b.	*In vivo* (mice)	[174]
	MiRNA-192 [corrected] and miRNA-204 Directly Suppress lncRNA HOTTIP and Interrupt GLS1-Mediated Glutaminolysis in Hepatocellular Carcinoma.	*In vitro* and *in vivo* (mice)	[173]
	Long noncoding RNA HOTTIP/HOXA13 expression is associated with disease progression and predicts outcome in hepatocellular carcinoma patients.	*In vitro*	[172]
Linc00152	LINC00152 promotes proliferation in hepatocellular carcinoma by targeting EpCAM *via* the mTOR signaling pathway.	*In vitro* and *in vivo* (mice)	[178]
NEAT1	Long non-coding RNA NEAT1 promotes hepatocellular carcinoma cell proliferation through the regulation of miR-129-5p-VCP-IκB.	*In vitro* and *in vivo* (mice)	[176]
	Long noncoding RNA NEAT1 promotes cell proliferation and invasion by regulating hnRNP A2 expression in hepatocellular carcinoma cells.	*In vitro* and *in vivo* (mice)	[177]
P21	lincRNA-p21 inhibits invasion and metastasis of hepatocellular carcinoma through Notch signaling-induced epithelial-mesenchymal transition.	*In vitro* and *in vivo* (nude mice)	[180]
	LincRNA-p21 inhibits invasion and metastasis of hepatocellular carcinoma through miR-9/E-cadherin cascade signaling pathway molecular mechanism.	*In vitro*	[181]
PCAT1	Upregulation of long non coding RNA PCAT-1 contributes to cell proliferation, migration and apoptosis in hepatocellular carcinoma.	*In vitro*	[182]
	Prognostic significance of long non-coding RNA PCAT-1 expression in human hepatocellular carcinoma.	*In vitro*	[183]
PRAL	Systemic genome screening identifies the outcome associated focal loss of long noncoding RNA PRAL in hepatocellular carcinoma	*In vitro* and *in vivo* (mice)	[44,209]
PVT1	Long non-coding RNA PVT1 serves as a competing endogenous RNA for miR-186-5p to promote the tumorigenesis and metastasis of hepatocellular carcinoma.	*In vitro*	[186]
	Oncofetal long noncoding RNA PVT1 promotes proliferation and stem cell-like property of hepatocellular carcinoma cells by stabilizing NOP2.	*In vitro* and *in vivo* (mice)	[185]

SPRY4-IT1	Overexpression of the long non-coding RNA SPRY4-IT1 promotes tumor cell proliferation and invasion by activating EZH2 in hepatocellular carcinoma	*In vitro*	[210]
TCF7	Long noncoding RNA lncTCF7, induced by IL-6/STAT3 transactivation, promotes hepatocellular carcinoma aggressiveness through epithelial-mesenchymal transition	*In vitro*	[200]
	The long noncoding RNA lncTCF7 promotes self-renewal of human liver cancer stem cells through activation of Wnt signaling.	*In vitro*	[201]
TUG1	Long non-coding RNA TUG1 is up-regulated in hepatocellular carcinoma and promotes cell growth and apoptosis by epigenetically silencing of KLF2.	*In vitro*	[195]
UCA1	HBx-upregulated lncRNA UCA1 promotes cell growth and tumorigenesis by recruiting EZH2 and repressing p27Kip1/CDK2 signaling	*In vitro*	[203]
	Upregulated lncRNA-UCA1 contributes to progression of hepatocellular carcinoma through inhibition of miR-216b and activation of FGFR1/ERK signaling pathway.	*In vitro* and *in vivo* (Mice)	[204]
XIST	Long non-coding RNA XIST regulates PTEN expression by sponging miR-181a and promotes hepatocellular carcinoma progression.	*In vitro*	[206]
	MicroRNA-92b promotes hepatocellular carcinoma progression by targeting Smad7 and is mediated by long non-coding RNA XIST	*In vitro* and *in vivo* (mice)	[207]
	Long non-coding RNA XIST promotes cell growth by regulating miR-139-5p/PDK1/AKT axis in hepatocellular carcinoma.	*In vitro* and *in vivo* (mice)	[208]

HOTTIP: HOXA transcript at the distal TIP; lncRNAs: Long non-coding RNAs; PCAT: Prostate cancer-associated transcript; HULC: Highly up-regulated in liver cancer; XIST: X-inactive specific transcript; TCF7: Transcription factor 4; TUG1: Taurine upregulated gene 1; EGFR: Epidermal growth factor receptor; CCAT1: Colon cancer associated transcript 1; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; UCA1: Urothelial carcinoma-associated 1; SPRY4-IT: Sprouty 4-intron transcript; SNHG: Small nucleolar rna host gene.

that could overcome AFP's accuracy in the future. Finally, all the work that is being done adds to the functional benefits of lncRNAs and the beneficial roles in diagnosis, treatment and prognosis of HCC.

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ORIGINAL ARTICLE

Basic Study

Hsa-miR-202-3p, up-regulated in type 1 gastric neuroendocrine neoplasms, may target *DUSP1*

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Abstract

AIM

To detect abnormal microRNA (miRNA) expression in type 1 gastric neuroendocrine neoplasms (g-NENs) and find potential target genes.

METHODS

Tumour tissues from patients with type 1 g-NENs were

used as experimental samples, and gastric mucosal tissues from the same patients obtained during gastroscopy review after several months were used as control samples. miRNA expression was examined with Agilent human miRNA chips and validated *via* RT-PCR. Three types of target gene prediction software (TargetScan, PITA, and microRNAorg) were used to predict potential target genes of the differentially expressed miRNAs, and a dual-luciferase reporter assay system was used for verification.

RESULTS

Six miRNAs were significantly upregulated or downregulated in the tumours compared to the control samples. Among them, miR-202-3p was extraordinarily upregulated. RT-PCR of seven sample sets confirmed that miR-202-3p was upregulated in tumour tissues. In total, 215 target genes were predicted to be associated with miR-202-3p. Among them, dual-specificity phosphatase 1 (DUSP1) was reported to be closely related to tumour occurrence and development. The dual-luciferase reporter assay showed that miR-202-3p directly regulated *DUSP1* in 293T cells.

CONCLUSION

miR-202-3p is upregulated in type 1 g-NEN lesions and might play important roles in the pathogenesis of type 1 g-NENs by targeting *DUSP1*.

Key words: MicroRNA; Type 1 g-NEN; Neuroendocrine neoplasm; Recurrence

Core tip: In this study, we have innovatively used chip technology to study microRNAs in type 1 gastric neuroendocrine neoplasms. We found miR-202-3p was extraordinarily upregulated in the tumours compared to the control samples. Interestingly, although miR-202 belongs to let-7, a famous cancer-suppressing family, some studies have reported its oncogenic potential in some tumours. Then, we found that *DUSP1* could be a target gene of miR-202-3p and was closely related to tumour occurrence and development. Finally, we successfully showed that the miR-202-3p directly regulated *DUSP1* in tool cells.

Dou D, Shi YF, Liu Q, Luo J, Liu JX, Liu M, Liu YY, Li YL, Qiu XD, Tan HY. Hsa-miR-202-3p, up-regulated in type 1 gastric neuroendocrine neoplasms, may target *DUSP1*. *World J Gastroenterol* 2018; 24(5): 573-582 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i5/573.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i5.573

INTRODUCTION

Gastric neuroendocrine neoplasms (g-NENs) are a rare malignancy mainly derived from enterochromaffin-like (ECL) cells and occasionally derived from other cells that secrete somatostatin, auxin, or serotonin[1]. Based on the National Cancer Institute Surveillance, Epidemiology, and End Results (SEER) cancer registry in the United States, the annual age-adjusted incidence of neuroendocrine tumours (NETs) was 1.09 per 100000 persons in 1973 and increased to 6.98 per 100000 persons by 2012[2]. The proportion of g-NENs among all NENs was 6%[3], 23%[4], 5%[5], and 7.4%[6] in the United States, Austria, Canada, and Taiwan, respectively.

Patients with g-NENs can be subdivided into four types according to their specific tumour aetiology, pathogenesis, and pathology. The clinicopathological features, treatment, and prognosis of different types are completely different[7,8]. The type 1 g-NEN is a well differentiated g-NEN related to chronic atrophic gastritis, with patients exhibiting hypergastrinaemia and achlorhydria. It is the most common type of g-NEN and accounts for approximately 70%-80% of g-NENs[9]. Metastatic type 1 g-NENs are extremely rare[10]; however, the median relapse-free survival is only 8 mo after endoscopic submucosal dissection (ESD) or endoscopic mucosal resection (EMR)[11]. These characteristics make the type 1 g-NEN a stubborn disease. Nevertheless, the molecular mechanism of type 1 g-NEN recurrence is not well understood, and the treatment targets are limited.

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that can regulate target genes through translational repression or mRNA transcript destabilization at the post-transcriptional level. It is estimated that approximately 300 miRNAs (1%-4% of the expressed human genes) exist in the human genome, and a single miRNA can regulate as many as 200 mRNAs[12]. miRNA regulation is linked to various tumours and plays different roles in different diseases, including both oncogenic and tumour suppressor roles[13,14]. miRNAs have the potential to act as biomarkers for patient stratification into different prognostic groups and may also be able to guide treatment decisions. Furthermore, the molecular mechanisms of tumour recurrence and metastasis can often be explained by miRNA activity[7,15,16].

At present, because of the low incidence of NENs, miRNA research related to this disease is not as common as that for other tumour types. However, several studies have shown a variety of results with regard to pancreatic and small intestinal NENs and small cell lung cancer. Roldo *et al*[17] reported differences in miRNA expression between pancreatic NENs and pancreatic acinar cell tumours. Li *et al*[18] reported that the progression of small intestinal NENs is related to a significant increase in miR-96/182/183/196/200. Miller *et al*[19] reported that miR-1 and miR-143-3p gene targets were upregulated in an existing small bowel NET dataset, which could contribute to disease progression, and showed that these miRNAs directly regulate FOSB and NUAK2 oncogenes.

There are a few previous reports regarding type 1 g-NEN-associated miRNAs, including a study from

Professor Pritchard *et al*, who reported that miR-222 expression was increased in the serum and gastric corpus mucosa of hypergastrinaemic INS-GAS mice and hypergastrinaemic patients with autoimmune atrophic gastritis and type 1 gastric NETs. miR-222 expression decreased in these patients following treatment with the CCK2R antagonist netazepide (YF476)[20].

Although there have been a few previous miRNA studies about (or including) type 1 g-NENs, there are still many other miRNAs and target genes waiting to be discovered and studied. The molecular mechanism of this disease remains largely unknown. This study assessed differences in miRNA expression between type 1 g-NEN tissues and non-tumour tissues and identified relevant target genes, with an aim to discover the possible molecular mechanism of type 1 g-NEN recurrence.

MATERIALS AND METHODS

Materials

This study was approved by the Ethics Committee of China-Japan Friendship Hospital. All patient procedures were performed after obtaining written informed consent.

Tissue samples were collected from patients with type 1 g-NENs at our hospital, and patients were included in the study based on the following criteria: (1) a clear pathological diagnosis of g-NENs; and (2) compliance with all of the following clinical characteristics of type 1 g-NENs: hypergastrinaemia, achlorhydria, and chronic atrophic gastritis[9].

To observe differences in miRNA expression between tumour and non-tumour tissues, tumour tissue obtained from the first endoscopic biopsy (before ESD or EMR) was selected for use as experimental samples and gastric mucosal tissue obtained from gastroscopy review (an average of 9 mo after ESD or EMR) was used as control samples. To ensure that the gastric mucosal tissue was tumour-free, the gastroscopy review had to show no recurrence based on both endoscopic and pathologic assessments. Each pair of experimental and control samples was taken from the same patient, reducing the possible impact of individual differences.

RNA extraction and miRNA expression profiling

Sections were prepared from each paraffin-embedded specimen. The specimens were prepared from tumour tissues or non-tumour tissues removed from the patients described above *via* endoscopic biopsy. Total RNA was extracted from the specimens using a RecoverAll Total Nucleic Acid Isolation Kit for FFPE (OE Biotech, Shanghai, China) according to the manufacturer's specifications. The RNA yield was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, United States) and an Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, United States), and the integrity was evaluated using agarose gel electrophoresis with ethidium bromide staining.

The miRNAs in the total RNA were labelled using a miRNA complete Labeling and Hyb Kit (Agilent Technologies Inc., Santa Clara, United States). Then, the miRNAs were hybridized at 55 ℃ for 20 h with an Agilent human miRNA microarray (8*60 K, Design ID: 070156), which included all mature human miRNAs available in the latest version of the miRBase database (Release 20). After the microarray was washed, the fluorescence intensity of the samples was scanned using a microarray scanner (Agilent p/n G4900DA), and the results were transformed into quantitative data. The normalized value was calculated as the ratio of the expression of each target miRNA to that of the reference 5S in the same sample. Fold changes in miRNA expression were obtained by comparing the ratio of miRNA expression in the tumour samples to that in the control samples. Three biological replicates were examined.

Validation analysis using qRT-PCR

The expression of miRNAs chosen for further study was validated *via* qRT-PCR analysis. Total RNA from the samples was purified in the manner described above, and the quality of total RNA was also monitored.

Quantification was performed *via* a two-step reaction process: reverse transcription (RT) and PCR. Each RT reaction consisted of 1 μg of RNA, 4 μL of miScriptHiSpec Buffer, 2 μL of Nucleotide Mix, and 2 μL of miScript Reverse Transcriptase Mix (Qiagen, GER) in a total volume of 20 μL. Reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, United States) for 60 min at 37 ℃, followed by heat inactivation of RT for 5 min at 95 ℃. The 20 μL RT reaction mix was then diluted 5-fold in nuclease-free water and stored at -20 ℃.

Real-time PCR was performed using a LightCycler 480 II Real-time PCR Instrument (Roche, SWI) with 10 μL of PCR reaction mixture that included 1 μL of cDNA, 5 μL of 2 × LightCycler 480 SYBR Green I Master (Roche, SWI), 0.2 μL, of universal primer (Qiagen, GER), 0.2 μL of miRNA-specific primer, and 3.6 μL of nuclease-free water. Reactions were incubated in a 384-well optical plate (Roche, SWI) at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s. Each sample was run in triplicate for analysis. At the end of the PCR cycles, a melting curve analysis was performed to validate the specific generation of the expected PCR product. The miRNA-specific primer sequences were designed and synthesized by Generay Biotech (Shanghai, China) based on the miRNA sequences obtained from the miRBase database (Release 20.0) and are shown in Table 1. Other primer sequences used (not shown in Table 1) are the universal primers from the kit (Qiagen, GER). We used 5S rRNA as an endogenous reference gene. The miRNA expression levels were normalized to that of 5S rRNA and calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1 Primer sequences

	Primer sequence
Hsa-miR-202-3p	5′-AGAGGTATAGGGCATGGGAA-3′
5S rRNA	5′-GGAGACCGCCTGGGAATA-3′

Table 2 Group design

Control	DUSP1 3′-UTR	Non-targeting control
WT	DUSP1 3′-UTR	miR-202-3p
MUTA	DUSP1 3′-UTR-muta	miR-202-3p

Target gene prediction and bioinformatic analysis

MiRNA target prediction can be performed through computational algorithms due to the base-pairing rules between miRNA and mRNA target sites, location of binding sequences within the target 3′-untranslated region (UTR), and conservation of target binding sequences within related genomes. We searched potential targets of certain miRNAs in TargetScan (http://www.targetscan.org/vert_71/), PITA (http://www.pita.ps/), and microRNAorg (http://www.microrna.org/microrna/home.do). Overlapping target genes predicted by the three different databases were further studied. By reviewing the published literature to understand the function of all the overlapping predicted target genes, we selected the target genes that may be associated with type 1 g-NENs for validation.

We then used DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp) to perform GO analysis, which is used to predict the potential functions of the gene products, including molecular function, biological process, and cellular component categories. Pathway analysis of the target genes was performed using the KEGG database, and a statistical test was used to calculate the significance of target gene enrichment in each pathway and obtain the FDR_bhvalue (*P*-value corrected by Benjamini-Hochberg method). This value can be used to provide some hints for selecting the target genes if $P < 0.05$ in any term.

Transfection and luciferase reporter assay

The putative interaction site between hsa-miR-202-3p and the dual-specificity phosphatase 1 (DUSP1) 3′-UTR was searched using TargetScan and microRNA.org. The 388 bp 3′UTR region of the *DUSP1* gene, including the binding site for miR-202, was amplified from 293T cells. The amplified fragment was cloned into a psiCHECK2 luciferase reporter vector (Promega, USA) at the *Xba* I site. A deletion in the miR-202-3p binding site of the *DUSP1* gene 3′-UTR was introduced using a Quik-Change Site-Directed Mutagenesis Kit (Stratagene, United States) following the manufacturer's instructions. The group design is shown in Table 2. The luciferase activity was corrected for transfection efficiency using a Renilla luciferase vector (Promega, United States).

In accordance with the manufacturer's instructions, Lipofectamine 2000 was used to cotransfect 293T cells with the miR-202-3p/non-targeting control mimic (purchased from Hanbio, Shanghai) using a psiCHECK2 vector containing either the wild-type or mutant-type 3′-UTR of *DUSP1*. After incubation in 5% CO_2 at 37 ℃ for 48 h, a luciferase reporter assay was performed using the Promega dual-luciferase reporter assay system according to the manufacturer's instructions. Each assay was performed in triplicate.

Statistical analysis

Statistical analyses of the qRT-PCR results were performed using SPSS software (Version 20.0; SPSS Inc., Chicago, IL, United States). All the data were from at least two independent experiments, with triplicate samples tested in each experiment, and the results are expressed as the means ± SD. The differences between groups were analysed by single-factor analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

RESULTS

miRNA expression profiles in type 1 g-NEN vs non-tumour gastric mucosal (NGM) samples

Samples from three patients (marked as A, B, and C) with type 1 g-NENs were collected following the procedures described in the 'Materials and Methods' section. Thus, we obtained three pairs of samples: three tumour samples (marked as A1, B1, and C1) and the corresponding three tumour-free samples (marked as A2, B2, and C2). An Agilent human miRNA microarray was used to evaluate the miRNA expression profiles in type 1 g-NEN and NGM tissues. We found one upregulated miRNA and five downregulated miRNAs (log2-fold change (FC) > 1.5; adjusted $P < 0.05$) in type 1 g-NEN *vs* NGM tissue (Table 3, Figures 1 and 2).

miRNA-202-3p overexpression in tumours is confirmed by qRT-PCR analysis

Because we were mainly looking for upregulated miRNAs in type 1 g-NENs, we chose miRNA-202 for the follow-up study. Seven pairs of samples obtained from different patients were used for the qRT-PCR analysis of miRNA-202-3p. The results validated our miRNA expression profile microarray results (Figure 3). Each pair of samples showed the same trend as the results mentioned above: miRNA-202-3p is upregulated in type 1 g-NENs.

miR-202-3p might target 215 genes, including DUSP1

Next, miR-202-3p target genes were predicted using three online available databases, and 427, 4038, and 666 potential target genes were predicted with TargetScan, microRNA.org, and PITA, respectively. There were 215 genes at the intersection of the three

Table 3 MiRNA expression profiles of type 1 g-neuroendocrine neoplasms *vs* non-tumour gastric mucosal tissues

Systematic_Name	*P* value	FC (abs)	Regulation
Hsa-miR-194-3p	0.022978	4.278965	Down
Hsa-miR-202-3p	0.014934	12.12983	Up
Hsa-miR-6752-3p	0.012949	6.846719	Down
Hsa-miR-6800-3p	0.026902	5.196331	Down
Hsa-miR-6889-3p	0.039335	15.72488	Down
Hsa-miR-933	0.008580	4.18559	Down

FC: Fold change.

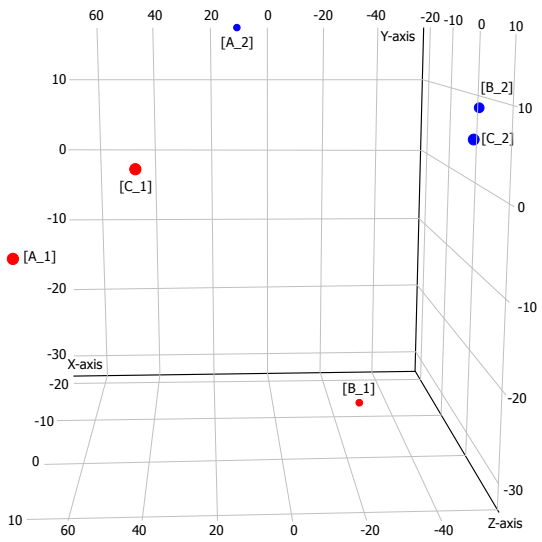

Figure 1 Principal component analysis plot of normalized miRNA expression values. PCA was performed using R Bioconductor DESeq2. A clear separation between the tumour (red) and non-tumour (blue) samples was observed. PCA: Principal component analysis.

predicted target gene sets (Figure 4). We considered potential target genes to exhibit an expression trend opposite to that of the miRNA, in accordance with the antiregulation paradigm (*i.e.*, upregulated miRNA and downregulated mRNA)[21]. As miR-202-3p is overexpressed in type 1 g-NENs, we speculated that it may play a role in promoting cancer by downregulating specific tumour suppressor genes. Enrichment analyses of GO terms and KEGG pathways were also performed. Unfortunately, the bioinformatic analysis did not show any terms with an FDR_bh less than 0.05, and thus it failed to provide hints for selecting target genes.

We searched a large number of studies on the 215 potential target genes mentioned above and found that *DUSP1* was reported to be a tumour suppressor gene in a variety of diseases and was associated with gastric tumours[22-26]. Therefore, we chose this gene for further validation experiments.

Direct targeting of DUSP1 by miR-202-3p

To verify whether miR-202-3p directly targets *DUSP1*, luciferase reporter assays were conducted. The results of a luciferase reporter assay are shown in Figure 5 and indicate that 293T cells cotransfected with the miR-202-3p mimic and wild-type 3′-UTR of *DUSP1* showed a notable decrease in luciferase activity compared with the control group ($P < 0.05$). However, 293T cells cotransfected with the miR-202-3p mimic and mutant-type 3′-UTR of *DUSP1* had the same luciferase activity as the control group. These results indicate that *DUSP1* is a target gene of miR-202-3p, and its expression can be negatively regulated by miR-202-3p.

DISCUSSION

Role of miRNA-202-3p

miR-202 is located at 10q26 (position 135061015 -135061124 on chromosome 10), and its mature single-stranded miRNA sequence is 5′-UUCCUAUGCAUAUAC UUCUUUG-3′[27]. The mature sequence is highly conserved in vertebrates, such as humans, rats, and mice. The two arms of a pre-miRNA are named -3p and -5p, and their properties and functions are basically the same when they are processed to produce miRNAs.

miR-202 belongs to the let-7 family, which was discovered by Reinhart *et al* in *Caenorhabditis elegans*. It is developed from a precursor molecule of approximately 70 nucleotides with a 21 nt-long stem-loop structure. According to current studies, let-7 is highly conserved, and its expression is often controlled in a temporal and tissue specific manner. There are currently 13 types of miRNAs in the let-7 family, including let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f -1, let-7f-2, let-7g, let-7i, miR-98, and miR-202. The main physiological functions of let-7 include regulating the growth and development of cells and organs, regulating cell proliferation and apoptosis, and participating in metabolic and stress responses[28]. Above all, let-7 is a widely accepted tumour suppressor miRNA. The expression of let-7 family members is downregulated in many carcinoma types[29].

Interestingly, miRNAs may play a diametrically opposed role in different tumours[30]. Although miR-202 belongs to this famous cancer-suppressing family and has been reported as an anti-oncomir in various tumours, such as lung and liver cancer, some studies have reported its oncogenic potential in other tumours in recent years. Researchers from Henan University reported that miR-202 played a promotive role in endometrial cell proliferation[31]. Yu *et al* reported that miR-202 was highly expressed in peripheral blood monocytes in patients with multiple myeloma[32]. Researchers in Washington state found that in eight cases of prostate cancer specimens, miR-202 was more highly expressed than in benign prostatic hyperplasia tissues[33]. British researchers observed 102 cases of breast cancer patients, benign breast disease patients, and healthy people and reported that the expression of miR-202 in tumour tissue was significantly higher and was associated with a worse prognosis[34]. The *Lancet Cancer Journal* reported upregulation of miR-202 in

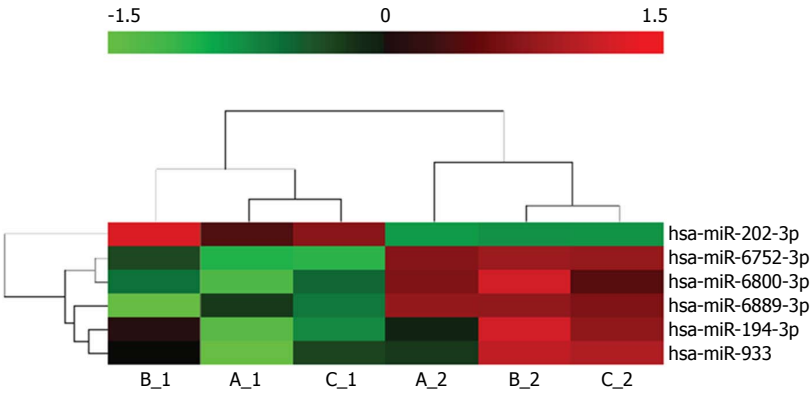

Figure 2 Hierarchical clustering analysis of differentially expressed miRNAs. Differentially expressed miRNAs shown in the rows of the heatmap were clustered using Euclidean distance and complete linkage. The colour scale at the top illustrates the expression value of a particular miRNA on a $\log_2$ scale.

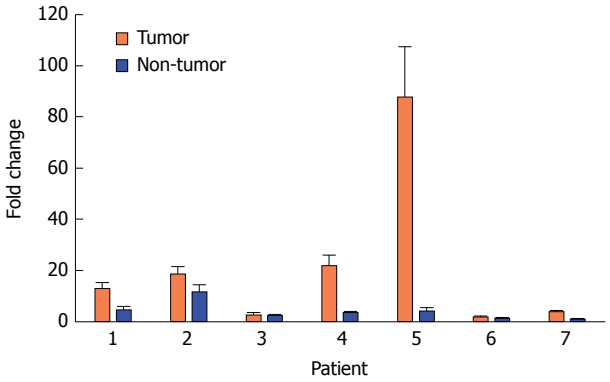

Figure 3 Quantitative RT-PCR analysis of miR-202-3p in type 1 g-neuroendocrine neoplasms and non-tumour gastric mucosal tissues. The value of the lowest expression sample was set to 1, and thus, the fold change values indicate the relative trend of change in the expression levels among different samples.

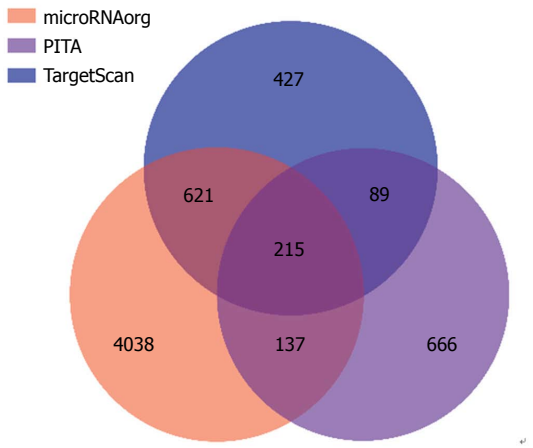

Figure 4 The intersection of the predicted genes of miR-202-3p.

gastric cancer. The researchers compared 160 pairs of gastric cancer tissues and matched paracancerous tissues. Finally, four miRNAs were found to be dramatically upregulated in intestinal-type gastric carcinoma, including miR-202[35].

Possible functions of DUSP1

Our results have shown that *DUSP1* is a target gene of miR-202-3p, and its expression can be negatively regulated by miR-202-3p. DUSP1, also known as mitogen-activated protein kinase phosphatase-1 (MKP-1), is an archetypal member of the dual-specificity phosphatases, which play an important role in inactivating different isoforms of mitogen-activated protein kinases (MAPKs)[36]. In recent years, DUSP1 has been studied in many fields. The functions of DUSP1 focus on cell proliferation, differentiation and transformation, stress responses, inflammation, cycle arrest, and apoptosis mainly by regulating MAPK signalling[37]. An increasing number of studies have discovered that its effects in tumours may be varied and complex. The role of *DUSP1* can be oncogenic in some tumours, while it can be anti-oncogenic in other tumours[38].

According to the literature, *DUSP1*, which is considered an oncogene, is upregulated in lung cancer[39], cholangiocarcinoma[40], and colorectal cancer[41], while in many other tumour types, such as endometrial cancer[24], liver cancer[22], and head and neck squamous cell carcinoma[25], it acts as an anti-oncogene. Researchers have found that total DUSP1 protein levels were decreased in 63.7% of breast cancer tissues compared with matched noncancerous breast tissues. Decreased DUSP1 protein levels were correlated with increased tumour stage, positive recurrence, and poor survival, even when using a multivariate Cox regression model[23]. An experiment showed that most of the apparently normal glands, benign prostatic hyperplasia, and low-grade prostatic intraepithelial neoplasia samples showed high DUSP1 expression. By contrast, DUSP1 expression levels were lower or even absent in high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma samples. The researchers also found an inverse correlation between DUSP1 expression and activation of both p65/NF-κa and p38 MAPK, and DUSP1 promoted apoptosis through a p38 MAPK-dependent mechanism[26].

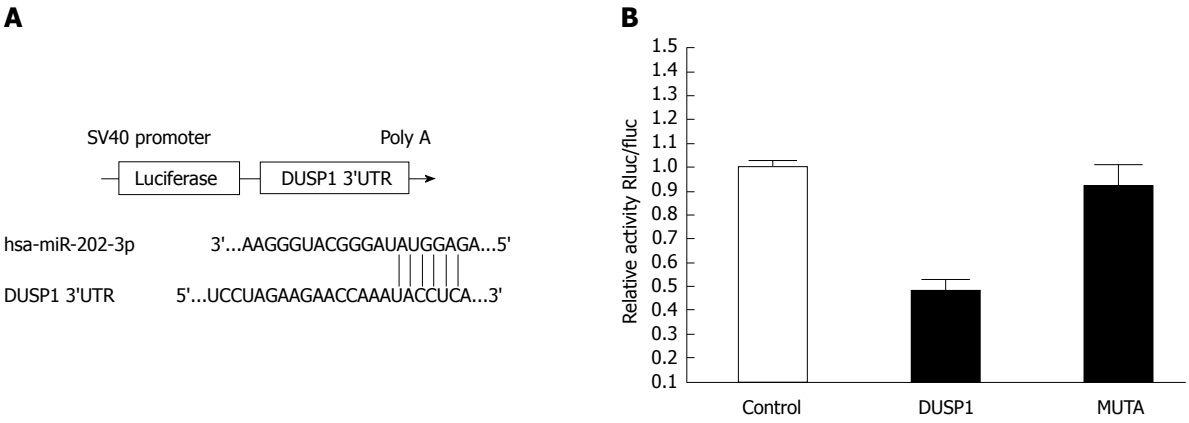

Figure 5 Luciferase reporter assay. The relative activity of the wild-type group was decreased by 52% compared with that of the control group. Compared with the wild-type group, the relative activity of the mutant group was increased by 44%. There was a significant difference between the wild-type group and the control group ($P < 0.05$), and the relative activity of the mutant group recovered to almost the same level as that of the control group ($P > 0.05$). The other panel in the figure shows the target site where hsa-miR-202-3p binds to the *DUSP1* 3'-UTR.

Because our experiments demonstrated that *DUSP1* and miR-202-3p are inversely regulated and miR-202-3p is highly expressed in g-NENs, we can speculate that DUSP1 expression is also low in type 1 g-NENs, and it is likely a tumour suppressor gene in these tumours.

Possible role of miR-202-3p/DUSP1 in the pathogenesis of type 1 g-NETs

Type 1 g-NETs arise in patients who have autoimmune atrophic gastritis. This kind of gastritis causes atrophy and a reduced number of acid secreting gastric parietal cells. This leads to achlorhydria and corresponding symptoms such as bloating, indigestion, and constipation. The high intra-gastric pH level triggers negative feedback that stimulates G cells to over-secrete gastrin and leads to hypergastrinaemia. Since gastrin has a nutritional effect on ECL cell growth, hypergastrinaemia can lead to ECL cell proliferation. In some patients, ECL cell proliferation will further develop into dysplasia and ultimately the formation of a type 1 g-NET[42-44].

Nevertheless, only a few autoimmune atrophic gastritis patients eventually develop type 1 g-NETs[45]. This indicates that there are still other factors in addition to hypergastrinaemia that promote progression from proliferation and dysplasia to tumour formation. Thus, many factors that may contribute to tumour formation have been proposed, such as mutations in *BCL-2*[46], *Reg*[47], *Mcl-2*[48], and *MEN-1*[49] genes, growth factor regulation, and bacterial infection. These factors may affect apoptosis, autophagy, proliferation, and differentiation, thereby promoting tumour formation[50].

In our study, all the control group samples were tumour-free gastric mucosa, but they all exhibited ECL cell proliferation. Therefore, we speculate that miR-202-3p/*DUSP1* plays a role in the process of ECL cell dysplasia and tumour formation (Figure 6). Because high miR-202-3p expression has been found in type 1 g-NETs, we can safely conclude that miR-202-3p acts as a tumour-promoting miRNA in the development of these tumours. The dual-luciferase reporter assay showed the relationship between miR-202-3p and *DUSP1*, confirming the negative regulation between them.

Moreover, the studies mentioned above have shown that *DUSP1* has a significant antitumour effect in a variety of tumours. Therefore, we speculate that *DUSP1* is a tumour suppressor gene in type 1 g-NENs. In normal situations, although hypergastrinaemia leads to ECL cell proliferation, the cells will be prevented from dysplasia if normal *DUSP1* expression is maintained. However, when *DUSP1* is downregulated by high miR-202 expression, ECL cells will have more opportunities to develop type 1 g-NETs.

Future work

MiRNA-mRNA interactions occur in a context-dependent, cell-type-specific manner[51,52]. Since g-NEN is a rare disease, no ready-made cell lines can be found, and thus, we had to validate the interaction between miRNA-202-3p and *DUSP1* in 293T cells (a well-established tool cell for dual-luciferase experiments). Our next step is to culture primary ECL cells to further validate our findings.

As type 1 g-NENs are tumours with a high recurrence rate and a short recurrence time, what concerns researchers the most is how to prevent this disease from recurrence. Because the recurrence mechanism is similar to the pathogenesis of type 1 g-NETs, we believe that treatment targeting miR-202-3p/*DUSP1* may help reduce the recurrence rate. In our hospital, we often use Chinese herbal medicine for patients with type 1 g-NETs after ESD/EMR to prevent recurrence. Our previous clinical observation has found that Chinese herbal medicine can extend the median disease-free survival to 15 mo (Tan HY, unpublished data). However, the mechanism of this phenomenon is unclear. We also plan to explore whether Chinese herbal medicine can regulate the expression of miRNA-202-3p/*DUSP1* using

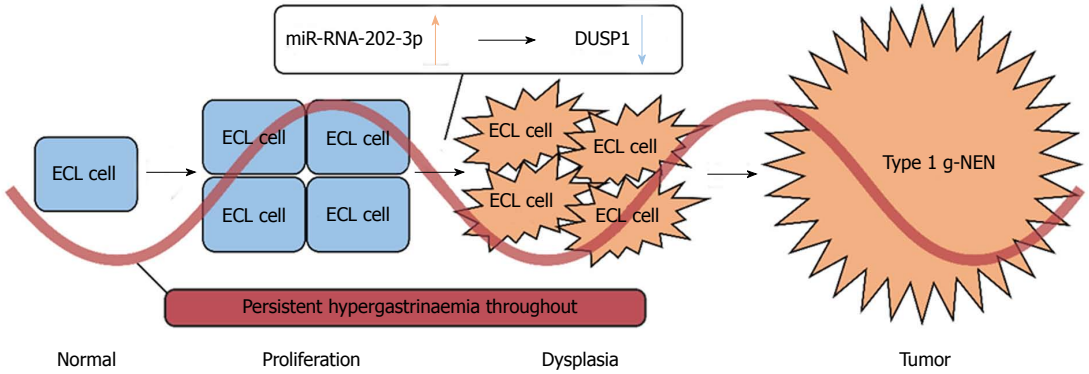

Figure 6 The possible role of miR-202-3p/DUSP1 in the pathogenesis of type 1 g-NETs.

cell models.

ARTICLE HIGHLIGHTS

Research background

Type 1g-NEN is a kind of rare malignant tumor. Because its recurrence rate is relatively high, the molecular mechanism of this disease urgently needs to be explored. MiRNAs play important roles in the occurrence and development of tumors. At present, studies on the role of miRNAs in type 1 g-NEN are quite few. This study may provide some potential therapeutic targets for the prevention of type 1 g-NEN recurrence.

Research motivation

The main topic of this study is the molecular mechanism of type 1 g-NENs. The key issue to be solved is which miRNAs and their target genes could affect the process of tumor recurrence. In future research, our study may help to explain the mechanism of some existing treatment and provide new therapeutic targets for type 1 g-NEN therapy.

Research objectives

The main objective of this study was to discover some type 1 g-NEN associated miRNAs and find their target genes. In this study, the differential miRNA expression between tumor lesions and tumor-free gastric mucosa was described, and the target gene of miRNA-202-3p was found. These may provide a basis for further revealing the molecular mechanism of type 1 g-NEN recurrence in the future.

Research methods

Four main technologies were used in this study. First, we used Agilent human miRNA chips to find the differential miRNA expression between tumor lesions and tumor-free gastric mucosa. This kind of chip is expensive, but covers all known human miRNAs. Second, the results of chips were validated *via* RT-PCR, which is a proven and reliable experimental method to obtain a more accurate conclusion. Third, we used bioinformatics to look for target genes on web (TargetScan, PITA, and microRNAorg). This technique can quickly help us narrow down the scope of target genes. Last, a dual-luciferase reporter assay system was used for verification of the target gene. This system can show clearly the influence of miRNAs on its target gene. All the above research methods have been rarely applied to g-NENs before.

Research results

The high expression of miR-202-3p in type 1 g-NENs was found, which indicates that miR-202-3p acts as a tumor-promoting miRNA. The dual-luciferase reporter assay system showed the relationship between miR-202-3p and *DUSP1*, confirming that the high expression of miR-202-3p leads to the downregulation of *DUSP1*. Therefore, we speculate that *DUSP1* is a tumor suppressor gene in type 1 g-NENs. Since g-NEN is a rare disease, there are no ready-made cell lines and we had to conduct the dual-luciferase experiment in 293T cells (a well-established tool cell for dual-luciferase experiment). Our next step is to carry out the primary culture of ECL cells to further validate our findings.

Research conclusions

In this study, we summarized the existing mechanisms of type 1 g-NENs and confirmed the difference of miRNA expression between tumor and non-tumor gastric mucosa. Our study found that miRNA-202-3p was overexpressed in type 1 g-NENs and *DUSP1* was its target gene and put forward an assumption: In normal situation, although hypergastrinaemia leads to ECL cell proliferation, the cells could be prevented from dysplasia if *DUSP1* is expressed normally. However, when the *DUSP1* is down-regulated by the highly expressed miR-202, ECL cells will have more opportunities to develop to type 1 g-NENs. This will help clinicians to further understand the molecular mechanism of the disease.

Research perspectives

Our next step is to carry out the primary culture of ECL cells to further validate our findings. Our previous clinical study has found that Chinese herbal medicine can extend the median disease-free survival (DFS). So, using cell models to explore whether Chinese herbal medicine can regulate the expression of miRNA-202-3p/*DUSP1* is the best method for the future research.

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ORIGINAL ARTICLE

Basic Study

Enteral nutrition combined with glutamine promotes recovery after ileal pouch-anal anastomosis in rats

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Author contributions: Xu YY and He AQ collected the data; Liu G conceived and designed the study; Li KY and He AQ contributed to the data analysis; Xu YY contributed to the interpretation of the data; Liu G participated in streamlining the study protocol; Xu YY, He AQ, Li KY, Liu J and Liu T proofread the study protocol; Li KY supervised the data collection process; Xu YY, He AQ, Liu G, Li KY, Liu J and Liu T contributed to drafting the manuscript; all authors contributed to the revision of the manuscript and approved the final version.

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Abstract

AIM

To assess the effect of enteral nutrition (EN) supplemented with glutamine on recovery after ileal pouch-anal anastomosis (IPAA) in rats, to provide an experimental basis for nutritional support in patients with ulcerative colitis (UC) after IPAA.

METHODS

Male Sprague-Dawley (SD) rats were randomly divided into three groups (n = 8) after IPAA operation using a microsurgical technique. From the third postoperative day, rats in the control group, EN group, and immune nutrition (IN) group were fed standard rat chow, short peptide EN, and short peptide EN combined with glutamine *ad libitum*, respectively. The rats' general condition was observed throughout the study. Serum levels of total protein (TP), albumin (ALB), prealbumin (PA), and transferrin (TF) were detected on the 30th postoperative day, using an automatic biochemical analyzer. The ileal pouch mucosa was stained with hematoxylin and eosin (HE), and occludin protein levels

were detected by immunohistochemistry.

RESULTS

The body weight of rats in the EN group (359.20 ± 10.06 g) was significantly higher than that in the control group (344.00 ± 9.66 g) ($P < 0.05$) and lower than that in the IN group (373.60 ± 9.86 g) ($P < 0.05$) on the 30th postoperative day. The levels of serum TP, ALB, PA, and TF in the EN group were significantly higher than those in the control group ($P < 0.01$ for all) and lower than those in the IN group ($P < 0.05$ for all). Histopathological score (EN: 0.80 ± 0.37; IN: 0.60 ± 0.40; control group: 2.29 ± 0.18) and expression level of occludin protein (EN: 0.182 ± 0.054; IN: 0.188 ± 0.048; control group: 0.127 ± 0.032) were significantly lower in the control group compared with the EN and IN groups ($P < 0.05$ for all), but there were no significant differences between the latter two groups ($P > 0.05$ for all).

CONCLUSION

EN combined with glutamine may effectively improve nutritional status after IPAA. Our results suggest a benefit of glutamine supplementation in EN for UC patients undergoing IPAA, although human studies are required to confirm this finding.

Key words: Enteral nutrition; Glutamine; Ileal pouch-anal anastomosis; Nutritional status; Recovery

Core tip: We assessed the effect of enteral nutrition (EN) supplemented with glutamine on recovery after ileal pouch-anal anastomosis (IPAA) in rats, to provide an experimental basis for nutritional support in patients with ulcerative colitis after IPAA. Male Sprague-Dawley rats underwent IPAA and were then fed standard rat chow, short peptide EN, or short peptide EN combined with glutamine from postoperative day 3. The rats' general condition was observed throughout the study, and serum levels of total protein, albumin, prealbumin, and transferrin were measured on the 30th postoperative day. The ileal pouch mucosa was stained with hematoxylin and eosin and occludin protein levels were measured by immunohistochemistry.

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INTRODUCTION

Proctocolectomy with ileal pouch-anal anastomosis (IPAA) has become the gold-standard surgical treatment for ulcerative colitis (UC)[1]. However, studies have shown that some patients who have undergone IPAA still have postoperative problems, such as malnutrition, frequent defecation, and severe pouchitis[2], which can cause surgical failure if the pouch needs to be discarded. Therefore, ways of providing nutrition and energy, promoting early recovery of patients, and maintaining the integrity of the pouch mucosa barrier and function have thus become key considerations for surgical treatment of UC.

In recent years, the IPAA rat model established by Chen *et al*[3] has shown many characteristics similar to human IPAA, and has become an effective and important *in vivo* model for the study of recovery and defense as well as immune mechanisms after IPAA.

Nutritional support includes parenteral nutrition (PN) and enteral nutrition (EN). At present, numerous studies have reported that giving EN to patients at an early stage following surgery or severe trauma can rapidly restore the integrity of the digestive tract. This prevents intestinal mucosal atrophy, enhances intestinal barrier function, promotes rehabilitation, and reduces complications and mortality[4,5]. In addition, enteric bacteria and endotoxins are prone to translocation if patients fast too long postoperatively[6]. Early EN is therefore recommended in patients with IPAA. However, general EN may lack certain specific nutrients and its ability to regulate immune function is thus limited, and the supplementation of nutritional therapy with specific nutrients with certain pharmacological effects may improve the patient's nutritional status, while protecting the integrity of mucosal barrier function.

Glutamine is a non-essential amino acid that can act as an energy source for the proliferation of intestinal lymphocytes, mucosal cells, and fibroblasts[7]. It also has many important physiological properties, including restoring intestinal permeability, preventing intestinal mucosal atrophy, protecting the barrier function of the intestinal mucosa, and improving nitrogen balance. Glutamine can also stimulate immune cells in a specific way, enhance immune function, and maintain a moderate immune response, which is therefore referred to as 'immune nutrition'. Li *et al*[8] reported that glutamine may be an effective intestinal mucosal protective agent when supplemented into EN. Rogero *et al*[9] also suggested that glutamine plays an important role in maintaining the integrity of the intestinal epithelial structure. Fujita *et al*[10] found that glutamine-supplemented EN reduced the translocation of bacteria in the intestinal contents and enhanced the barrier function of the intestinal mucosa in a pig model.

Clinical research into nutritional support for UC patients after IPAA is currently lacking; however, it is important to explore appropriate postoperative nutritional support to address the issue of postoperative malnutrition. The purpose of this study was to investigate the effects of EN supplemented with different nutrients on recovery, nutritional status, and mucosal

barrier function of the ileal pouch in IPAA rats. These results will provide an experimental basis for nutritional treatment of UC patients after IPAA.

MATERIALS AND METHODS

Animals

Specific pathogen-free (SPF) male Sprague-Dawley (SD) rats aged 10-12 wk and weighing 320-350 g were purchased from the Laboratory Animal Center of the Military Medical Science Academy of the Chinese People's Liberation Army. They were housed in controlled environmental conditions of ventilation (wind speed, 0.1-0.2 m/s), room temperature (20-25 ℃), and humidity (40%-70%) for 1 wk. Rats had access to natural light and were provided with standard rat chow and running water *ad libitum* prior to surgery. The animal use protocol was reviewed and approved by the Animal Ethical and Welfare Committee.

Rat model

All rats underwent IPAA using a microsurgical technique. They were then divided into three groups (n = 8 each): a control group fed standard rat chow and tap water (The detailed compositions of standard rat chow were rice, bran, corn, soybean cake, vitamins, minerals, salt, *etc*), an EN group fed short peptide EN (Milupa GmbH, Germany), and an IN group fed short peptide EN combined with 0.4 g/(kg/d) glutamine (YaoYou Pharmaceutical Co., Ltd, ChongQing, China), *ad libitum*. All groups were given from the third postoperative day.

General condition

The rats' general status was observed and recorded daily from the first to the 30th postoperative day. Observations included mental state (burnout, laziness, and/or irritability) and fur condition (glossiness and messiness). Body weight was measured at 10 am every day to produce a weight-change curve. Stool characteristics were evaluated and feces were scored using the Bristol Stool Form Scale[11]: type 1, separate hard lumps, like nuts; type 2, sausage-shaped but lumpy; type 3, like a sausage or snake but with cracks on its surface; type 4, like a sausage or snake, smooth and soft; type 5, soft blobs with clear-cut edges; type 6, fluffy pieces with ragged edges, a mushy stool; and type 7, watery, no solid pieces. The fecal score was summarized every 5 d.

Sample collection and tissue processing

Ileal pouch tissue was harvested from rats under anesthesia on the 30th postoperative day, with specimens taken from the same location in the pouches in all rats. Specimens were rinsed with ice-cold saline and then fixed in 4% neutral formalin solution (Jiayu Chemical Co. Ltd, Jinan, China). Blood samples (3 mL) were taken from the abdominal aorta using a 5 mL syringe, placed in an anticoagulant biochemical tube, reversed, and mixed. After centrifugation, the supernatant was collected, packed separately, and stored at -20 ℃ in a refrigerator for cryopreservation.

Nutritional parameters

Serum levels of total protein (TP), albumin (ALB), prealbumin (PA), and transferrin (TF) were detected in defrosted blood samples using an automatic biochemical analyzer (Johnson and Johnson, United States).

Pathology scores

Histological scoring was performed by hematoxylin and eosin (HE) staining. Tissues previously fixed in formaldehyde solution were cut into 0.5 cm pieces, dehydrated in graded ethanol solutions, embedded in paraffin, and stained with HE. The ileal pouch tissue was then evaluated according to the histopathological scoring criteria described by Shebani *et al*[12] (Table 1).

Expression level of occludin protein

Occludin protein in the ileal pouch mucosa was detected by immunohistochemical staining. Paraffin sections (approximately 5 μm thick) were dewaxed, hydrated, and immersed in boiling citrate buffer (Scientan, Beijing, China; 0.01 mol/L, pH 6.0) for thermal antigen repair. They were then cooled and washed twice with phosphate buffered solution (PBS; Scientan, Beijing, China; 0.01 mol/L, pH 7.4). Sections were immersed in 5% bovine serum albumin and then incubated with rabbit anti-occludin antibody (bs-10011R; Bioss, Beijing, China), followed by incubation with biotinylated goat anti-mouse IgG. A DAB kit (AR1022; Boster, Wuhan, China) was used for color rendering, and sections were lightly stained with hematoxylin (Hua Liang, Fushan, China) and finally observed under a microscope.

The optical density of the immunohistochemical images was measured using Image-Pro Plus 6.0 software, according to the Chinese reference guidelines. The 'irregular' tool was used to delineate the measuring area, and the results were then calculated and analyzed.

Statistical analysis

All statistical analyses were performed using SPSS 19.0. Measurement data are presented as mean ± SD. Data analysis was carried out using one-way ANOVA, and comparisons between two among the three groups were made using the Student-Newman-Keuls method. A P-value < 0.05 was considered statistically significant.

RESULTS

General condition

Rats in all groups responded well to surgery, with no signs of burnout, laziness, or irritability (Figure 1A-C). The time to first defecation in the control, EN, and IN

Table 1 Histological scoring criteria

Score	Erosion	Ulceration	Villous atrophy	Edema in the lamina propria
0	Negative	None	None	None
1	Focal erosion	Focal ulceration of the mucosa at ½ superficial regions	Mild	Positive
2	Erosion is observed In many regions	Total mucosal ulceration at multiple foci	Moderate	
3	Extensive erosion	Extensive mucosal ulceration extending to muscularis mucosa or beyond	Severe, villous flattening	

Intra-epithelial inflammation was evaluated by counting the lymphocytes in 100 epithelial cells at the tips of the villi. Abscess formation and submucosal inflammation were also evaluated.

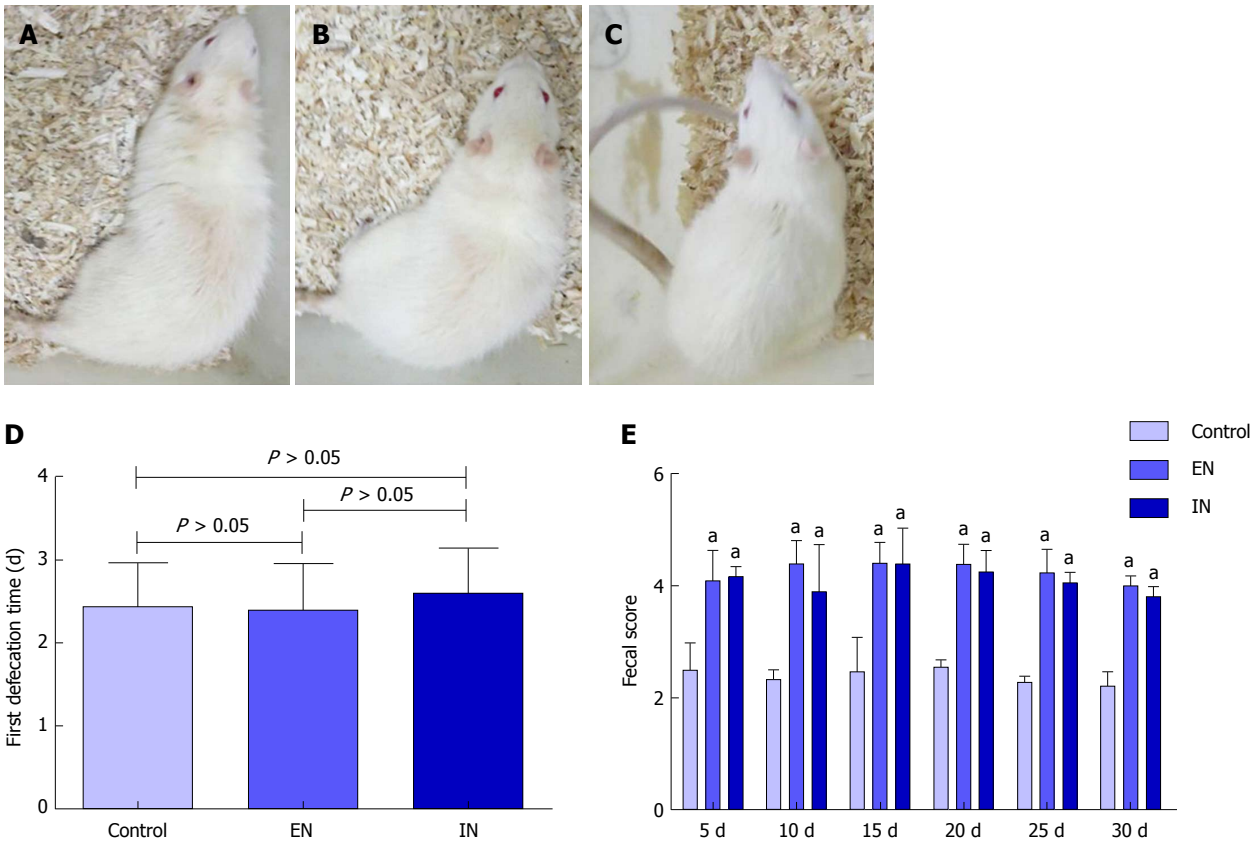

Figure 1 General condition of rats. Mental states of rats in the (A) Control, (B) EN, and (C) IN groups are shown, and they reacted sensitively, with no signs of burnout, laziness, or irritability; D: There was no significant difference in the time to first defecation among the three groups (F = 0.32, P = 0.73); E: The fecal scores were significantly higher in the EN and IN groups compared with the control group at 5, 10, 15, 20, 25, and 30 d postoperatively (P < 0.05 for all), but there were no significant differences between the EN and IN groups (P > 0.05 for all). Bars represent mean ± SD, n = 8. ^{a}P < 0.05 *vs* Control. EN: Enteral nutrition; IN: Immune nutrition.

groups was 2.43 ± 0.53, 2.40 ± 0.55, and 2.60 ± 0.54 d after IPAA, respectively. There was no significant difference in the first defecation time among the three groups (F = 0.32, P = 0.73) (Figure 2D). The fecal scores in the EN and IN groups were significantly higher than those in the control group at 5, 10, 15, 20, 25, and 30 d postoperatively (P < 0.05 for all), but there were no differences between the EN and IN groups (P > 0.05 for all).

Nutritional status

There were no significant differences in body weight among the three groups pre-operatively (F = 0.57, P = 0.57) (Figure 2A), but body weight showed a short-term decrease postoperatively. The lowest body weight was reached at 9.43 ± 1.81, 7.80 ± 0.84, and 4.60 ± 1.71 d postoperatively in the control, EN, and IN groups, respectively (F = 20.98, P < 0.01), and the weight decreases were 81.29 ± 12.30, 59.81 ± 4.15, and 26.26 ± 8.04 g, respectively (F = 79.18, P < 0.01). The time to the lowest body weight and body weight decrease were both greater in the control compared with the EN (P < 0.05) and IN (P < 0.05) groups, and were both greater in the EN compared with the IN group (P < 0.05) (Figure 2B and C). Body weights of rats in the control, EN, and IN groups recovered to 344.00 ± 9.66, 359.20 ± 10.06, and 373.60 ± 9.86 g, respectively, on the 30th day postoperatively (F =

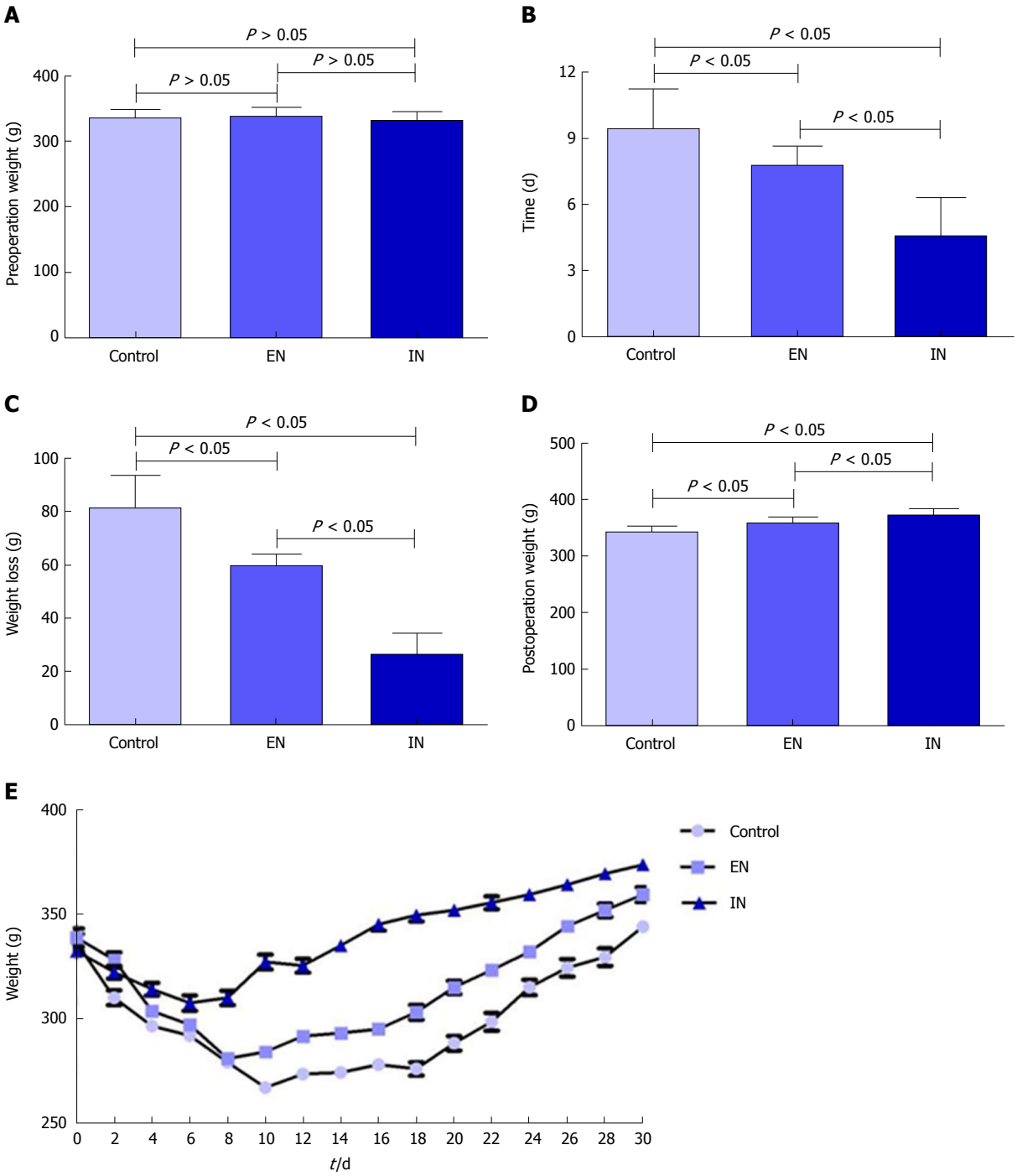

Figure 2 Body weight changes in rats during the study period. A: Body weights were similar in all three groups pre-operatively (F = 0.57, P = 0.57); B Time to minimum weight was significantly longer in the control group compared with the EN (P < 0.05) and IN (P < 0.05) groups, and longer in the EN group compared with the IN group (P < 0.05); C: The weight decline was greater in the control group compared with the EN (P < 0.05) and IN (P < 0.05) groups, and greater in the EN compared with the IN group (P < 0.05); D: Body weight at 30 d postoperatively was significantly higher in the EN group compared with the control group (P < 0.05) and significantly lower compared with the IN group (P < 0.05); E: Weight-change curve for rats over 30 d postoperatively. Bars represent mean ± SD, n = 8. EN: Enteral nutrition; IN: Immune nutrition.

18.02, P < 0.01). Body weight was significantly lower in the control group than in the EN an IN groups (P < 0.05), and in the EN group than in the IN group (P < 0.05) (Figure 2D).

Serum levels of TP, ALB, PA, and TF were significantly different among the three groups (F = 13.51, P < 0.01; F = 17.25, P < 0.01; F = 37.98, P < 0.01; F = 36.41, P < 0.01, respectively). Levels in the control group were significantly higher than those in the EN (P < 0.05 for all) and IN (P < 0.05 for all) groups, while levels in the EN group were higher than those in the IN group (P < 0.05 for all) (Figure 3).

Mucosal barrier function of the ileal pouch

HE staining showed that the integrity of the mucosal villi in the ileal pouch was disrupted in the control group.

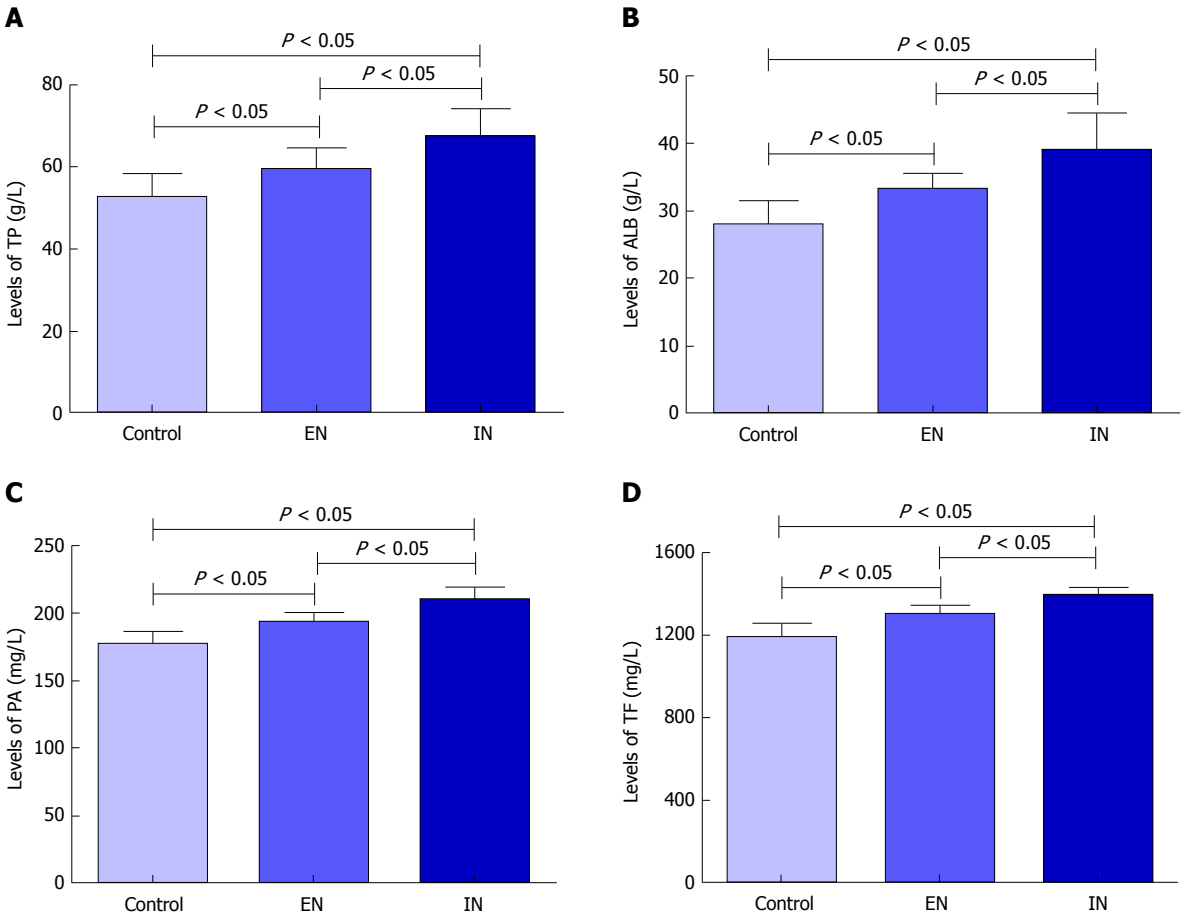

Figure 3 Changes in serum protein levels. A: Serum TP level was significantly higher in the EN group compared with the control group ($P < 0.05$) and significantly lower compared with the IN group ($P < 0.05$); B: Serum ALB level was significantly higher in the EN group compared with the control group ($P < 0.05$) and significantly lower compared with the IN group ($P < 0.05$); C: Serum PA level was significantly higher in the EN group compared with the control group ($P < 0.05$) and significantly lower compared with the IN group ($P < 0.05$); D: Serum TF level was significantly higher in the EN group compared with the control group ($P < 0.05$) and significantly lower compared with the IN group ($P < 0.05$). Bars represent mean ± SD, $n = 8$. EN: Enteral nutrition; IN: Immune nutrition; TP: Total protein; ALB: Albumin; PA: Prealbumin; TF: Transferrin.

The villus stroma was loose and irregular, and the villus epithelium showed necrosis, shedding, and atrophy, as well as edema in the lamina propria (Figure 4A). The morphology of the pouch mucosa in the EN (Figure 4B) and IN (Figure 4C) groups was largely normal: the villus structure was intact and its arrangement was neat, the epithelial cells were arranged regularly, and there was occasional interstitial edema. The pathological scores in the EN (0.80 ± 0.37) and IN (0.60 ± 0.40) groups were significantly higher than that in the control group (2.29 ± 0.18, $F = 62.15$, $P < 0.01$) ($P < 0.05$ for both). However, there was no significant difference between the EN and IN groups ($P > 0.05$) (Figure 4D).

Immunohistochemical staining revealed that expression levels of occludin protein in the ileal pouch mucosa in the EN (0.182 ± 0.054) and IN (0.188 ± 0.048) groups were significantly higher than that in the control group (0.127 ± 0.032, $F = 4.34$, $P = 0.02$) ($P < 0.05$ for both), but there was no significant difference between the EN and IN groups ($P > 0.05$) (Figure 5).

DISCUSSION

UC patients lose nutrition because of the nature of the disease and its related clinical manifestations. In addition, restrictions on the types of food that can be eaten safely can result in food intolerances. As a result, about 23.4% of UC patients are malnourished[13]. During IPAA, the colon and rectum are removed and the ileal pouch is connected to the anus[3]. However, although the digestive tract is reconstructed, its digestive and absorptive functions are impaired, while the patient's nutritional status is further worsened by stress caused by the surgery and anesthesia[14]. Effective means of providing nutritional support for UC patients and thus accelerating their recovery after IPAA have thus become an important question. The current study, based on a stable model of IPAA in rats fed different EN diets, showed that short peptide EN and glutamine could effectively improve nutritional status, protect the mucosal barrier of the ileal pouch, and accelerate the recovery of rats after IPAA operation.

The results of this study showed that the time to first defecation and mental state of rats fed short peptide EN with different nutrients after IPAA were similar to those of control rats, but the fecal scores were higher. This effect may be associated with the increased absorption efficiency of short peptide

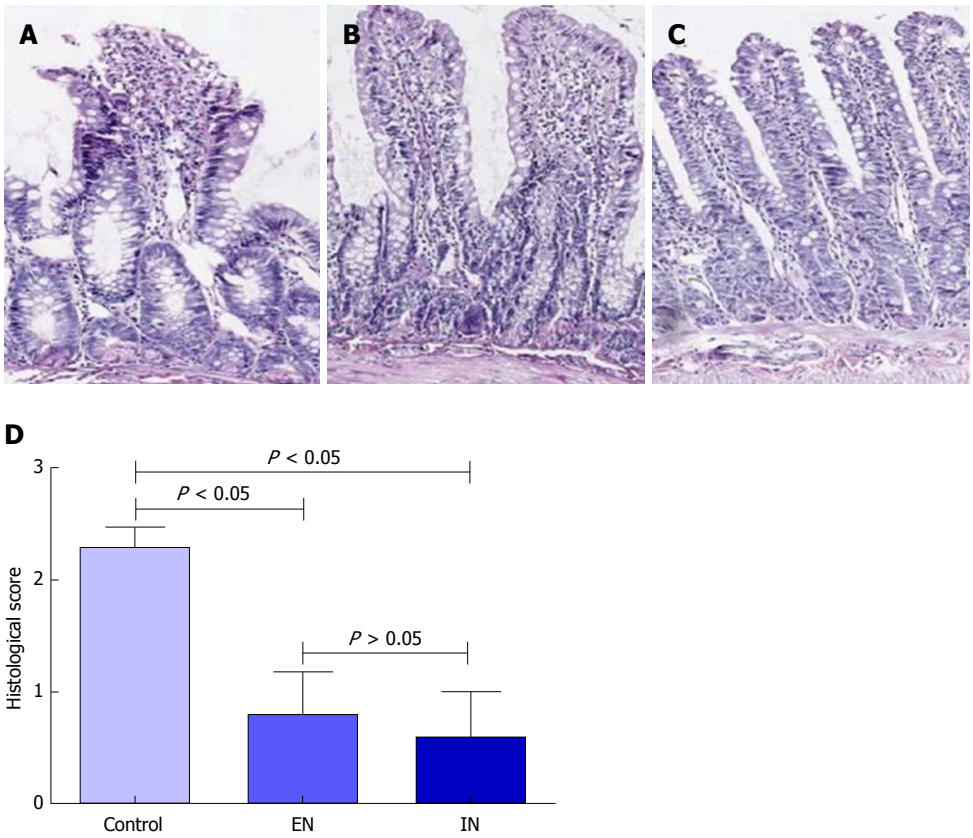

Figure 4 Hematoxylin and eosin staining of the ileal pouch mucosa. A: The integrity of the pouch's mucosal villi was disrupted in the control group. The villus stroma was loose and irregular, and the villus epithelium showed necrosis, shedding, and atrophy, as well as edema in the lamina propria. The morphology of the pouch mucosa in the (B) EN and (C) IN groups was largely normal; the villus structure was intact and its arrangement was neat, the epithelial cells were arranged regularly, and there was occasional interstitial edema; D: Pathological scores were higher in the EN and IN groups compared with the control group ($P < 0.05$ for both). However, there was no significant difference between the EN and IN groups ($P > 0.05$ for both). Bars represent mean ± SD, $n = 8$. EN: Enteral nutrition; IN: Immune nutrition.

nutrients in the small intestine[15]. Glutamine can serve as an energy source for intestinal epithelial cells and provide nutrients[7], while short peptide nutrition has the characteristics of low residue, defecation balance, and a low need for digestive juices[16].

The levels of plasma proteins play an important role in the assessment of nutrition[17]. The main indices include TP, ALB, and protein A/G; however, progress in the development of detection technologies means that other visceral proteins, such as PA and TF, have also emerged as candidates for assessing nutritional status[18]. TP can reflect malnutrition caused by chronic disease, while ALB is an important indicator of nutritional status that can effectively reflect disease severity and the relationship between protein consumption and intake. PA is a relatively sensitive indicator of acute changes in nutritional status in the short term, and is the gold standard for monitoring and evaluating the nutritional status of patients[19,20]. TF often falls along with decreases in PA and ALB in the acute reaction phase, and can be used as an indicator of nutritional status[21,22].

The results of this study showed that serum levels of TP, PA, ALB, and TF were significantly improved in the IN group postoperatively, compared with the control and EN groups. Changes in body weight, which is another indicator of nutritional status, are consistent with serum protein levels. These findings demonstrated that glutamine could effectively improve nutritional status after IPAA in rats. Glutamine is a non-essential amino acid in the body in the stress state; it can prevent the excessive decomposition of muscle, promote protein synthesis, and protect the intestinal mucosa. After IPAA, the rats were stressed and the demand for nutrients was thus greatly increased, but the synthesis of nutrients could not meet their needs. EN and postoperative diets generally have a lack of glutamine, and supplementing the diet with glutamine after IPAA could increase the synthesis of tissue proteins, meet the nutritional needs, and improve nutritional status. Liu *et al*[23] found that glutamine supplementation improved the nutritional status of patients with acute pancreatitis, while Wischmeyer *et al*[24] also reported that glutamine had a positive effect on recovery after IPAA. Our results are consistent with these previous reports.

In addition to the functions of digestion, absorption, and peristalsis, the intestinal tract is also involved in immune regulation[25,26], hormone secretion[27], and mucosal barrier function. The intestinal barrier plays an

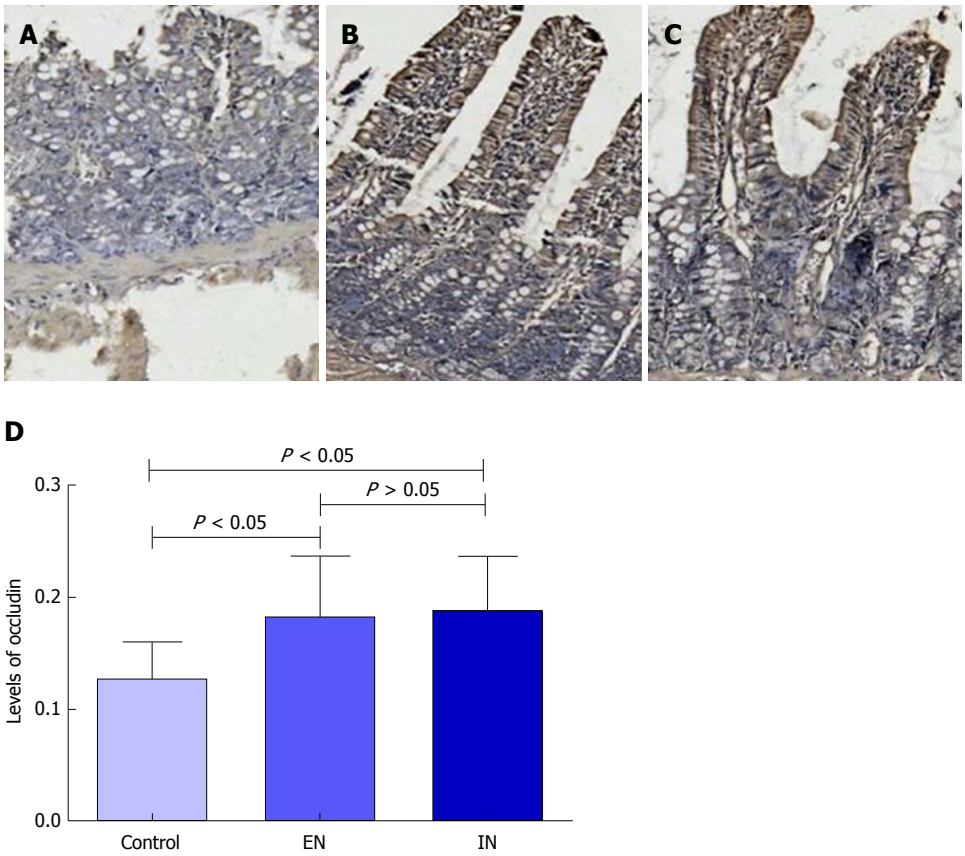

Figure 5 Immunohistochemical staining of the ileal pouch mucosa. Immunohistochemical staining in the (A) control, (B) EN, and (C) IN groups are shown. D: Expression levels of occludin protein in the EN and IN groups were significantly higher compared with the control group ($P < 0.05$ for both), but there was no significant difference between the EN and IN groups ($P > 0.05$ for both). Bars represent mean ± SD, $n = 8$. EN: Enteral nutrition; IN: Immune nutrition.

important role in the maintenance of intestinal function[28]. Occludin is an intercellular tight junction protein, and numerous studies have shown that occludin plays an important role in the intestinal mucosal barrier[29,30] and can be used as an indicator of mucosal barrier function in the ileal pouch[31]. In our study, occludin levels were higher in the EN and IN groups compared with the control group, suggesting that short peptide EN and glutamine could enhance the mucosal barrier function of the ileal pouch by increasing the expression of occludin.

In addition, the histological score and occludin level in the IN group were higher than those in the EN group, indicating that glutamine could increase the expression level of occludin. Numerous animal experiments have demonstrated that glutamine supplementation can prevent intestinal mucosal atrophy, as well as restore intestinal villus height and crypt depth. Wang *et al*[7] reported that glutamine could regulate the expression of tight junction proteins. A possible mechanism for this is as follows: (1) glutamine can supply energy for the proliferation and differentiation of intestinal epithelial cells[32]; (2) stimulate heat shock proteins and thus promote cell growth[33]; and (3) promote cell proliferation by regulating the ERK1 and the JNK signaling pathways[34].

In conclusion, feeding short peptide EN supplemented with glutamine can accelerate postoperative recovery after IPAA in rats. It can also improve nutritional status, which has important implications for the nutritional support of patients with UC after IPAA. In terms of nutritional support, EN can be used in patients with UC at the early postoperative stage, and glutamine may be added as appropriate to improve the nutritional status of patients and speed up their recovery.

ARTICLE HIGHLIGHTS

Research background

Glutamine is a nutrient active in the immune system, and may influence recovery after surgery. However, clinical research into nutritional support for ulcerative colitis (UC) patients after ileal pouch-anal anastomosis (IPAA) is currently lacking. Therefore, it is important to explore appropriate postoperative nutritional support to address the issue of postoperative malnutrition.

Research motivation

The purpose of this study was to investigate the effects of enteral nutrition (EN) supplemented with different nutrients on recovery, nutritional status, and mucosal barrier function of the ileal pouch in IPAA rats. These results will provide an experimental basis for nutritional treatment of ulcerative colitis (UC) patients after IPAA.

Research objectives

To assess the effect of EN supplemented with glutamine on recovery after IPAA in rats, to provide an experimental basis for nutritional support in patients with UC after IPAA.

Research methods

Male Sprague-Dawley (SD) rats were randomly divided into three groups (n = 8) after IPAA operation using a microsurgical technique. From the third day postoperatively, rats in the control group, EN group, and immune nutrition (IN) group were fed standard rat chow, short peptide EN, and short peptide EN combined with glutamine *ad libitum*, respectively. The rats' general condition was observed throughout the study. Serum levels of total protein, albumin, prealbumin, and transferrin were detected on the 30th day postoperatively, using an automatic biochemical analyzer. The ileal pouch mucosa was stained with hematoxylin and eosin, and occludin protein levels were detected by immunohistochemistry.

Research results

The body weight of rats in the EN group was significantly higher than that in the control group ($P < 0.05$) and lower than that in the IN group ($P < 0.05$) on the 30th day postoperatively. The levels of serum TP, ALB, PA, and TF in the EN group were significantly higher than those in the control group ($P < 0.01$ for all) and lower than those in the IN group ($P < 0.05$ for all). Histopathological scores and expression levels of occludin protein were significantly lower in the control group compared with the EN and IN groups ($P < 0.05$ for all), but there were no significant differences between the latter two groups ($P > 0.05$ for all).

Research conclusions

Feeding short peptide EN supplemented with glutamine can accelerate postoperative recovery after IPAA in rats. It can also improve nutritional status, which has important implications for the nutritional support of patients with UC after IPAA. In terms of nutritional support, EN can be used in patients with UC at the early postoperative stage, and glutamine may be added as appropriate to improve the nutritional status of patients and speed up their recovery.

Research perspectives

In the future, we will further study on the nutritional support following IPAA procedure, such as enteral nutrition supplemented with probiotics, to improve the postoperative life quality.

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ORIGINAL ARTICLE

Basic Study

Promotion of Sema4D expression by tumor-associated macrophages: Significance in gastric carcinoma

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Abstract

AIM

To study the role of semaphorin 4D (Sema4D) expression promoted by tumor-associated macrophages (TAMs) in gastric carcinoma cells and its clinical significance in the invasion and metastasis of gastric carcinoma.

METHODS

CD68 and Sema4D expression was analyzed in gastric carcinoma and adjacent normal tissues from 290 patients using the immunohistochemical streptavidin-peroxidase method, and their relationships with clinicopathological features were evaluated. Human M2 macrophages were induced *in vitro* and co-cultured in non-contact with gastric carcinoma SGC-7901 cells. Changes in the secretory Sema4D level in the SGC-7901 cell supernatant were measured using an enzyme-linked immunosorbent assay. The effects of TAMs on SGC-7901 cell invasion and migration were assessed with invasion and migration assays, respectively.

RESULTS

CD68 and Sema4D protein expression was significantly higher in gastric carcinoma tissues than in adjacent normal tissues (71.7% *vs* 33.8% and 74.5% *vs* 42.8%, respectively; $P < 0.01$). CD68 and Sema4D protein expression was significantly associated with histological differentiation, TNM stage, and lymph node metastasis ($P < 0.05$), and their expression levels were positively correlated with one another ($r = 0.467$, $P < 0.01$). In the *in vitro* experiment, secretory Sema4D protein expression was significantly increased in the supernatant of SGC-7901 cells co-cultured with TAMs compared with the blank control (1224.13 ± 29.43 *vs* 637.15 ± 33.84, $P < 0.01$). Cell invasion and metastasis were enhanced in the Transwell invasion and migration assays ($P < 0.01$).

CONCLUSION

TAMs promote the invasion and metastasis of gastric carcinoma cells possibly through upregulated secretory Sema4D protein expression. Combined detection of TAM markers, CD68 and Sema4D, in gastric carcinoma tissue shows potential to predict the trend of gastric carcinoma progression.

Key words: Gastric carcinoma; CD68; Semaphorin 4D; Tumor-associated macrophages

Core tip: This study explored the role and clinical significance of semaphorin 4D (Sema4D) expression promoted by tumor-associated macrophages (TAMs) in gastric carcinoma cells. By using immunohistochemical streptavidin-peroxidase method on tissue species and gastric carcinoma cells in non-contact co-culture with human M2 macrophages *in vitro*, we found that Sema4D protein expression was significantly higher in gastric carcinoma tissues than in adjacent normal tissues, and TAMs promoted the invasion and metastasis of gastric carcinoma cells possibly through upregulated Sema4D protein expression.

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INTRODUCTION

Gastric carcinoma is one of the most common malignancies. The incidence of gastric carcinoma currently ranks fourth in malignant tumors worldwide after only lung cancer, breast cancer, and colon cancer, and the mortality of gastric carcinoma is ranked second among cancers[1,2]. The tumor microenvironment plays a major role in the invasion and metastasis of gastric carcinoma. Tumor-associated macrophages (TAMs) are an important component of the tumor microenvironment. TAMs are recruited in the hypoxic microenvironment of various solid tumors and are involved in multiple steps of tumor progression[3,4]. Macrophages can be divided into two classes according to differences in the activation method, surface markers, and functions as follows: M1 macrophages with classic activation, and M2 macrophages with selective activation. Numerous TAMs are present in the inflammatory environment of tumor and are recognized as M2 macrophages[5]. CD68 is an important marker molecule of TAMs[6]. TAMs have been shown to enhance the infiltration and metastasis ability of tumor cells by expressing certain growth factors and cytokines[7]. TAMs also suppress immune responses in the microenvironment and promote tumor progression by facilitating tumor angiogenesis and lymphangiogenesis[8].

Semaphorin 4D (Sema4D) is an important member of the semaphorin subfamily and plays a major role in the nervous and immune systems. Sema4D is expressed at high levels in various tumor tissues, including head and neck squamous cell carcinoma, prostate cancer, and colon cancer, and its role in promoting tumor angiogenesis is becoming a hot research topic[9-12]. Sema4D is another important proangiogenic factor following vascular endothelial growth factor (VEGF)[13]. In the present study, we examined the expression of the TAM markers CD68 and Sema4D in gastric carcinoma and adjacent normal tissues using immunohistochemical assays and analyzed their clinical significance. We also evaluated Sema4D expression in gastric carcinoma cells and the changes in the cellular invasion and metastasis abilities through co-culture of TAMs and gastric carcinoma cells *in vitro*. We explored the effect of TAMs on Sema4D expression in gastric carcinoma tissues and its role in the development and progression of gastric carcinoma to provide a new theoretical reference for the prevention and treatment of this cancer.

MATERIALS AND METHODS

Patients

A total of 290 patients with gastric carcinoma confirmed by a histopathological diagnosis from January to December 2012 with complete medical record data were collected from Shandong Provincial Hospital, the Affiliated Hospital of Qingdao University, and Weifang People's Hospital in China. None of the patients received radiotherapy or chemotherapy prior to surgery or had a hereditary family medical history. The patients included 158 men and 132 women aged 30 to 81 years (median age, 55 years). Regarding the pathological type, 78 patients had well differentiated adenocarcinoma, 113 patients had moderately differentiated adenocarcinoma, and 99 patients had

poorly differentiated adenocarcinoma. Based on the 7th edition of the International Union Against Cancer (UICC) tumor, node, metastasis (TNM) staging system, 105 patients had stage Ⅰ/Ⅱ disease, and 185 patients had stage Ⅲ/Ⅳ disease. A total of 195 patients had lymph node metastasis, and 95 patients did not have lymph node metastasis. Normal adjacent tissue was collected at a 3-cm distance from the lesion as the control. Two pathologists determined the pathological type and tumor grade. In case of disagreement in the diagnosis, a third pathologist reviewed the case, and the final diagnosis was made through discussion.

Immunohistochemical reagents and experimental methods

Reagents and methods: The monoclonal rabbit anti-Sema4D antibody, anti-CD68 antibody, and streptavidin-peroxidase (SP) kit were purchased from Abcam, United States. All paraffin-embedded tissue blocks were cut into 4-μm thick continuous sections. Immunohistochemical staining using the SP method was performed following the kit instructions after positioning by hematoxylin and eosin staining. Briefly, the sections were subjected to conventional deparaffinization, 3% H_2O_2 inactivation of endogenous enzymes, antigen retrieval by heating, and blocking of non-specific staining with rabbit serum. The primary antibody (1:100 dilution), biotin-labeled secondary antibody, and enzyme-labeled streptavidin were successively added, and the sections were incubated at 37 ℃ for 25 min. After color development with 3,3-diaminobenzidine, the sections were slightly counterstained with hematoxylin, conventionally dehydrated, cleared with xylene, mounted with neutral resin, observed under an inverted microscope, and photographed. The primary antibody was substituted with phosphate-buffered saline (PBS) as a negative control. Known positive sections provided by the reagent company were used as positive controls. Each sample was re-stained once.

Result interpretation: The sections were reviewed according to the criteria of Birner *et al*[14]. The protein staining intensity and the percentage of positive cells in the total cell count were analyzed semi-quantitatively. The criteria for determining positive expression were as follows: (1) Positive expression was mainly shown as brownish-yellow or brown particles in the cell membrane, cytoplasm, or nucleus. The intensity of positive staining was scored as follows: cells without staining, 0 points; cells stained light yellow, 1 point; cells stained yellow, 2 points; and cells stained brown, 3 points; (2) Five different fields of view were selected at random under a high-resolution electron microscope to count the numbers of total cells and positive cells. Scores were recorded based on the percentage of positive cells as follows: <5% positive cells, 0 points; 5%-25% positive cells, 1 point; 26%-50% positive cells, 2 points; 51%-75% positive cells, 3 points; and > 75% positive cells, 4 points. The final score was the sum of the scores obtained using the above two criteria. A total score of 0-2 points was considered negative expression and 3-7 points was considered positive expression.

Gastric carcinoma cell lines and major reagents and materials used to induce M2 macrophages

The gastric carcinoma SGC-7901 cell line and the human monocyte THP-1 cell line were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences. The monoclonal rabbit anti-CD68 antibody and fluorescein (FITC)-labeled goat anti-rabbit secondary IgG were purchased from Abcam, United States. The Sema4D ELISA kit was purchased from BD, United States. Matrigel was purchased from Sigma, United States. Six-well Transwells with 0.4-μm and 8-μm pore polycarbonate membrane inserts were purchased from Corning, United States. Phorbol ester (PMA), interleukins (IL)-4 and -13, and Giemsa dye were purchased from Zsbio, Beijing, China. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco, United States.

Conventional cell culture: Gastric carcinoma SGC-7901 cells and human THP-1 mononuclear cells were cultured in RPMI 1640 medium containing 10% deactivated FBS at 37 ℃ with 5% CO_2 and saturated humidity. The cells were passaged every 2-3 d.

***In vitro* activation of human M2 macrophages:** Suspended human THP-1 mononuclear cells were centrifuged and resuspended with RPMI 1640 medium containing 10% FBS. The cells were counted and then seeded at a density of 7.5×10^5 cells/well into 6-well culture plates. Human THP-1 macrophages were treated with 320 nmol/L of PMA for 6 h, followed by 20 ng/mL of IL-4 and 20 ng/mL of IL-13 for 18 h, for a total of 24 h in culture. Thereafter, the medium was aspirated using a pipette. The cells were washed with PBS three times and resuspended in serum-free RPMI 1640 medium.

Immunofluorescence for identification of cells: The medium was aspirated from the 6-well plates used for TAM culture. The cells were fixed with 4% paraformaldehyde at room temperature, clarified with 0.3% Triton X-100, and blocked at room temperature with 1% bovine serum albumin. Subsequently, the cells were incubated with an anti-CD68 monoclonal antibody (1:100 dilution) at 4 ℃ overnight, followed by incubation with a FITC-labeled secondary antibody at room temperature in the dark. After nuclear staining, the cells were mounted and observed. The primary antibody was substituted with PBS as the negative

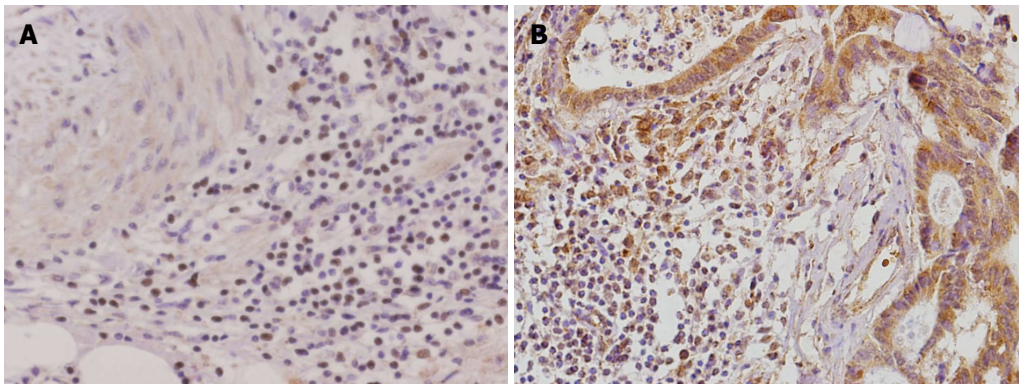

Figure 1 CD68 and Sema4D expression in gastric carcinoma (streptavidin-peroxidase, × 400). A: Positive staining for CD68 in gastric carcinoma tissues; B: Positive staining for Sema4D in gastric carcinoma tissues.

control.

Transwell non-contact co-culture of M2 macrophages and gastric carcinoma cells

Invasion assay: The upper compartment of a six-well Transwell chamber was coated with 200 μL of diluted matrigel (matrigel:PBS = 1:9) and incubated in a 37 ℃ incubator for 30 min. Gastric carcinoma SGC-7901 cells were seeded at a density of 2 × 10^5 cells/well into the upper compartment of the Transwell chamber (8-μm pore size) and cultured in serum-free RPMI 1640 medium. Tumor-associated M2 macrophages were implanted into the lower compartment as an experimental group, and serum-free RPMI 1640 medium was used as a blank control group. The cells were cultured at 37 ℃ in a 5% CO_2 incubator for 18 h and then fixed and stained. Three replicate wells were set for each group, and the experiment was repeated three times. The cells were continuously counted in five fields of view selected at random under high magnification (200 ×), and the mean number was calculated.

Migration assay: The Transwell did not need to be coated with matrigel. The remaining procedures were identical to those used in the invasion assay.

ELISA assay: Gastric carcinoma SGC-7901 cells were seeded into the lower compartment of a 6-well Transwell chamber (0.4-μm pore size) at a density of 2 × 10^5 cells/well. Tumor-associated M2 macrophages were implanted into the upper compartment as an experimental group, and serum-free RPMI 1640 medium was used as a blank control group. Three replicate wells were set up for each group. The cells were cultured at 37 ℃ in a 5% CO_2 incubator for 18 h and then fixed and stained. At the end of the culture period, the upper compartment was removed. The SGC-7901 cell supernatant was discarded, and the cells were washed three times with PBS, followed by the addition of fresh medium. The cell supernatants of the two groups were collected 18 h later, centrifuged at 1000 *g*/min for 15 min, and frozen at -80 ℃. The supernatant was thawed in a 37 ℃ water bath 30 min prior to the ELISA. The procedure followed the instructions of the Sema4D ELISA kit.

Statistical analysis

Data were analyzed using SPSS 18.0 software. Clinical data were analyzed using the χ^2 test or Fisher's exact probability test with a four-fold table, and correlations were tested by Spearman's rank correlation analysis. Count data, including cell counts in the invasion and migration assays and the Sema4D concentration in the cell supernatant estimated by ELISA, were analyzed using an independent sample *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

CD68 and Sema4D protein expression in gastric carcinoma

Immunohistochemical staining showed that CD68 was mainly concentrated in the interstitium of gastric carcinoma tissues. CD68 mainly infiltrated interstitial cells in gastric carcinoma tissues, which was stained as a brown color. Sema4D protein expression was mainly localized in the cytoplasm and nuclei of gastric carcinoma cells and the cytoplasm of tumor interstitial cells (mainly TAMs), which stained a brown color. CD68 and Sema4D expression was significantly higher in gastric carcinoma tissues than in adjacent normal tissues [71.7% (208/290) *vs* 33.8% (98/290), χ^2 = 83.703; 74.5% (216/290) *vs* 42.8% (124/290), χ^2 = 60.161; $P < 0.01$ for both, Figure 1].

Correlation analysis between CD68 and Sema4D expression and clinicopathological features of gastric carcinoma

Positive staining for CD68 and Sema4D was correlated with histological differentiation type, TNM stage, and lymph node metastasis ($P < 0.05$), but not with age,

Table 1 Relationship between CD68 and Sema4D expression and clinicopathological features of patients *n* (%)

Clinicopathological feature	CD68-positive			Sema4D-positive		
	Positive rate	χ^2	*P* value	Positive rate	χ^2	*P* value
Age (yr)		0.761	0.383		3.538	0.060
≤ 50	103/149 (69.0)			104/149 (70.0)		
> 50	104/141 (73.8)			112/141 (79.4)		
Gender		0.524	0.469		2.194	0.139
Male	105/151 (69.5)			113/151 (77.2)		
Female	102/139 (73.4)			114/139 (71.2)		
Histological differentiation type		18.511	0.000		30.696[1]	0.000[1]
Well differentiated adenocarcinoma	41/78 (52.5)			40/78 (51.3)		
Moderately differentiated adenocarcinoma	88/113 (87.9)			96/113 (85.0)		
Poorly differentiated adenocarcinoma	78/99 (78.8)			80/99 (80.8)		
Tumor diameter (cm)		3.120	0.077		1.189	0.276
< 5	116/153 (75.8)			118/153 (77.1)		
≥ 5	91/137 (66.4)			98/137 (71.5)		
TNM stage		6.142	0.013		20.639	0.000
Ⅰ / Ⅱ	58/105 (55.2)			60/105 (57.1)		
Ⅲ / Ⅳ	149/185 (80.5)	1.000		156/185 (84.3)		
Lymph node metastasis		7.375	0.007		6.319	0.012
Yes	149/195 (76.4)			154/195 (80.0)		
No	58/95 (61.1)			62/95 (65.3)		

[1]Well differentiated group compared with the moderately and poorly differentiated groups. TNM: Tumor, node, metastasis.

Table 2 CD68 and Sema4D expression in gastric carcinoma and adjacent tissues

Group	*n*	Sema4D	
		Negative	Positive
CD68			
Negative	83	50	33
Positive	207	24	183

gender, or tumor size (Table 1, $P > 0.05$).

Correlation between CD68 and Sema4D protein expression in gastric carcinoma tissues

Sema4D expression was positive in 183 of the 207 gastric carcinoma tissues with positive CD68 expression (88.4%, 183/237). Of the 83 gastric carcinoma tissues with negative CD68 expression, Sema4dD showed positive expression in only 33 (39.8%, 33/83) cases. Spearman's correlation analysis revealed a positive correlation between CD68 and Sema4D expression in gastric carcinoma tissues ($r = 0.467$, $P < 0.01$, Table 2).

Induction and identification of TAMs

Suspended human THP-1 mononuclear cells were treated with PMA, IL-4, and IL-13, followed by adherent growth. CD68 was expressed in the cytoplasm of macrophages, and positive material in the cytoplasm emitted green fluorescence after staining (Figure 2). When the primary antibody was substituted with PBS, the cytoplasm showed a negative result (Figure 2).

Cell morphological observation

After co-culture with TAMs, gastric carcinoma SGC-7901 cells showed great morphological changes. The cells in the blank control group showed an epithelial cell structure that was cubic, with a blunt edge and a compact arrangement, and the cell confluence was high. Gastric carcinoma SGC-7901 cells in the co-culture group presented a narrow, long, interstitial cell-like shape that was long spindle, with pseudopodium elongation, loose cell arrangement, and even individual cell migration, and the confluence between the cells decreased (Figure 3).

TAMs promote the invasion and migration of gastric cancer SGC-7901 cells

Migration assay: The number of migrating cells in the co-culture group was significantly higher than that in the control group (111.80 ± 11.82 *vs* 71.27 ± 6.44, $P < 0.01$, Figure 4).

Invasion assay: The numbers of invasive SGC-7901 cells that passed through the matrigel were 120.40 ± 8.10 in the co-culture group and 76.67 ± 8.63 in the control group; the difference between the two groups was significant ($P < 0.01$, Figure 5). Thus, TAMs enhanced the *in vitro* invasion and metastasis abilities of the SGC-7901 cells.

Expression of the secretory Sema4D protein in the supernatants from different cultures

At the end of co-culture, we measured the expression of the secretory Sema4D protein in the SGC-7901 cell supernatants of the control and co-culture groups using the Sema4D ELISA kit. The Sema4D level in the blank control group was 637.15 ± 33.84 pg/mL, and the level in the co-culture group was 1224.13 ± 29.43 pg/mL; the difference between the two groups was

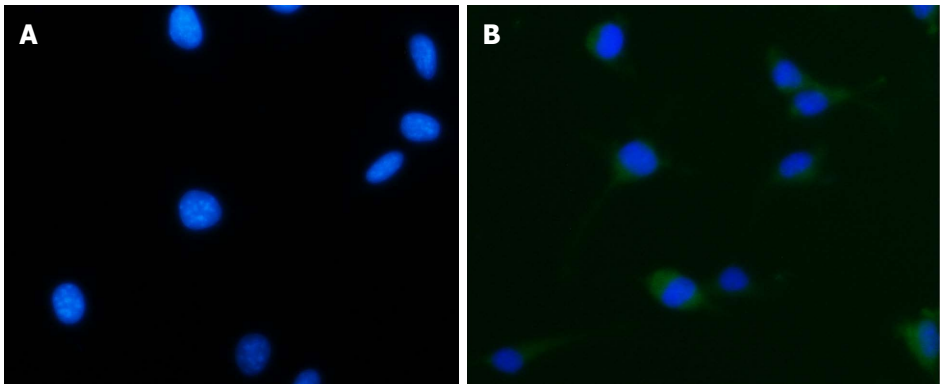

Figure 2 Immunofluorescence staining of cells (400 ×). A: CD68-negative cells; B: CD68-positive cells.

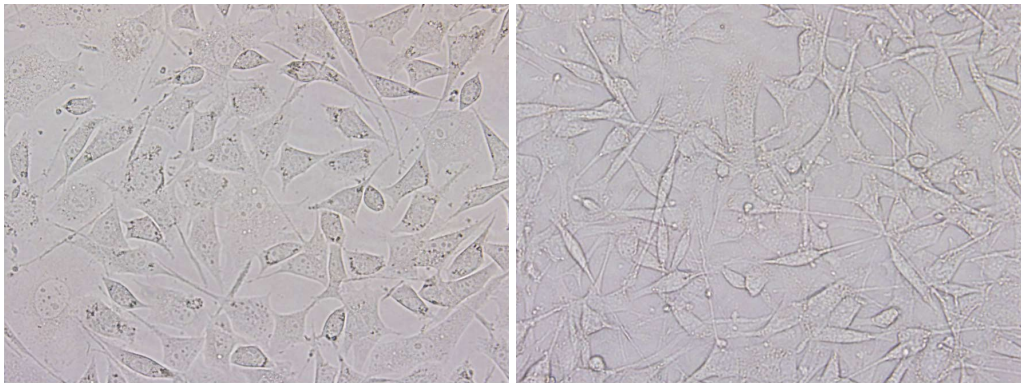

Figure 3 Morphological changes of gastric carcinoma SGC-7901 cells. A: Control; B: Co-culture group (400 ×).

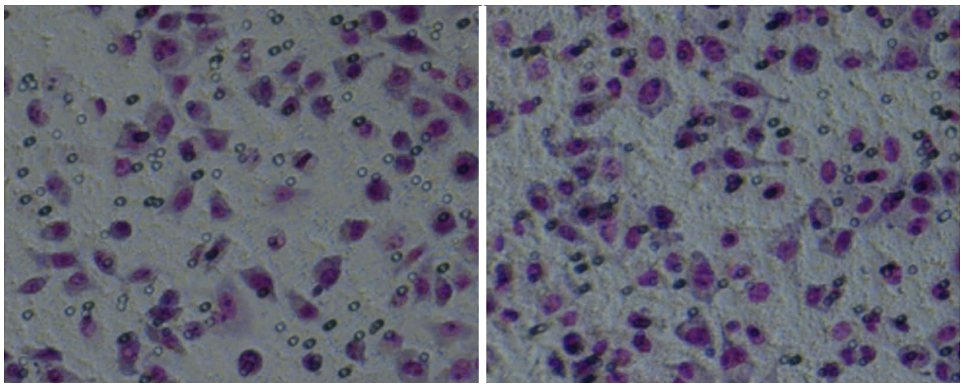

Figure 4 Tumor-associated macrophages promote SGC-7901 cell migration (200 ×, $P < 0.01$).

significant ($P < 0.01$, Figure 6).

DISCUSSION

Distant metastasis and recurrence are the leading causes of death in patients with gastric carcinoma and are critical factors that affect the clinical efficacy and prognosis[15]. Gastric carcinoma is an immunogenic tumor, and the immune microenvironment of the tumor interstitium plays a major role in the development and progression of gastric carcinoma[16]. A dynamic balance exists between normal cells and their surrounding microenvironment, both of which jointly act to regulate cell activity, proliferation, differentiation, apoptosis, and the secretion and expression of related cytokines[17]. Once this balance is broken, the normal microenvironment changes to allow the malignant transformation of the cells. With the malignant growth of tumor, tumor cells continue to invade outward to fetch nutrients, and the surrounding microenvironment gradually evolves into a special environment with tissue hypoxia, acidosis, numerous growth factors,

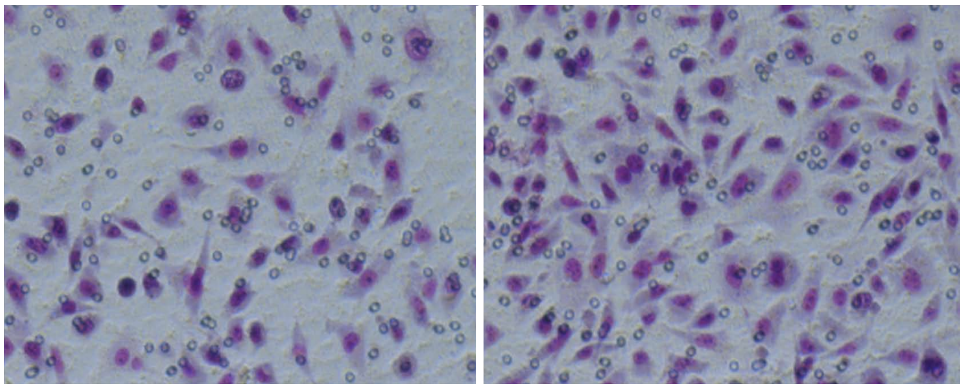

Figure 5 Tumor-associated macrophages promote SGC-7901 cell invasion (200 ×, $P < 0.01$).

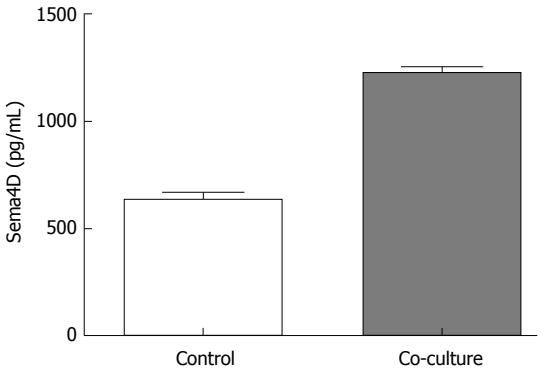

Figure 6 Expression of Sema4D protein in SGC-7901 cell supernatants of the control and co-culture experimental groups.

and proteolysis[18]. This microenvironment and the tumor cells mutually stimulate each other, which plays an important role in tumor invasion, migration, and metastasis[19-21].

The tumor microenvironment is a chronic inflammatory environment composed of inflammatory cells, endothelial cells, fibroblasts, and extracellular matrix[22]. Macrophages are an essential component of the innate immune system as well as a class of important cells involved in the chronic inflammatory response. Macrophages *in vivo* can acquire specific phenotypes from the local environment and show different functions, including phagocytosis, antigen presentation, antibacterial activity, cytotoxicity, tissue remodeling, and secretory functions[23]. TAMs are important inflammatory cells in the tumor microenvironment that are formed by monocytes in the peripheral circulating blood that migrate to the tumor microenvironment to proliferate and differentiate under the action of tumor-derived cytokines and chemokines[3]. TAMs infiltrate the interstitium of many malignancies and interact with tumor cells. TAMs also secrete growth factors and various cytokines, including matrix metalloproteinase (MMP)-2, MMP-9, VEGF, and TNF-α, to enhance the invasion and metastasis abilities of tumor cells, facilitate angiogenesis and lymphangiogenesis, and induce the immunosuppressive state of the microenvironment, ultimately promoting tumor development and progression[24]. TAMs are closely related to tumor development, progression, and prognosis[25]. CD68 is a cytoplasmic protein which is the most reliable TAMs marker[6]. In the present study, TAMs extensively infiltrated the interstitial gastric carcinoma tissues, and significantly higher expression was observed in gastric carcinoma tissues than in adjacent normal tissues. Patients with lymph node metastasis presented higher expression than those without. A lower histological differentiation level was associated with a higher positive rate of CD68 expression. No significant correlation was found with other clinicopathological features, such as patient age or tumor size. These findings suggest that TAMs are closely associated with the prognosis of gastric carcinoma.

Sema4D, also known as CD100, is an important member of the class Ⅳ semaphorin subfamily that was originally identified as a semaphorin affecting neurodevelopment[26]. Further studies found that SEMA4D could bind to the receptors cluster of differentiation 72 and Plexin B and thus play an important role in the nervous system, immune system, thrombosis, and tumor neovascularization[27]. Sema4D is another important proangiogenic factor after VEGF and is highly expressed in various tumor tissues, including head and neck squamous cell carcinoma, prostate cancer, colon cancer, breast cancer, and lung cancer[28]. Sema4D expression level is positively correlated with tumor progression level, metastasis, and resistance to radiotherapy and chemotherapy; it plays a major role in tumor development, progression, adhesion, metastasis, and invasive growth[29]. Sema4D plays a critical role in tumor angiogenesis and vascular maturation and can increase the tumorigenicity of tumor cells. In the Sema4D-deficient microenvironment, the tumorigenicity of tumor cells and their invasion and metastasis abilities are seriously weakened[30]. In the present study, we found that Sema4D was highly expressed in gastric carcinoma tissues and was closely

associated with histological differentiation type, TNM stage, and lymph node metastasis. A significant positive correlation was found between positive CD68 and Sema4D expression in gastric carcinoma tissues by Spearman's correlation analysis. This finding indicates that TAMs in gastric carcinoma tissues have a synergistic relationship with Sema4D production in gastric carcinoma, which may be an important factor promoting the invasion and metastasis of gastric carcinoma.

Based on the clinical research results, we conducted Transwell non-contact co-culture assays with TAMs and gastric cancer SGC-7901 cells. SGC-7901 cells showed significant morphological changes, with the cell body changing from the cubic shape with a blunt edge into a long spindle; meanwhile, the pseudopodium increased to varying degrees and became slender, the cell confluence declined, and individual cell migration was observed. The epithelial cell characteristics were lost, and more interstitial cell characteristics were observed, suggesting that the epithelial-mesenchymal transition (EMT) occurred to a certain degree. The EMT is the first step by which tumor cells isolated from the primary lesion achieve metastatic invasion and thus is a critical step[31]. The EMT promotes the loss of epithelial cell polarity, reduces cell adhesion, and enhances cell motility; these biological changes lead to damage to the polarity and tight junctions of the cells. Additionally, the EMT alters the cytoskeleton remodeling process, thereby enhancing tumor cell invasiveness and contributing to the acquisition of the ability to form distant metastases[31,32]. The invasion and migration assays showed that SGC-7901 cells in the co-culture group had significantly enhanced invasion and migration abilities compared to the cells in the control group. This result suggested that intercellular adhesion was reduced and that the infiltration and invasion abilities were enhanced after the morphological changes of SGC-7901 cells.

TAMs and Sema4D are highly expressed in many tumors and are associated with tumor development, progression, angiogenesis, invasion, and metastasis. Research on their functions and related mechanisms makes progress every day. Combined detection of CD68 and Sema4D proteins shows potential for predicting the progression trend of gastric carcinoma and determining the patient prognosis. Continuous in-depth study of their mechanisms is bound to inject new vitality into the prevention and treatment of tumors.

ARTICLE HIGHLIGHTS

Research background

Gastric carcinoma is one of the most common malignancies and its mortality is ranked second among cancers. The tumor microenvironment plays a major role in the invasion and metastasis of gastric carcinoma. Tumor-associated macrophages (TAMs) are an important component of the tumor microenvironment. TAMs have been shown to enhance the infiltration and metastasis ability of tumor cells by expressing certain growth factors and cytokines. TAMs also suppress immune responses in the microenvironment and promote tumor progression by facilitating tumor angiogenesis and lymphangiogenesis.

Semaphorin 4D (Sema4D) is an important member of the semaphorin subfamily and plays a major role in the nervous and immune systems. Sema4D is expressed at high levels in various tumor tissues, including head and neck squamous cell carcinoma, prostate cancer, and colon cancer, and its role in promoting tumor angiogenesis is becoming a hot research topic. Sema4D is another important proangiogenic factor following vascular endothelial growth factor.

Research motivation

The expression of Sema4D in gastric cancer and its relationship with TAMs have not been explored. If TAMs can promote Sema4D expression and influence the characters of gastric cancer cells, this will be greatly helpful to inject new vitality into the prevention and treatment of gastric cancer.

Research objectives

In the present study, we explored the effect of TAMs on Sema4D expression in gastric carcinoma tissues and its role in the development and progression of gastric carcinoma to provide a new theoretical reference for the prevention and treatment of this cancer.

Research methods

By using the immunohistochemical method, the expression of the TAM markers CD68 and Sema4D in gastric carcinoma and adjacent normal tissues from 290 patients and their relationships with clinical significance were analyzed. *In vitro*, changes of the secretory Sema4D level in SGC-7901 cells were measured using ELISA, and the effect of TAMs on SGC-7901 cell invasion and migration was assessed with invasion and migration assays, respectively.

Research results

CD68 and Sema4D protein expression was significantly higher in gastric carcinoma tissues than in adjacent normal tissues. CD68 and Sema4D protein expression was significantly associated with histological differentiation type, TNM stage, and lymph node metastasis, and their expression levels were positively correlated with one another. In the *in vitro* experiment, secretory Sema4D protein expression was significantly increased in the supernatant of SGC-7901 cells co-cultured with TAMs compared with the blank control. Cell invasion and metastasis were enhanced in the Transwell invasion and migration assays. However, the mechanism by which TAMs promote gastric cancer cells to express Sema4D is not clearly now, so we should explore the signal pathway and other mechanism in the next step to make this more clearly.

Research conclusions

In our study we found that TAMs promoted the invasion and metastasis of gastric carcinoma cells possibly through upregulated secretory Sema4D protein expression. Combined detection of TAM markers, CD68 and Sema4D, in gastric carcinoma tissue shows potential to predict the trend of gastric carcinoma progression. TAMs and Sema4D are highly expressed in many tumors and are associated with tumor development, progression, angiogenesis, invasion, and metastasis. Research on their functions and related mechanisms makes progress every day. Combined detection of CD68 and Sema4D proteins shows potential for predicting the progression trend of gastric carcinoma and determining the patient prognosis. Continuous in-depth study of their mechanisms is bound to inject new vitality into the prevention and treatment of tumors.

Research perspectives

By this study, we can learn that TAMs and Sema4D are highly expressed in gastric carcinoma and are associated with tumor development, progression, angiogenesis, invasion, and metastasis. Combined detection of CD68 and Sema4D proteins shows potential for predicting the progression trend of gastric carcinoma and determining the patient prognosis. Research on their functions and related mechanisms will make great progress in anti-tumor field. Continuous in-depth study of their mechanisms is bound to inject new vitality into the prevention and treatment of tumors.

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ORIGINAL ARTICLE

Case Control Study

Impact of SNP-SNP interactions of DNA repair gene *ERCC5* and metabolic gene *GSTP1* on gastric cancer/atrophic gastritis risk in a Chinese population

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Author contributions: Yuan Y conceived and designed the experiments and revised the manuscript; Sang L, Sun LP, Xu Q and Lv Z performed the experiments; Sang L, Lv Z and Sun LP analyzed the data; Sang L wrote the paper.

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Abstract

AIM

To investigate the interactions of the DNA repair gene excision repair cross complementing group 5 (*ERCC5*) and the metabolic gene glutathione S-transferase pi 1 (*GSTP1*) and their effects on atrophic gastritis (AG) and gastric cancer (GC) risk.

METHODS
Seven *ERCC5* single nucleotide polymorphisms (SNPs) (rs1047768, rs2094258, rs2228959, rs4150291, rs4150383, rs751402, and rs873601) and *GSTP1* SNP rs1695 were detected using the Sequenom MassARRAY platform in 450 GC patients, 634 AG cases, and 621 healthy control subjects in a Chinese population.

RESULTS
Two pairwise combinations (*ERCC5* rs2094258 and rs873601 with *GSTP1* rs1695) influenced AG risk ($P_{interaction}$ = 0.008 and 0.043, respectively), and the ERCC5 rs2094258-GSTP1 rs1695 SNP pair demonstrated an antagonistic effect, while *ERCC5* rs873601-*GSTP1* rs1695 showed a synergistic effect on AG risk OR = 0.51 and 1.79, respectively). No pairwise combinations were observed in relation to GC risk. There were no cumulative effects among the pairwise interactions (*ERCC5* rs2094258 and rs873601 with *GSTP1* rs1695) on AG susceptibility (P_{trend} > 0.05). When the modification effect of *Helicobacter pylori* (*H. pylori*) infection was evaluated, the cumulative effect of one of the aforementioned pairwise interactions (*ERCC5* rs873601-*GSTP1* rs1695) was associated with an increased AG risk in the case of negative *H. pylori* status (P_{trend} = 0.043).

CONCLUSION
There is a multifarious interaction between the DNA repair gene *ERCC5* SNPs (rs2094258 and rs873601) and the metabolic gene *GSTP1* rs1695, which may form the basis for various inter-individual susceptibilities to AG.

Key words: Excision repair cross complementing group 5; Glutathione S-transferase pi 1; Atrophic gastritis; Gastric cancer; Single nucleotide polymorphisms

Core tip: We detected seven excision repair cross complementing group 5 (*ERCC5*) single nucleotide polymorphisms (SNPs) and a glutathione S-transferase pi1 (*GSTP1*) SNP using the Sequenom MassARRAY platform in a Chinese population and used them to investigate their interactions and their effects on atrophic gastritis and gastric cancer risk. The results showed a multifarious interaction between the DNA repair gene *ERCC5* SNPs (rs2094258 and rs873601) and the metabolic gene *GSTP1* rs1695. In addition, the cumulative effect of one pairwise interaction (*ERCC5* rs873601-*GSTP1* rs1695) was associated with an increased atrophic gastritis risk in the case of negative *H. pylori* status when the modification effect of *H. pylori* infection was evaluated.

Sang L, Lv Z, Sun LP, Xu Q, Yuan Y. Impact of SNP-SNP interactions of DNA repair gene *ERCC5* and metabolic gene *GSTP1* on gastric cancer/atrophic gastritis risk in a Chinese population. *World J Gastroenterol* 2018; 24(5): 602-612 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i5/602.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i5.602

INTRODUCTION

In light of the study of gastric cancer (GC) pathogenesis, there is increasing evidence to suggest that the interactions between various inherited susceptibility genes may affect the risk of GC development in individuals[1]. Single nucleotide polymorphisms (SNPs), as one of the most general forms of genetic variation, play a key role in predicting cancer risk in individuals and are widely applied to study tumor incidence and prognostic evaluation. However, they are inadequately utilized for studies of various genes in intricate diseases such as cancer[2], and the presently investigated polymorphisms for each single gene may not entirely reveal a definite phenotype[1]. Some studies have shown that interactions among genes are more significant than solitary genes in determining cancer susceptibility[3,4].

Numerous epidemiological studies have shown that inherited polymorphisms involved in xenobiotic metabolism and DNA repair are related to GC[1,5]. These genes are acknowledged as risk-modifier indicators, especially those whose allelic polymorphisms are accountable for the repair of oxidative stress induced DNA damage and/or the impaired metabolism of exogenous carcinogens. Excision repair cross complementing group 5 (*ERCC5*) is a critical element of the nucleotide excision repair (NER) pathway, and the *ERCC5* gene is mapped to a region on chromosome 13q33 and comprises 15 exons[6]. It encodes a structure-specific endonuclease that has multiple functions during NER[7]. Its main role is to identify and shear damage to the DNA chain 3' terminus[8]. Its gene mutation may lead to abnormal cell proliferation and differentiation and increased cancer susceptibility. SNPs of *ERCC5* linked with GC susceptibility have been reported, including rs2094258, rs751402, rs2296147, rs1047768, rs873601, rs2227869, and rs17655[6,9-15]. We previously analyzed six SNPs of the *ERCC5* gene in 2686 subjects from northern China and found that the selected polymorphisms of the ERCC5 gene were not significantly associated with atrophic gastritis (AG)/GC risk[16]. Glutathione S-transferase (GST) is an important member of the phase Ⅱ metabolic enzymes, including GSTM1, GSTT1, and glutathione S-transferase pi1 (GSTP1)[17], which can affect detoxification processes and increase individual susceptibility to cancers[18]. The GSTP1 Ile105Val polymorphism produces the amino acid replacement of Ile (105) with Val *via* the change of A (Ile) to G (Val) in exon 5, which diminishes enzyme catalytic activity[6] and indirectly stimulates DNA repair and protection of the cell genome[7,8]. Our previous study also identified SNP rs1695 in *GSTP1*, which appears to drastically change the susceptibility of individuals to

GC[9]. This finding is consistent with previous studies[10,11].

Although some studies have found that *ERCC5* SNPs and *GSTP1* polymorphisms were related to GC risk, there are limited data on the effects of gene-gene interactions, and some results are equivocal[12,13]. Additionally, given the vital impact of environmental factors on the susceptibility to GC and our previous findings regarding gene interaction and environmental factors[4,14,16], we explored possible two-dimensional gene interactions among inherited polymorphisms in the DNA repair gene *ERCC5* (rs1047768, rs2094258, rs2228959, rs4150291, rs4150383, rs751402, and rs873601) and the metabolic gene *GSTP1* (rs1695), as well as the three-dimensional interactions between SNP-SNP and environmental factors in diverse stages of gastric carcinogenesis to assess the possibility of predicting GC risk and the identification of a combination of biomarkers for precancerosis and GC.

MATERIALS AND METHODS

Study population

In all, 1705 subjects were included in the present study, comprising 621 healthy controls, 634 cases of AG, and 450 cases of GC. All registered individuals originated from a Screening Program for Gastric Diseases or hospitals in Zhuanghe and Shenyang of Liaoning Province, China between 2002 and 2013, as previously described[16]. Metadata for every participant was collected using a standardized questionnaire survey and stored in a spreadsheet, including gender, age, history of illness, status of smoking, and alcohol consumption. Every participant signed a written informed consent from, according to the Declaration of Helsinki and its later revision. We collected peripheral venous blood from all participants, and experienced endoscopists simultaneously performed gastroscopic examination. All subjects received histopathological diagnosis according to the updated Sydney System[15] and the World Health Organization criteria, independently, by two gastrointestinal pathologists. This project was approved by the Human Ethics Review Committee of China Medical University (Shenyang, China).

SNP selection and genotyping assay

Briefly, as described in our previous study[16], we extracted *ERCC5* genotype data from the HapMap Chinese Han Beijing population (http://www.HapMap.org). Tag SNPs were derived from pairwise linkage disequilibrium information to maximally capture (r^2 > 0.8) common or rare variants [minor allele frequency (MAF) > 0.05] using Haploview 4.2 (http://www.broadinstitute.org/mpg/haploview). FastSNP Search was used to predict potential SNP function. Finally, a total of seven *ERCC5* SNPs (rs1047768, rs2094258, rs2228959, rs4150291, rs4150383, rs751402, and rs873601) were chosen in this study. In addition, *GSTP1* rs1695 was selected according to our previous study and literature references[9-11]. Genomic DNA was isolated from blood samples using a routine phenol-chloroform method and then diluted to working concentrations (50 ng/μL) for genotyping. Samples were placed randomly in 384-well plates and blinded for disease status. Selected SNP genotyping was performed using the Sequenom MassARRAY platform (Sequenom, San Diego, CA, United States) according to the manufacturer's instructions[16]. The average genotyping rate was 99.3% and the results of all duplicated samples were 100% consistent.

Assessment of Helicobacter pylori serology

Helicobacter pylori (*H. pylori*) immunoglobulin G levels was tested using an enzyme-linked immunosorbent assay (ELISA kit, Biohit, Helsinki, Finland) according to the manufacturer's instructions, as previously described[16]. *H. pylori* positivity was defined as a numerical reading exceeding 34 enzyme immune units.

Statistical analysis

Statistical analyses in the study were completed by applying SPSS 17.0 software (SPSS Inc., Chicago, IL, United States). We used the χ^2 test to calculate the differences in demographic characteristics and genotypes between cases and controls. The two- or three-dimensional interaction effects among SNP-SNP with or without environmental factors were estimated using multivariate logistic regression models. General linear regression modeling was used to assess the trends with an increasing number of mutation genotypes in the cumulative effect. Associations were evaluated by odds ratios (ORs) and 95% confidence intervals (CIs) adjusted by sex, age, and *H. pylori* infection status except for being stratified by *H. pylori* infection status. Two-sided *P*-values < 0.05 were considered statistically significant.

RESULTS

Demographic and geographic characteristics

The distribution characteristics of gender, age, and *H. pylori* infection status of all participants are shown in Table 1. No significant differences were found in the gender or age distribution among the case and control groups. The study subjects consisted of 634 AG patients, 450 GC patients, and two control groups, including 620 and 535 for AG and GC cases, matched by gender and age, respectively. Additionally, there were significantly higher *H. pylori* infection rates (59.5% and 49.6%, respectively) in the AG and GC groups compared to the two matched control groups (27.1% and 26.7%, respectively, *P* < 0.001).

Pairwise interactions between the ERCC5 SNPs and GSTP1 rs1695 polymorphism

We primarily examined SNP-SNP two-dimensional interaction effects in the main effect analysis using a full-factor model. Two pairwise SNP combinations

Table 1 Baseline characteristics of the subjects *n* (%)

Variable	AG *vs* CON		GC *vs* CON	
	CON	AG	CON	GC
	n = 620	*n* = 634	*n* = 535	*n* = 450
Gender	*P* = 0.492		*P* = 0.588	
Male	362 (58.4)	358 (56.5)	363 (67.9)	298 (66.2)
Female	258 (41.6)	276 (43.5)	172 (32.1)	152 (33.8)
Age	*P* = 0.845		*P* = 0.235	
mean ± SD	54.7 ± 9.1	54.8 ± 9.0	55.6 ± 9.2	56.3 ± 10.1
Median	54	55	56	57
Range	17-85	16-82	17-85	26-84
H. pylori infection status	*P* < 0.001		*P* < 0.001	
Positive	168 (27.1)	377 (59.5)	143 (26.7)	223 (49.6)
Negative	452 (72.9)	257 (40.5)	392 (73.3)	227 (50.4)

AG: Atrophic gastritis; GC: Gastric cancer; CON: Controls.

were found that could affect AG risk, but no pairwise combination was found in relation to GC risk. The results indicated that the *ERCC5* rs2094258 and rs873601 polymorphisms with *GSTP1* rs1695 polymorphism could engender interaction effects for AG risk ($P_{interaction}$ = 0.008 and 0.043 respectively, Table 2). The *ERCC5* rs2094258-*GSTP1* rs1695 SNP pair demonstrated an antagonistic effect, while ERCC5 rs873601-GSTP1 rs1695 showed a synergistic effect on AG risk (OR = 0.51 and1.79, respectively, Table 2). No significant differences were observed among other SNP-SNP interactions (P > 0.05).

Epistatic effect of two-way interactions

We further investigated epistatic effects between pairs of *ERCC5* rs2094258 and rs873601 polymorphisms with *GSTP1* rs1695. For *ERCC5* rs2094258 and *GSTP1* rs1695, the AG/AA genotypes of rs2094258 and AA genotype of rs1695 were related to an increased risk of AG, but GA/GG genotypes of rs1695 were associated with a reduced risk of AG (OR = 1.523 and 0.678, respectively). For *ERCC5* rs873601 and *GSTP1* rs1695, AA genotype of rs873601 resulted in a reduced risk of AG, only in the presence of AA genotype of rs1695 (OR = 0.678) (Table 3). These findings illustrated that *ERCC5* rs2094258 and rs873601, individually, had no main effect but did display epistatic interactions with *GSTP1* rs1695.

Cumulative effect of the interacting factors of ERCC5 SNPs-GSTP1 rs1695

We also investigated the cumulative effect among the interacting SNPs of *ERCC5* rs2094258 and rs873601 with *GSTP1* rs1695, but neither had a statistically significant relationship to AG risk (P > 0.05, Table 4). We further analyzed the cumulative effect of interacting SNPs modified by *H. pylori*. The *ERCC5* rs873601-*GSTP1* rs1695 SNP pair had significant differences in AG risk among the subgroups with negative *H. pylori* infection status (P_{trend} = 0.043). Moreover, AG risk was significantly reduced while one or two mutation genotypes were present (OR = 0.66, 95%CI: 0.37-1.16, and OR = 0.73, 95%CI: 0.53-1.02, respectively).

Three-dimensional analysis of the effect of interactions of ERCC5 SNPs-GSTP1 rs1695-environmental factors on AG risk

To explore the influence of environmental factors on the interaction, we further explored probable three-dimensional interactions among *ERCC5* SNPs (rs2094258 and rs873601), *GSTP1* rs1695, and environmental factors (smoking, alcohol consumption, and *H. pylori* infection status). We found no significant three-dimensional interactions with regard to AG risk (P > 0.05, Supplementary Table 1).

DISCUSSION

GC is an outcome of the interaction between multiple genes and environmental factors and is considered a multistep and multifactor process involving different carcinogen metabolic and DNA repair pathways[19,20]. Currently, researchers are concentrating more on the gene-gene interaction effect rather than a single-gene effect. In this study, we examined the possible interaction effect of DNA repair gene *ERCC5* SNPs and the metabolic detoxification gene *GSTP1* polymorphism. We first found new two-pair SNP interactions among *ERCC5* SNPs and the *GSTP1* polymorphism (*ERCC5* rs2094258-*GSTP1* rs1695 and *ERCC5* rs873601-GSTP1 rs1695), which could alter the susceptibility to AG compared to host genetic effects alone. Moreover, the cumulative effect resulting from two-way interaction of *ERCC5* rs873601-*GSTP1* rs1695 was shown to differ in a stratified analysis of *H. pylori* infection status. The change from no cumulative effect to significant difference in AG risk in the case of negative *H. pylori* status indicated that *H. pylori* infection status could modify the cumulative effect mentioned above for the interacting SNPs. Genetic polymorphisms may explain partial individual deviations in disease risk, but a more multifarious condition involving numerous gene-gene interactions and gene-environment characteristics must

Table 2 Impact of two-way interactions between *ERCC5* polymorphisms and *GSTP1* rs1695 on risk of atrophic gastritis and gastric cancer[1]

Gene	Genotype	Number of participants	GSTP1 rs1695			
			AA	GA + GG	AA + GA	GG
AG *vs* CON (n = 634 *vs* 620)						
ERCC5 rs1047768	TT	No. of cases/controls	231/200	124/116	338/307	17/9
		OR (95%CI)	1 (Ref.)	0.93 (0.68-1.27)	1 (Ref.)	1.72 (0.75-3.91)
	TC + CC	No. of cases/controls	177/188	102/116	270/289	9/15
		OR (95%CI)	0.82 (0.62-1.08)	0.76 (0.55-1.06)	0.85 (0.68-1.07)	0.55 (0.24-1.26)
			$P_{interaction}$ = 0.317		$P_{interaction}$ = 0.683	
			Interaction index = 0.88		Interaction index = 1.13	
	TT + TC	No. of cases/controls	376/345	207/214	559/538	24/21
		OR (95%CI)	1 (Ref.)	0.89 (0.70-1.13)	1 (Ref.)	1.10 (0.61-2.00)
	CC	No. of cases/controls	32/43	19/18	49/58	2/3
		OR (95%CI)	0.68 (0.42-1.10)	0.97 (0.50-1.87)	0.81 (0.55-1.21)	0.64 (0.11-3.86)
			$P_{interaction}$ = 0.296		$P_{interaction}$ = 0.531	
			Interaction index = 0.88		Interaction index = 0.88	
ERCC5 rs2094258	GG	No. of cases/controls	132/162	93/84	214/234	11/12
		OR (95%CI)	1 (Ref.)	1.36 (0.94-1.98)	1 (Ref.)	1.00 (0.43-2.32)
	GA + AA	No. of cases/controls	276/226	133/148	394/362	15/12
		OR (95%CI)	1.50 (1.12-2.00)	1.10 (0.79-1.53)	1.19 (0.94-1.50)	1.37 (0.63-2.99)
			$P_{interaction}$ = 0.008		$P_{interaction}$ = 0.842	
			Interaction index = 0.51		Interaction index = 1.13	
	GG + GA	No. of cases/controls	337/328	195/204	508/510	24/22
		OR (95%CI)	1 (Ref.)	0.93 (0.73-1.19)	1 (Ref.)	1.10 (0.61-1.98)
	AA	No. of cases/controls	71/60	31/28	100/86	2/2
		OR (95%CI)	1.15 (0.79-1.68)	1.08 (0.63-1.84)	1.17 (0.85-1.60)	1.00 (0.14-7.15)
			$P_{interaction}$ = 0.594		$P_{interaction}$ = 0.620	
			Interaction index = 0.83		Interaction index = 0.58	
ERCC5 rs2228959	CC	No. of cases/controls	371/346	198/215	548/539	21/22
		OR (95%CI)	1 (Ref.)	0.86 (0.67-1.09)	1 (Ref.)	0.94 (0.51-1.73)
	CA + AA	No. of cases/controls	37/42	28/17	60/57	5/2
		OR (95%CI)	0.82 (0.52-1.31)	1.54 (0.83-2.86)	1.04 (0.71-1.52)	2.46 (0.48-12.73)
			$P_{interaction}$ = 0.103		$P_{interaction}$ = 0.435	
			Interaction index = 2.00		Interaction index = 2.11	
	CC + CA	No. of cases/controls	408/383	224/231	606/590	26/24
		OR (95%CI)	1 (Ref.)	0.91 (0.72-1.15)	1 (Ref.)	1.06 (0.60-1.86)
	AA	No. of cases/controls	0/5	2/1	2/6	0/0
		OR (95%CI)	NA	1.88 (0.17-20.79)	0.32 (0.07-1.61)	NA
			$P_{interaction}$ = NA		$P_{interaction}$ = 0.720	
			Interaction index = NA		Interaction index = 1.12	
ERCC5 rs4150291	AA	No. of cases/controls	347/332	193/205	517/516	23/21
		OR (95%CI)	1 (Ref.)	0.90 (0.70-1.15)	1 (Ref.)	1.09 (0.60-2.00)
	AT + TT	No. of cases/controls	61/56	33/27	91/80	3/3
		OR (95%CI)	1.04 (0.70-1.54)	1.17 (0.69-1.99)	1.14 (0.82-1.57)	1.00 (0.20-4.97)
			$P_{interaction}$ = 0.667		$P_{interaction}$ = 0.679	
			Interaction index = 1.17		Interaction index = 0.68	
	AA + AT	No. of cases/controls	406/382	225/232	605/590	26/24
		OR (95%CI)	1 (Ref.)	0.91 (0.73-1.15)	1 (Ref.)	1.06 (0.60-1.86)
	TT	No. of cases/controls	2/6	1/0	3/6	0/0
		OR (95%CI)	0.31 (0.06-1.56)	NA	0.49 (0.12-1.96)	NA
			$P_{interaction}$ = NA		$P_{interaction}$ = 0.703	
			Interaction index = NA		Interaction index = 1.12	
ERCC5 rs4150383	GG	No. of cases/controls	365/344	197/202	539/526	23/20
		OR (95%CI)	1 (Ref.)	0.92 (0.72-1.18)	1 (Ref.)	1.12 (0.61-2.07)
	GA + AA	No. of cases/controls	43/44	29/30	69/70	3/4
		OR (95%CI)	0.92 (0.59-1.44)	0.91 (0.54-1.55)	0.96 (0.68-1.37)	0.73 (0.16-3.29)
			$P_{interaction}$ = 0.720		$P_{interaction}$ = 0.894	
			Interaction index = 1.15		Interaction index = 1.12	
	GG + GA	No. of cases/controls	406/387	226/231	606/594	26/24
		OR (95%CI)	1 (Ref.)	0.93 (0.74-1.17)	1 (Ref.)	1.06 (0.60-1.87)
	AA	No. of cases/controls	2/1	0/1	2/2	0/0
		OR (95%CI)	1.91 (0.17-21.11)	NA	0.98 (0.14-6.98)	NA
			$P_{interaction}$ = NA		$P_{interaction}$ = 0.695	
			interaction index = NA		interaction index = 1.13	
ERCC5 rs751402	CC	No. of cases/controls	191/173	97/104	281/266	7/11
		OR (95%CI)	1 (Ref.)	0.85 (0.60-1.19)	1 (Ref.)	0.60 (0.23-1.58)

	CT + TT	No. of cases/ controls	203/198	124/118	308/303	19/13
		OR (95%CI)	0.93 (0.70-1.23)	0.95 (0.69-1.32)	0.96 (0.76-1.21)	1.38 (0.67-2.86)
			$P_{interaction}$ = 0.196		$P_{interaction}$ = 0.109	
			Interaction index = 1.39		Interaction index = 2.84	
	CC + CT	No. of cases/ controls	355/324	193/196	526/500	22/20
		OR (95%CI)	1 (Ref.)	0.90 (0.70-1.15)	1 (Ref.)	1.05 (0.56-1.94)
	TT	No. of cases/ controls	39/47	28/26	63/69	4/4
		OR (95%CI)	0.76 (0.48-1.19)	0.98 (0.56-1.71)	0.87 (0.60-1.25)	0.95 (0.24-3.81)
			$P_{interaction}$ = 0.488		$P_{interaction}$ = 0.886	
			Interaction index = 1.31		Interaction index = 1.13	
ERCC5 rs873601	GG	No. of cases/ controls	126/109	57/59	178/165	5/3
		OR (95%CI)	1 (Ref.)	0.84 (0.54-1.30)	1 (Ref.)	1.55 (0.36-6.57)
	GA + AA	No. of cases/ controls	282/279	169/173	430/431	21/21
		OR (95%CI)	0.87 (0.64-1.19)	0.85 (0.61-1.18)	0.93 (0.72-1.19)	0.93 (0.49-1.76)
			$P_{interaction}$ = 0.197		$P_{interaction}$ = 0.770	
			Interaction index = 1.44		Interaction index = 0.78	
	GG + GA	No. of cases/ controls	321/279	167/177	473/441	15/15
		OR (95%CI)	1 (Ref.)	0.82 (0.63-1.07)	1 (Ref.)	0.93 (0.45-1.93)
	AA	No. of cases/ controls	87/109	59/55	135/155	11/9
		OR (95%CI)	0.69 (0.50-0.96)	0.93 (0.62-1.39)	0.81 (0.62-1.06)	1.14 (0.47-2.78)
			$P_{interaction}$ = 0.043		$P_{interaction}$ = 0.488	
			Interaction index = 1.79		Interaction index = 1.55	
GC *vs* CON (n = 450 *vs* 535)						
ERCC5 rs1047768	TT	No. of cases/ controls	142/162	78/105	204/259	16/8
		OR (95%CI)	1 (Ref.)	0.85 (0.59-1.23)	1 (Ref.)	2.54 (1.07-6.05)
	TC + CC	No. of cases/ controls	128/164	102/104	211/255	19/13
		OR (95%CI)	0.89 (0.65-1.23)	1.12 (0.79-1.59)	1.05 (0.81-1.36)	1.86 (0.89-3.85)
			$P_{interaction}$ = 0.101		$P_{interaction}$ = 0.594	
			interaction index = 1.56		interaction index = 0.73	
	TT + TC	No. of cases/ controls	247/292	158/193	374/467	31/18
		OR (95%CI)	1 (Ref.)	0.97 (0.74-1.27)	1 (Ref.)	2.15 (1.18-3.91)
	CC	No. of cases/ controls	23/24	22/16	41/47	4/3
		OR (95%CI)	0.80 (0.46-1.39)	1.63 (0.84-3.16)	1.09 (0.70-1.69)	1.67 (0.37-7.49)
			$P_{interaction}$ = 0.115		$P_{interaction}$ = 0.640	
			Interaction index = 2.07		Interaction index = 0.66	
ERCC5 rs2094258	GG	No. of cases/ controls	110/131	66/77	165/197	11/11
		OR (95%CI)	1 (Ref.)	1.02 (0.67-1.55)	1 (Ref.)	1.19 (0.51-2.82)
	GA + AA	No. of cases/ controls	160/195	114/132	250/317	24/10
		OR (95%CI)	0.98 (0.70-1.36)	1.03 (0.72-1.47)	0.94 (0.72-1.23)	2.87 (1.33-6.17)
			$P_{interaction}$ = 0.923		$P_{interaction}$ = 0.134	
			Interaction index = 0.97		Interaction index = 2.47	
	GG + GA	No. of cases/ controls	226/275	158/185	352/441	32/19
		OR (95%CI)	1 (Ref.)	1.04 (0.79-1.37)	1 (Ref.)	2.11 (1.18-3.79)
	AA	No. of cases/ controls	44/51	22/24	63/73	3/2
		OR (95%CI)	1.05 (0.68-1.63)	1.12 (0.61-2.04)	1.08 (0.75-1.56)	1.88 (0.31-11.31)
			$P_{interaction}$ = 0.801		$P_{interaction}$ = 0.834	
			Interaction index = 0.90		Interaction index = 0.81	
ERCC5 rs2228959	CC	No. of cases/ controls	247/295	164/194	380/470	31/19
		OR (95%CI)	1 (Ref.)	1.01 (0.77-1.32)	1 (Ref.)	2.02 (1.12-3.63)
	CA + AA	No. of cases/ controls	23/31	16/15	35/44	4/2
		OR (95%CI)	0.89 (0.50-1.56)	1.27 (0.62-2.63)	0.98 (0.62-1.57)	2.47 (0.45-13.58)
			$P_{interaction}$ = 0.491		$P_{interaction}$ = 0.813	
			Interaction index = 1.40		Interaction index = 1.26	
	CC + CA	No. of cases/ controls	268/322	180/208	413/509	35/21
		OR (95%CI)	1 (Ref.)	1.04 (0.80-1.35)	1 (Ref.)	2.05 (1.18-3.58)
	AA	No. of cases/ controls	2/4	0/1	2/5	0/0
		OR (95%CI)	0.60 (0.11-3.31)	NA	0.49 (0.10-2.55)	NA
			$P_{interaction}$ = NA		$P_{interaction}$ = NA	
			Interaction index = NA		Interaction index = NA	
ERCC5 rs4150291	AA	No. of cases/ controls	222/276	143/182	338/440	27/18
		OR (95%CI)	1 (Ref.)	0.98 (0.74-1.29)	1 (Ref.)	1.95 (1.06-3.61)
	AT + TT	No. of cases/ controls	48/50	37/27	77/74	8/3
		OR (95%CI)	1.19 (0.77-1.84)	1.70 (1.01-2.89)	1.36 (0.96-1.92)	3.47 (0.91-13.18)
			$P_{interaction}$ = 0.385		$P_{interaction}$ = 0.818	
			Interaction index = 1.37		Interaction index = 1.20	
	AA + AT	No. of cases/ controls	267/321	177/209	410/509	34/21
		OR (95%CI)	1 (Ref.)	1.02 (0.79-1.32)	1 (Ref.)	2.01 (1.15-3.52)
	TT	No. of cases/ controls	3/5	3/0	5/5	1/0
		OR (95%CI)	0.72 (0.17-3.05)	NA	1.24 (0.36-4.32)	NA

			$P_{interaction}$ = NA		$P_{interaction}$ = NA	
			Interaction index = NA		Interaction index = NA	
ERCC5 rs4150383	GG	No. of cases/ controls	237/288	168/180	373/451	32/17
		OR (95%CI)	1 (Ref.)	1.13 (0.86-1.49)	1 (Ref.)	2.28 (1.24-4.16)
	GA + AA	No. of cases/ controls	33/38	12/29	42/63	3/4
		OR (95%CI)	1.06 (0.64-1.74)	0.50 (0.25-1.01)	0.81 (0.53-1.22)	0.91 (0.20-4.08)
			$P_{interaction}$ = 0.060		$P_{interaction}$ = 0.497	
			Interaction index = 0.43		Interaction index = 0.55	
	GG + GA	No. of cases/ controls	270/325	180/208	415/512	35/21
		OR (95%CI)	1 (Ref.)	1.04 (0.81-1.35)	1 (Ref.)	2.06 (1.18-3.59)
	AA	No. of cases/ controls	0/1	0/1	0/2	0/0
		OR (95%CI)	NA	NA	NA	NA
			$P_{interaction}$ = NA		$P_{interaction}$ = NA	
			Interaction index = 0.90		Interaction index = NA	
ERCC5 rs751402	CC	No. of cases/ controls	114/149	82/90	180/229	16/10
		OR (95%CI)	1 (Ref.)	1.19 (0.81-1.75)	1 (Ref.)	2.04 (0.90-4.59)
	CT + TT	No. of cases/ controls	142/161	89/109	212/259	19/11
		OR (95%CI)	1.15 (0.83-1.61)	1.07 (0.74-1.55)	1.04 (0.80-1.36)	2.20(1.02-4.74)
			$P_{interaction}$ = 0.453		$P_{interaction}$ = 0.945	
			Interaction index = 0.81		Interaction index = 0.96	
	CC + CT	No. of cases/ controls	225/275	156/174	348/432	33/17
		OR (95%CI)	1 (Ref.)	1.10 (0.83-1.45)	1 (Ref.)	2.41 (1.32-4.40)
	TT	No. of cases/ controls	31/35	15/25	44/56	2/4
		OR (95%CI)	1.08 (0.65-1.81)	0.73 (0.38-1.42)	0.98 (0.64-1.48)	0.62 (0.11-3.41)
			$P_{interaction}$ = 0.409		$P_{interaction}$ = 0.241	
			Interaction index = 0.69		Interaction index = 0.32	
		No. of cases/ controls	79/91	55/53	122/141	12/3
ERCC5 rs873601	GG	OR (95%CI)	1 (Ref.)	1.20 (0.74-1.94)	1 (Ref.)	4.62 (1.28-16.76)
		No. of cases/ controls	191/235	125/156	293/373	23/18
	GA + AA	OR (95%CI)	0.94 (0.66-1.34)	0.92 (0.63-1.35)	0.91 (0.68-1.21)	1.48 (0.76-2.87)
			$P_{interaction}$ = 0.901		$P_{interaction}$ = 0.217	
			Interaction index = 0.96		Interaction index = 0.40	
		No. of cases/ controls	205/232	139/156	317/375	27/13
	GG + GA	OR (95%CI)	1 (Ref.)	1.01 (0.75-1.36)	1 (Ref.)	2.46 (1.25-4.84)
		No. of cases/ controls	65/94	41/35	98/139	8/8
	AA	OR (95%CI)	0.78 (0.54-1.13)	0.88 (0.56-1.37)	0.83(0.62-1.12)	1.18 (0.44-3.18)
			$P_{interaction}$ = 0.477		$P_{interaction}$ = 0.384	
			Interaction index = 1.25		Interaction index = 0.57	

[1]*P* for interaction, logistic regression adjusted for gender, age, and *H. pylori* infection status; Statistically significant associations were highlighted in bold ($P < 0.05$). CON: Controls; AG: Atrophic gastritis; GC: Gastric cancer; NA: Not available; GSTP1: Glutathione S-transferase pi 1; ERCC5: Excision repair cross complementing group 5.

be mentioned.

A combination of SNPs would produce synergistic or antagonistic effects compared to an SNP, which could change the susceptibility to disease[21,22]. Individually, two *ERCC5* SNPs (rs2094258 and rs873601; unpublished data) showed no effect on either AG or GC risk ($P > 0.05$). However, our findings revealed a main effect on AG risk while these polymorphisms interacted with *GSTP1* rs1695 ($P_{interaction}$ = 0.008 and 0.043, respectively). The pairwise ERCC5 rs2094258-*GSTP1* rs1695 and *ERCC5* rs873601-GSTP1 rs1695 combinations had an OR of 0.51 and 1.79, respectively, for AG risk in the above two-way interaction analysis. In all, this evidence suggests that polymorphisms harbored in *ERCC5* (rs2094258 and rs873601) had a synergistic or antagonistic effect with *GSTP1* rs1695, which could alter the risk of an individual towards AG. According to the potential mechanism of SNP-SNP interactions, the *ERCC5* gene, as an NER pathway gene, may be responsible for repairing DNA damage from biological and environmental mutagens or regular cellular metabolism. In addition, *GSTP1* as an important phase Ⅱ metabolizing xenobiotic enzyme might promote DNA damage repair through an exogenous metabolic detoxification pathway. When exogenous or endogenous carcinogens cause damage to DNA, the metabolic gene *GSTP1* removes some harmful substances through the detoxification effect and then promotes DNA damage repair to protect against carcinogenic progression. The DNA repair gene *ERCC5* can identify and incise a DNA wound on the 3′ terminus to ensure reliable repair of DNA damage[23]. The interaction polymorphisms can produce a superposition or counteracting effect. This may partly explain the epigenetic heritability loss of precancerosis risk and suggests novel insight into the multifactorial etiology of AG risk with regard to DNA repair gene *ERCC5* and xenobiotic metabolic gene *GSTP1* pathways. However, further independent studies of the molecular mechanism of SNP-SNP interactions must be performed in the future.

The SNP-SNP interaction effect of the two genes was observed as epistasis in the absence of a significant main effect[24]. The epistasis was more pronounced than one sole susceptibility gene in terms of main effects,

Table 3 Epistatic effect of pair-wise interacting factors on the risk of atrophic gastritis and gastric cancer

Interacted pair-wise SNPs	Comparison	Subset	AG *vs* CON		GC *vs* CON	
			P value	OR (95%CI)	*P* value	OR (95%CI)
ERCC5 rs2094258 interacted with *GSTP1* rs1695	*ERCC5* rs2094258 GG *vs* AG + AA	GSTP1 rs1695 AA	0.006	1.523 (1.125-2.062)	0.948	0.989 (0.704-1.388)
		GSTP1 rs1695 GA + GG	0.205	0.766 (0.508-1.157)	0.820	0.951 (0.619-1.462)
	GSTP1 rs1695 AA *vs* GA + GG	ERCC5 rs2094258 GG	0.148	1.343 (0.901-2.002)	0.808	1.055 (0.685-1.625)
		ERCC5 rs2094258 AG + AA	0.014	0.678 (0.497-0.926)	0.799	1.045 (0.745-1.465)
ERCC5 rs873601 interacted with *GSTP1* rs1695	*ERCC5* rs873601 GA + GG *vs* AA	GSTP1 rs1695 AA	0.025	0.678 (0.483-0.952)	0.148	0.756 (0.517-1.105)
		GSTP1 rs1695 GA + GG	0.380	1.230 (0.775-1.951)	0.872	0.961 (0.592-1.560)
	GSTP1 rs1695 AA *vs* GA + GG	ERCC5 rs873601 GA + GG	0.054	0.758 (0.571-1.005)	0.955	0.991 (0.730-1.346)
		ERCC5 rs873601 AA	0.226	1.356 (0.828-2.222)	0.542	1.183 (0.690-2.027)

All tests were adjusted by age, sex, and *H. pylori* infection. Statistically significant associations were highlighted in bold ($P < 0.05$). GC: Gastric cancer; AG: Atrophic gastritis; CON: Controls; ERCC5: Excision repair cross complementing group 5; GSTP1: Glutathione S-transferase pi 1.

which embody the effect of multipart interaction[4]. Multiple studies have revealed a relationship between epistasis and cancer risk[25,26]. Our previous findings also indicated epistasis by combining individual SNPs, which had no effect on disease risk at a single locus[4,14]. In the present study, for *ERCC5* rs2094258 and *GSTP1* rs1695, the AG/AA genotype of rs2094258 and AA genotype of rs1695 were related to an increased risk of AG, but GA/GG genotypes of rs1695a were associated with a reduced risk of AG. For *ERCC5* rs873601 and *GSTP1* rs1695, AA genotype of rs873601 resulted in a reduced risk of AG, only in the presence of AA genotype of rs1695. Thus, there is still little direct evidence to reveal a specific functional association among the polymorphisms of *ERCC5* and *GSTP1*. In light of previous research findings, we hypothesized an interaction effect between the DNA repair gene and xenobiotic metabolism gene by various signal pathways, and our discoveries regarding the interactions of the DNA repair *ERCC5* gene and xenobiotic metabolism *GSTP1* gene pathways may reveal the above assumption. Further synthetic and functional research on these two gene pathways will be performed to assess the interaction effect of susceptibility genes that directly affect gastric carcinogenesis.

Gastric carcinogenesis is also affected by environmental factors, in addition to genetic factors. *H. pylori* is considered a class Ⅰ carcinogen by the World Health Organization and displays carcinogenic effects mediated by poisonous components[27,28]. Our previous studies have shown that the GSTP1 Val/Val genotype with smoking, alcohol consumption, or *H. pylori* IgG (+) could considerably increase AG and GC risk, and the NER SNPs (XPA rs2808668, DDB2 rs326222, rs3781619, rs830083, and XPC rs2607775) had interactive effects with alcohol consumption and smoking on AG or GC risk. In the present study, the cumulative effect was observed to be changed in subgroups with negative *H. pylori* infection status, which could imply an effect modification by *H. pylori* infection. Moreover, AG risk was significantly reduced, while one or two mutation genotypes were present (OR = 0.66 and 0.73, respectively). This suggested that *H. pylori* should be eliminated first for positive patients, which may be beneficial for reducing susceptibility to AG. However, we further analyzed the effect of three-dimensional interactions of the *ERCC5* SNPs (rs2094258 and rs873601)-*GSTP1* rs1695-environmental factors on the risk of AG, but no interaction effect was observed among them in terms of AG risk. This may be because our present sample size was relatively small, and some genotypes were scarce. In addition, there was a significant difference in *H. pylori* infection rates between the case groups and two matched control groups ($P < 0.001$). Although we performed all tests with an adjustment for *H. pylori* infection status except for those stratified by *H. pylori*, it still may be a limitation of the study. Nevertheless, it suggested that environmental factors were indispensable, although they cannot cause cancer or precancerosis alone. Genetic susceptibility may play a vital role in gastric carcinogenesis. Further large-sample and comprehensive study of the function of environmental factors in SNP-SNP interactions of *ERCC5* and *GSTP1* is necessary and may partially remedy probable false-negative results in the study.

The present study has several limitations. First, even if our study included a relatively large sample, prospective studies consisting of larger-scale sample and multicenter surveys are necessary to validate the results of SNP-SNP interaction effects shown here. Second, since we only included one metabolic gene polymorphism (*GSTP1* rs1695) of the GSTs in this study, further studies should involve other functional tagSNPs, such as GSTM1 and/or GSTT1, which should participate in SNP-SNP interactions between the DNA repair gene and xenobiotic metabolism gene pathways. In addition, the functions and mechanisms of the mentioned SNPs of the *ERCC5* gene and *GSTP1* gene pathways were not investigated and will require additional functional and molecular experiments to clarify.

In conclusion, we found for the first time that two pairwise interacting DNA repair gene *ERCC5* SNPs (rs2094258 and rs873601) and metabolic gene GSTP1 rs1695 polymorphism combinations were related to increased or reduced AG risk. Moreover, the results

Table 4 Cumulative effect of the interacting factors of ERCC5 SNPs-GSTP1 rs1696 on atrophic gastritis risk

No. of interacting genotypes	Total population			*H. pylori*-negative subpopulation			*H. pylori*-positive subpopulation		
	Cases/controls	P^1 value	OR (95%CI)	Cases/controls	P^2 value	OR (95%CI)	Cases/controls	P^2 value	OR (95%CI)
ERCC5 rs2094258-GSTP1 rs1695 on AG risk									
0	132/162		1 (Ref.)	57/115		1 (Ref.)	75/47		1 (Ref.)
1	369/310	0.008	1.48 (1.11-1.98)	216/79	0.125	1.35 (0.92-1.97)	216/79	0.017	1.72 (1.10-2.69)
2	133/148	0.782	1.05 (0.74-1.49)	47/106	0.655	0.90 (0.56-1.44)	86/42	0.363	1.270.76-2.14)
		P_{trend} = 0.528			P_{trend} = 0.720			P_{trend} = 0.349	
ERCC5 rs873601-GSTP1 rs1695on AG risk									
0	321/279		1 (Ref.)	138/206		1 (Ref.)	183/73		1 (Ref.)
1	254/286	0.013	0.73 (0.57-0.94)	99/201	0.061	0.73 (0.53-1.02)	155/85	0.090	0.72 (0.49-1.05)
2	59/55	0.670	0.911 (0.60-1.40)	20/45	0.150	0.66 (0.37-1.16)	39/10	0.241	1.57 (0.74-3.31)
		P_{trend} = 0.156			P_{trend} = 0.043			P_{trend} = 0.907	

[1]Adjusted by sex, age, and *H. pylori* infection; [2]Adjusted by sex and age. Statistically significant associations were highlighted in bold ($P < 0.05$). CON: Controls; AG: Atrophic gastritis; ERCC5: Excision repair cross complementing group 5; GSTP1: Glutathione S-transferase pi 1.

also demonstrated a significant difference in the cumulative effect in the *H. pylori*-negative subgroup on AG risk. The conclusions inferred from the present study about the effect of interactions between genetic polymorphisms may be conductive to proposing further studies to discover gene-gene interactions between DNA repair genes with xenobiotic metabolic gene pathways in gastric carcinogenesis.

ARTICLE HIGHLIGHTS

Research background

Previous studies suggested that the interactions between various inherited susceptibility genes may affect carcinogenesis in individuals. Single nucleotide polymorphisms (SNPs) are widely applied to the research of tumor incidence and prognostic evaluation.

Research motivation

We aimed to assess gene interactions amongst inherited polymorphisms between DNA repair gene excision repair cross complementing group 5 (*ERCC5*) SNPs and glutathione S-transferase pi1 (*GSTP1*) rs1695 to explore their possibility of predicting gastric cancer (GC) risk and identify combination biomarkers for precancerosis and GC.

Research objectives

The objective was to investigate the impact of interactions of the DNA repair gene *ERCC5* with metabolic gene *GSTP1* on atrophic gastritis (AG) and GC risk.

Research methods

Seven *ERCC5* SNPs (rs1047768, rs2094258, rs2228959, rs4150291, rs4150383, rs751402, and rs873601) and *GSTP1* rs1695 SNP were detected using the Sequenom MassARRAY platform in 450 GC patients, 634 AG cases, and 621 healthy control subjects in a Chinese population.

Research results

Two pairwise combinations (*ERCC5* rs2094258 and rs873601 with *GSTP1* rs1695) influenced AG risk, and the *ERCC5* rs2094258-GSTP1 rs1695 SNP pair demonstrated an antagonistic effect while ERCC5 rs873601-GSTP1 rs1695 shown a synergistic effect on AG risk. When the effect modification of *Helicobacter pylori* (*H. pylori*) infection was evaluated, the cumulative effect of one aforementioned pairs-way interaction (ERCC5 rs873601-GSTP1 rs1695) showed a risk in the case

of negative status of *H. pylori* infection.

Research conclusions

DNA repair gene ERCC5 SNPs (rs2094258 and rs873601) and metabolic gene GSTP1 rs1695 polymorphism combinations were related to an increased or reduced AG risk. Moreover, the results also demonstrated a significant difference in the cumulative effect on AG risk in the *H. pylori*-negative subgroup.

Research perspectives

The interaction effects between genetic polymorphisms may be conductive to proposing further studies to discover gene-gene interactions between DNA repair genes and xenobiotic metabolic genes in gastric carcinogenesis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Incidence of hepatocellular carcinoma in patients with chronic liver disease due to hepatitis B or C and coinfected with the human immunodeficiency virus: A retrospective cohort study

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Abstract

AIM

To assess the incidence of hepatocellular carcinoma (HCC) in chronic liver disease due to hepatitis B virus (HBV) or hepatitis C virus (HCV) coinfected with human immunodeficiency virus (HIV).

METHODS

A retrospective cohort study was performed, including patients with chronic liver disease due to HBV or HCV, with and without HIV coinfection. Patients were

selected in the largest tertiary public hospital complex in southern Brazil between January 2007 and June 2014. We assessed demographic and clinical data, including lifestyle habits such as illicit drug use or alcohol abuse, in addition to frequency and reasons for hospital admissions via medical records review.

RESULTS

Of 804 patients were included (399 with HIV coinfection and 405 monoinfected with HBV or HCV). Coinfected patients were younger (36.7 ± 10 *vs* 46.3 ± 12.5, $P < 0.001$). Liver cirrhosis was observed in 31.3% of HIV-negative patients and in 16.5% of coinfected ($P < 0.001$). HCC was diagnosed in 36 patients (10 HIV coinfected and 26 monoinfected). The incidence density of HCC in coinfected and monoinfected patients was 0.25 and 0.72 cases per 100 patient-years (95%CI: 0.12-0.46 *vs* 0.47-1.05) (long-rank $P = 0.002$), respectively. The ratio for the HCC incidence rate was 2.98 for HIV-negative. However, when adjusting for age or when only cirrhotic are analyzed, the absence of HIV lost statistical significance for the development of HCC.

CONCLUSION

In this study, the presence of HIV coinfection in chronic liver disease due to HBV or HCV showed no relation to the increase of HCC incidence.

Key words: Hepatocellular carcinoma; Chronic hepatitis; Human immunodeficiency virus; Coinfection; Cirrhosis

Core tip: We conducted a retrospective cohort study with 804 patients with chronic viral hepatitis B or C, with and without human immunodeficiency virus (HIV) coinfection (399 HIV-coinfected and 405 monoinfected with HBV or HCV). The main objective was to assess the incidence of hepatocellular carcinoma in HIV-coinfected patients. Hepatocellular carcinoma (HCC) was observed in 36 patients (10 HIV-positive and 26 HIV-negative). When adjusted for age, the role of HIV was no longer statistical significant for the development of HCC. Moreover, when analyzing cirrhotic patients only, the HCC incidence had no difference between the groups. Only age and alcohol use were associated with risk of developing HCC.

Marcon PS, Tovo CV, Kliemann DA, Fisch P, de Mattos AA. Incidence of hepatocellular carcinoma in patients with chronic liver disease due to hepatitis B or C and coinfected with the human immunodeficiency virus: A retrospective cohort study. *World J Gastroenterol* 2018; 24(5): 613-622 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i5/613.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i5.613

INTRODUCTION

Liver disease performs an important public health issue with high costs for healthcare systems and causing a decrease in quality of life, representing the eighth cause of death in Brazil[1]. Among liver diseases, primary liver cancer receives particular attention for being the sixth most common malignant neoplasm worldwide and the second most common cause of death by cancer[2]. Hepatocellular carcinoma (HCC) is the most frequent neoplasm, and is responsible for 70%-90% of cases[2-4]. The American Cancer Society estimated 39200 new diagnoses for 2016, with 27170 deaths, affecting mostly men[5].

In general, 80%-90% of HCC cases are related to cirrhosis, regardless of etiology[2,3,6-10]. However, there is an important geographic variation with regard to HCC etiology. In endemic areas for the hepatitis B virus (HBV), such as in parts of Africa and Asia, HBV is the most common cause[8,10,11]. In the United States and in many countries of Europe, the main etiological factor is the hepatitis C virus (HCV)[12-17].

In Brazil, some authors found chronic HCV infection followed by alcohol liver disease as the most frequent causes of HCC[18,19]. Similar findings can be seen in a study conducted in Latin America[20].

The increase in HCC incidence in the past decade, along with the tendency that this increase will continue, is closely related to time of exposure to HBV and HCV[11]. Thus, the role of coinfection with the human immunodeficiency virus (HIV) has gained prominence, since the introduction of highly active retroviral therapies (HAART) against HIV has presented significant improvements in patient survival[21-35]. In this scenario, liver disease has become one of the main causes of hospitalization and death in HIV-positive patients, with HCC as a prominent factor. This phenomena is closely related to an increase in alcohol consumption and with the high prevalence of HBV and HCV in this population, which share similar transmission routes with HIV[34,36-40].

Current data suggests an unfavorable evolution both in the natural history of liver disease and in the outcome after hepatitis treatment in coinfected patients[41,42], suggesting that the incidence of cirrhosis and HCC is significantly higher in this population[34]. On the other hand, some authors found conflicting data. Recently, a study with 148 cirrhotic patients due to HCV, coinfected or not with HIV and monitored for an average period of 43 mo showed no significant difference in HCC incidence between HIV-positive and HIV-negative patients[43].

Thus, we believe it is extremely important to have data clarifying the profile of this association in our environment. Therefore, the main objective of this study is to assess the incidence of HCC in patients with chronic liver disease due to HBV or HCV and coinfected with HIV.

MATERIALS AND METHODS

Study design

A retrospective cohort study was conducted with individuals with chronic viral hepatitis B or C, with and without HIV coinfection. Patients were selected from

the viral hepatitis notification bank at the epidemiology department of Hospital Nossa Senhora da Conceição, a tertiary public care center in Porto Alegre - Brazil, between January 2007 and June 2014.

Initially, eligible patients were those with documented chronic infection by HBV or HCV. Chronic infection by HBV was considered in the presence of positive HBsAg and/or polymerase chain reaction (PCR) for HBV DNA for longer than six months. HCV chronic infection was defined by detection of the anti-HCV antibody and PCR for HCV RNA in the plasma for longer than six months. These individuals were split into two groups, according to their HIV infection status. Patients considered as infected by HIV were those with detection of anti-HIV antibodies in the plasma, repeated and confirmed via molecular method or Western blot.

Individuals under 18 years of age, patients with insufficient data on their medical records, those who did not attend monitoring exams, pregnant women, those with other types of hepatitis or who did not have at least one annual appointment during the monitoring period were excluded from the study.

All patients who were coinfected with HIV and met the inclusion criteria were included in the study; for patients who were only infected with HBV or HCV, a simple random draw was conducted using IBM® SPSS software version 22.0, for later data collection of selected patients (Figure 1).

We assessed demographic and clinical data, including lifestyle habits such as illicit drug use or alcohol abuse, in addition to frequency and reasons for hospital admissions via medical records review. The diagnosis of diabetes mellitus (DM) or glucose intolerance was done according the criteria set by the American Diabetes Association[44].

Specific treatments for viral hepatitis were investigated, with the following criteria being considered as adequate responses to HBV and sustained virological response (SVR) to HCV: HBV treatment with any oral antiviral or conventional interferon, with negative viral load after 12 mo from the start of monitoring; HCV treatment with conventional or pegylated interferon, associated or not to ribavirin and to boceprevir or telaprevir, with negative viral load after at least 12 wk from the end of treatment[34,45].

In patients coinfected with HIV, the use of HAART was assessed, with the negativation of the HIV viral load being considered as adequate response to the treatment. The occurrence of opportunistic infections was also investigated.

All patients were investigated for presence of liver cirrhosis and its complications. The cirrhosis diagnosis was established in accordance with the association of clinical and laboratory findings, abdominal and/or endoscopic images and histopathological in cases of doubt.

The HCC diagnosis was based on the typical radiologic aspect in at least one image exam (CT or MRI), following the criteria established by the European Association for the Study of the Liver (EASL)[8]. The inconclusive cases were referred for biopsy of the suspected hepatic lesion, followed by anatomopathological examination for diagnostic confirmation. In the confirmed cases of HCC, the extent of the disease was assessed according to the Milan criteria[46]. Additionally, we determined the alpha-fetoprotein value and the Child-Pugh and Model for End-Stage Liver Disease scores (MELD) at the time of HCC diagnosis.

Ethical considerations

This project was submitted to and approved by the Ethical Committees of Universidade Federal de Ciências da Saúde de Porto Alegre and of Hospital Nossa Senhora da Conceição in Porto Alegre, Brazil, following the ethical precepts of the Helsinki Declaration, revised in 2013[47]. This is a retrospective cohort study, which did not present direct intervention in the patients and with the data analyzed only in numbers; thus the informed consent statement was waived.

Statistical analysis

Continuous variables were described as mean ± SD. When Gaussian assumptions were violated, we used median and interquartile range (25 percentile to 75 percentile). Categorical variables were expressed by frequencies and percentages. The comparison of means was performed by Student's *t*-test, and in the case of asymmetric data, by its non-parametric substitute (Mann-Whitney *U* test). The comparison of categorical variables was performed by chi-square test or by Fisher's exact test when appropriate. The time for the event was calculated from the first event of medical care at the institution due to HIV and/or viral hepatitis until the final event, considered as HCC or death. The incidence of events (HCC or death) was estimated by the Kaplan-Meier method according to the presence or absence of HIV coinfection. The comparison between groups was performed by the log-rank test. Additionally, we elaborated a Cox proportional hazards model, with which we obtained the gross and adjusted rate ratios (hazard ratio) for potential confounders, followed by their respective confidence intervals. For all tests, the level of bicaudal significance of $\alpha/2 = 0.05$ was considered. Data was stored in Microsoft® Office Excel 2010 and statistically analyzed by IBM® SPSS 22.0 software. The statistical methods of this study were reviewed by Mario B. Wagner from Universidade Federal do Rio Grande do Sul - Brazil.

RESULTS

A total of 6567 medical records of patients referred to tertiary care center with viral hepatitis were analyzed; of these, 804 patients were included in the study (399 coinfected with HIV/HBV or HCV and 405 monoinfected with HBV or HCV) (Figure 1).

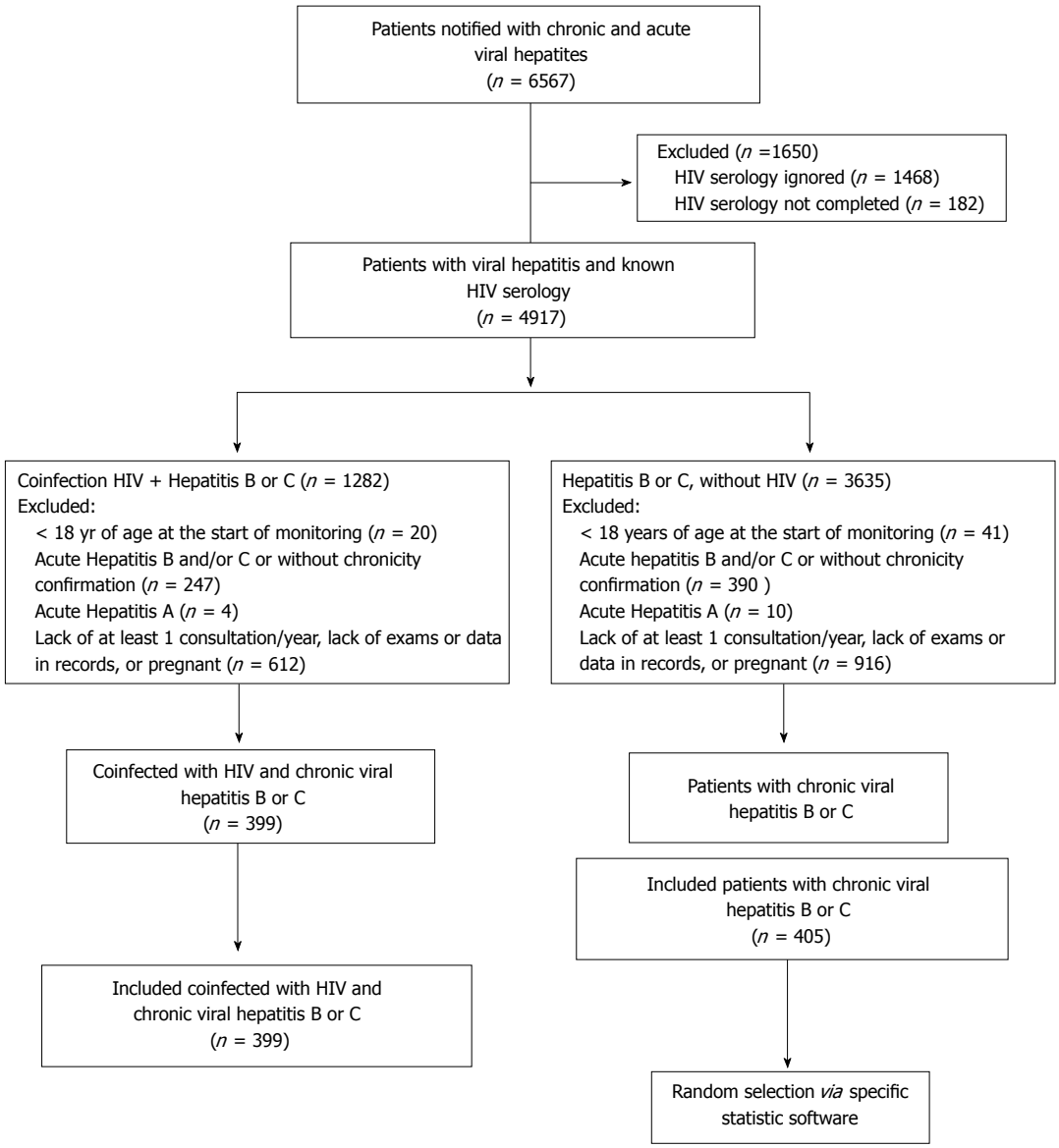

Figure 1 Fluxogram. HIV: Human immunodeficiency virus.

The general characteristics of patients are shown in Table 1, where it can be seen that those coinfected with HIV were younger, more often male and less frequently Caucasian, besides showing a lower frequency of DM. In this group of patients, the use of illicit drugs was more frequent, although there was no difference regarding alcohol abuse. There was also a higher frequency of HBV in the coinfected patients, with higher rates of treatment, but with lower response rates (54.8% *vs* 92.3%, $P = 0.012$). In turn, HIV-negative patients had a higher frequency of HCV and a higher rate of treatments performed, with SVR seen in 56.2% of these cases (56.2% *vs* 44.3%, $P = 0.083$).

Most of the coinfected patients received HAART (94.2%), with only 59% of them presenting adequate response to treatment. It was observed that approximately 64% of the coinfected patients presented some type of opportunistic infection during the monitoring period and had a larger number of hospitalizations than the HIV-negative patients [3 (1-5) *vs* 1 (0-3), $P < 0.001$]. On the other hand, the presence of hepatic cirrhosis was higher in HIV-negative patients.

When only patients with cirrhosis were analyzed, coinfected patients were also younger (40.1 ± 10.4 years *vs* 50.9 ± 11.8 years, $P < 0.001$), not Caucasian (77.3% *vs* 92%, $P = 0.006$), with less occurrences of diabetes (18.2% *vs* 39.5%, $P = 0.004$) and more occurrences of alcohol abuse (64.6% *vs* 42.6%, $P = 0.021$). There was no statistical difference regarding the presence of HBV and HCV among the groups. Regarding the treatment of HBV, it was not possible to make considerations due to the small number of cases evaluated. Regarding HCV treatment, it was observed that in both groups, less than 50% of the patients received treatment, with a higher rate of SVR in the monoinfected group (48.8% *vs* 17.6%, $P = 0.039$). Regarding cirrhosis complications, no differences were observed in the incidence of ascites, spontaneous bacterial peritonitis, hepatic

Table 1 Characteristics of patients with chronic liver disease due to hepatitis B or C virus, with and without human immunodeficiency virus

Characteristics	HIV+ (*n* = 399)	HIV- (*n* = 405)	*P* value
Age, yr	36.7 ± 10	46.3 ± 12.5	< 0.001
Male gender	249 (62.4)	197 (48.6)	< 0.001
Caucasian	285 (71.4)	352 (86.9)	< 0.001
	n = 390	*n* = 343	
Diabetes or glucose intolerance	57 (14.6)	100 (29.2)	< 0.001
	n = 247	*n* = 184	
Illicit drugs	161 (65.2)	41 (22.3)	< 0.001
	n = 283	*n* = 269	
Alcohol	119 (42.0)	96 (35.7)	0.138
	n = 399	*n* = 404	
Hepatitis B	82 (20.6)	49 (12.1)	0.002
	n = 81	*n* = 35	
Treatment	77 (95.1)	13 (37.1)	< 0.001
	n = 395	*n* = 399	
Hepatitis C	333 (84.3)	361 (90.5)	0.010
	n = 292	*n* = 296	
Genotype 1	192 (65.8)	157 (53.0)	0.005
Genotype 2	19 (6.5)	17 (5.7)	
Genotype 3	79 (27.1)	119 (40.2)	
Other[1]	2 (0.7)	3 (1.0)	
	n = 325	*n* = 338	
Treatment	102 (31.4)	151 (44.7)	< 0.001
	n = 399	*n* = 405	
Hospital admissions	359 (90.0)	249 (61.5)	< 0.001
	n = 399	*n* = 399	
Cirrhosis	66 (16.5)	125 (31.3)	< 0.001

Data presented as average ± SD or *n* (%). [1]Other related genotypes (genotype 4, genotype associations between 1 and 4, 1 and 2, 3 and 4). HIV: Human immunodeficiency virus.

encephalopathy, hepatorenal syndrome and variceal bleeding when comparing patients with and without HIV. The development of ascites (in about 60% of coinfected cirrhotic patients and 50% of HIV negative) was the most frequent complication in both groups. In cirrhotic patients, the frequency of hospital admissions was higher in the coinfected patients [3 (2-5) *vs* 2 (0-4), *P* = 0.047], but there was no statistical difference between the groups when analyzing the percentage of admissions due to hepatic causes (61.3% *vs* 76.1%, *P* = 0.072).

In general, patients were monitored for a median time of 10.54 years (95%CI: 9.58-11.50, *P* = 0.005). Coinfected patients were monitored for a median time 12.03 years (95% CI: 10.92 - 13.15), while monoinfected patients were monitored for a median time of 8.57 years (95%CI: 7.19-9.94), *P* = 0.005. The total follow-up was 7.498.8 patient-years (3.901.9 patient-years in HIV-positive patients and 3.596.9 patient-years in HIV-negative patients).

All patients who developed HCC had liver cirrhosis at the time of diagnosis. These patients' characteristics are presented in Table 2, where it can be observed that the co-infected population was younger and had higher alpha-fetoprotein levels. There was no difference in other aspects evaluated.

The development of HCC was observed in 36 patients - 10 cases in HIV-positive patients and 26 in HIV-negative patients, resulting in a cumulative incidence 2.5% e 6.4%, respectively. The incidence density of HCC in coinfected and monoinfected patients was 0.25 cases per 100 patient-years (95%CI: 0.12-0.46) and 0.72 cases per 100 patient-years (95%CI: 0.47-1.05) (long-rank *P* = 0.002), respectively (Table 3). The ratio of incidence rates of HCC of HIV negative when compared to HIV positive was 2.98. When adjusted for age, the role of HIV is no longer statistical significant for the development of HCC (Table 3).

When analyzing cirrhotic patients only, we observed an HCC incidence density of 1.54 cases per 100 patient-years (95%CI: 0.74-2.83) in coinfected patients and 2.31 cases per 100 patient-years (95%CI: 1.51-3.38) in monoinfected patients (long-rank p = 0.202) (Table 4). The HCC incidence rate in this case becomes 1.60, with no statistical significance (95%CI: 0.77-3.32, *P* = 0.207) (Table 4).

Among the factors analyzed in cirrhotic patients, the only ones that presented statistical significance for the risk of developing HCC were age and alcohol use; in this sample, DM was unable to demonstrate such association (Table 5).

DISCUSSION

The arrival of HAART against HIV in the 90s directly impacted the natural history of HIV infection. The improvement in the immunity and, consequently, the survival of these patients led to a decrease in diseases

Table 2 Characteristics of patients with hepatocellular carcinoma[1]

Characteristics	HIV+ (n = 10)	HIV- (n = 26)	P value
Age, yr	42.1 ± 8.3	53.7 ± 10.6	0.004
Male gender	8 (80.0)	18 (69.2)	0.689
Caucasian	9 (90.0)	24 (92.3)	> 0.999
Diabetes or glucose intolerance	2 (20.0)	10 (41.7)	0.432
	n = 9	n = 22	
Alcohol	7 (77.8)	10 (45.5)	0.132
	n = 10	n = 26	
Hepatitis B	1 (10.0)	2 (7.7)	> 0.99
Hepatitis C	9 (90.0)	24 (92.3)	> 0.99
Child- Pugh	n = 8	n = 25	
A	2 (25.0)	11 (44.0)	0.735
B	5 (62.5)	11 (44.0)	
C	1 (12.5)	3 (12.0)	
	n = 7	n = 22	
MELD	13.7 ± 5.1	10.9 ± 4.2	0.157
	n = 10	n = 26	
Milan criteria[2]	3 (30.0)	14 (53.8)	0.157
	n = 10	n = 24	
Alpha-fetoprotein, ng/mL	276 (23-18750)	20 (4-113)	0.038

Data presented as average ± SD or n (%). [1]All patients who developed hepatocellular carcinoma in this study had liver cirrhosis; [2]Patients with hepatocellular carcinoma satisfying the following criteria: single lesion < 5 cm or up to three lesions < 3 cm, without vascular tumor invasion and/or distant metastasis. HIV: Human immunodeficiency virus; MELD: Model for End-Stage Liver Disease.

Table 3 Incidence of hepatocellular carcinoma in patients with chronic liver disease due to hepatitis B or C, with and without human immunodeficiency virus

HIV	n	HCC	%	Patient-years	Rate × 100 patient-years	RR	95%CI	P value
+	399	10	2.5	3963.80	0.25	-	-	-
-	405	26	6.4	3624.50	0.72	2.98	1.43-6.18	0.003
Model 2: adjusted for age						1.29	0.58-2.87	0.529
Model 3: adjusted for age and DM						1.27	0.56-2.88	0.571
Model 4: adjusted for age, DM and alcohol						1.23	0.52-2.95	0.638

HIV: Human immunodeficiency virus; HCC: Hepatocellular carcinoma; RR: Rate ratio; DM: Diabetes mellitus.

related to the human immunodeficiency syndrome (which would invariably lead to death within only a few months). This has led to an increase in the incidence of diseases not directly related to HIV, especially neoplasms and liver disease[24,34,35,38,42,43,48,49]. In this scenario, HBV, HCV and alcohol abuse - factors often related to HIV infection - deserve attention[9,49-51].

In recent years, evidence suggests that HIV infection might be related to negative consequences on the progression of liver diseases, particularly increasing the risk of HCC. Puoti *et al*[51] observed an unfavorable evolution of liver disease in patients with HIV, with an increase in HCC cases, mainly associating this diagnosis with HCV infection. In 2008, a case-control study associated the development of HCC in coinfected patients with low CD4 levels, demonstrating the influence of HIV-related immunodeficiency on the development of this neoplasm[52]. Beretta *et al*[42] (2011) reported a younger profile of patients coinfected with HIV and HCC patients, with a clear deterioration in the survival rates of these patients, inferring that the presence of HIV would accelerate the process of carcinogenesis. Several other authors have also suggested that the presence of HIV may increase the risk of developing HCC, even after treatment for viral hepatitis, raising questions about the role of the HIV virus or the drugs involved in its treatment in hepatocarcinogenesis, something that has not been properly clarified[34,48,53,54].

The present study found no significant association between the presence of HIV and the development of HCC. When evaluating the cohort as a whole, we found lower cumulative incidence rates in coinfected patients (2.5% *vs* 6.4%). However, the group of monoinfected patients were 10 years older than coinfected patients, which suggests a longer time of infection/exposure to viral hepatitis. This is in line with the findings of Beretta *et al*[42] (2011). On the other hand, when we corrected for age, no difference was observed in the incidence between HIV-positive and HIV-negative patients. Similarly, when we analyzed the subgroup with liver cirrhosis only, no difference in the incidence of HCC between monoinfected and coinfected patients was seen.

Table 4 Incidence of hepatocellular carcinoma in cirrhotic patients

HIV	*n*	HCC	%	Patient-years	Rate of 100 patient-years	RR	95%CI	*P* value
+	66	10	15.2	649.75	1.54	-	-	-
-	125	26	20.8	1125.88	2.31	1.60	0.77-3.32	0.207

Table 5 Incidence of hepatocellular carcinoma in cirrhotic patients adjusted for Human immunodeficiency virus, age, diabetes mellitus and alcohol

Characteristic	RRc	95%CI	*P* value	RRa	95%CI	*P* value
HIV	1.60	0.77-3.32	0.207	0.60	0.23-1.62	0.313
Age (by 10 yr)	1.80	1.75-1.86	< 0.001	2.20	2.12-2.30	0.001
DM	0.96	0.48-1.95	0.913	0.97	0.44-2.14	0.930
Alcohol	1.29	0.64-2.62	0.480	2.31	1.02-5.23	0.046

HIV: Human immunodeficiency virus; HCC: Hepatocellular carcinoma; RRc: Crude rate ratio; RRa: Adjusted rate ratio; DM: Diabetes mellitus.

Other authors have also been unable to demonstrate a higher incidence of HCC in patients coinfected with HIV and HBV/HCV. Smukler *et al*[55] observed that despite an increase in the incidence of certain neoplasms in HIV-positive patients after the introduction of HAARTs, the incidence of HCC had no significant changes. Later, Kramer *et al*[56], in a retrospective cohort study, found no association between coinfection and HCC, regardless of the use of HAART. Likewise, García-García *et al*[57], in a study with over 1000 patients, comparing monoinfected patients with HCV and coinfected patients with HIV/HCV, observed a higher incidence of HCC in the group of patients without HIV. More recently, Benedetto *et al*[43] found similar results when, in a prospective cohort study with cirrhotic patients, they investigated 69 patients monoinfected with HCV and 79 patients coinfected with HIV/HCV. Patients in this study were monitored for an average of 43 mo, with an incidence density of 1.54 cases per 100 patient-years in the coinfected group and 3.03 cases per 100 patient-years in the monoinfected group, which are very similar numbers to those found in the present study, with the difference that our average monitoring was 126 mo. The Argentine study also found younger patients coinfected with HIV, which leads us to speculate that the time of exposure to hepatic injury factors, such as HBV and HCV, is much more relevant to the development of HCC than the presence of HIV *per se*.

Alcohol abuse is traditionally associated with the development and worsening of liver disease, both in HIV-positive and HIV-negative patients[34,51,58]. Data from the United States shows a prevalence of up to 35% of alcohol abuse in HIV-infected patients[34]. In the present study, the importance of this association was confirmed, as alcohol abuse was a risk factor for the development of HCC in cirrhotic patients.

In the present study, although around 94% of HIV-positive patients received some type of HAART, only 59% obtained an adequate response to treatment, probably due to the abandonment of the proposed therapy. This also explains the high incidence of opportunistic infections (64% presented some type of opportunistic infection during monitoring) as well as the large number of hospital admissions for these individuals.

Some limitations of the present study should be mentioned, such as those inherent to retrospective studies and regarding data collection from potentially incomplete medical records. In addition, because of the low HBV and HCC number , some analyzes were impaired, such as the subanalysis of the incidence of HCC categorized by the presence of HBV or HCV separately or by the SVR after treatment of hepatitis.

In conclusion, the data suggests that HIV coinfection in patients with chronic hepatitis related to HBV or HCV does not play a relevant role in the development of HCC.

ARTICLE HIGHLIGHTS

Research background

Following the introduction of highly effective antiviral therapy (HAART) against human immunodeficiency virus (HIV), the survival of affected patients improved considerably. In this scenario, liver diseases have gained prominence, especially viral hepatitis and hepatocellular carcinoma (HCC). Current data suggest an unfavorable evolution both in the natural history of liver disease and in the outcome after hepatitis treatment in coinfected patients.

Research motivation

The data about the outcomes in HIV/viral hepatitis association are conflicting, especially with regard to the incidence of HCC, mainly in South America. Thus, we believe it is extremely important to have data clarifying the profile of this association in our environment.

Research objectives

The main objective of this study is to assess the incidence of HCC in patients with chronic liver disease due to HBV or HCV and coinfected with HIV. These data are extremely important in order to implement more appropriate policies of HCC screening and prevention in this population.

Research methods

A retrospective cohort study was conducted with individuals with chronic viral hepatitis B or C, with and without HIV coinfection. Patients were selected from the viral hepatitis notification bank at the epidemiology department of Hospital Nossa Senhora da Conceição, a tertiary public care center in Porto Alegre - Brazil, between January 2007 and June 2014. These individuals were split into two groups, according to their HIV infection status. Individuals under 18 years of age, patients with insufficient data on their medical records, those who did not

attend monitoring exams, pregnant women, those with other types of hepatitis or who did not have at least one annual appointment during the monitoring period were excluded from the study. We assessed demographic and clinical data, including lifestyle habits, specific treatments for viral hepatitis, HAART use in patients coinfected with HIV, presence of liver cirrhosis and its complications and the HCC diagnosis.

Research results

A total of 6567 medical records of patients referred to this tertiary care center with viral hepatitis were analyzed; of these, 804 patients were included in the study (399 coinfected with HIV/HBV or HCV and 405 monoinfected with HBV or HCV). In general, patients were monitored for a median time of 10.54 years (95%CI: 9.58-11.50, P = 0.005). The total follow-up was 7498.8 patient-years (3901.9 patient-years in HIV-positive patients and 3596.9 patient-years in HIV-negative patients). The development of HCC was observed in 36 patients - 10 cases in HIV-positive patients and 26 in HIV-negative patients. All patients who developed HCC had liver cirrhosis at the time of diagnosis. The incidence density of HCC in coinfected and monoinfected patients was 0.25 cases per 100 patient-years (95%CI: 0.12-0.46) and 0.72 cases per 100 patient-years (95%CI: 0.47-1.05) (long-rank P = 0.002), respectively. The ratio of incidence rates of HCC of HIV negative when compared to HIV positive was 2.98. When adjusted for age, the role of HIV is no longer statistical significant for the development of HCC.

Research conclusions

The present study found no significant association between the presence of HIV and the development of HCC. The data from this study suggests that the time of exposure to hepatic injury factors, such as HBV and HCV, is much more relevant to the development of HCC than the presence of HIV *per se*.

Research perspectives

The present study demonstrates the need to study more deeply the consequences of the association of HIV with chronic liver diseases, especially in the era of HAART. The data presented here indicate the need for further prospective studies to better evaluate the consequences of HIV/viral hepatitis coinfection.

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ORIGINAL ARTICLE

Retrospective Study

Impaired granulocyte-macrophage colony-stimulating factor bioactivity accelerates surgical recurrence in ileal Crohn's disease

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Informed consent statement: Adult patients are recruited in a consecutive fashion by the Washington University Digestive Diseases Research Tissue Procurement Facility and provide verbal and written consent for chart abstraction, blood, stool, tissue biopsies and/or surgical waste collection with analysis for research purposes and for their information to be stored in the hospital database. The IRB at Washington University approved this consent procedure.

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Abstract

AIM

To examine the relationship between elevated granulocyte-macrophage colony-stimulating factor (GM-CSF) auto-antibodies (Ab) level and time to surgical recurrence after initial surgery for Crohn's disease (CD).

METHODS

We reviewed 412 charts from a clinical database at tertiary academic hospital. Patients included in the study had ileal or ileocolonic CD and surgical resection of small bowel or ileocecal region for management of disease. Serum samples were analyzed for serological assays including GM-CSF cytokine, GM-CSF Ab, ASCA IgG and IgA, and genetic markers including SNPs rs2066843, rs2066844, rs2066845, rs2076756 and rs2066847 in NOD2, rs2241880 in ATG16L1, and rs13361189 in IRGM. Cox proportional-hazards models were used to assess the predictors of surgical recurrence.

RESULTS

Ninety six percent of patients underwent initial ileocecal resection (ICR) or ileal resection (IR) and subsequently 40% of patients required a second ICR/IR for CD. GM-CSF Ab level was elevated at a median of 3.81 mcg/mL. Factors predicting faster time to a second surgery included elevated GM-CSF Ab [hazard ratio (HR) 3.52, 95%CI: 1.45-8.53, P = 0.005] and elevated GM-CSF cytokine (HR = 2.48, 95%CI: 1.31-4.70, P = 0.005). Factors predicting longer duration between first and second surgery included use of Immunomodulators (HR = 0.49, 95%CI: 0.31-0.77, P = 0.002), the interaction effect of low GM-CSF Ab levels and smoking (HR = 0.60, 95%CI: 0.45-0.81, P = 0.001) and the interaction effect of low GM-CSF cytokine levels and ATG16L1 (HR = 0.65, 95%CI: 0.49-0.88, P = 0.006).

CONCLUSION

GM-CSF bioavailability plays a critical role in maintaining intestinal homeostasis. Decreased bioavailability coupled with the genetic risk markers and/or smoking results in aggressive CD behavior.

Key words: Inflammatory bowel disease; Granulocyte-macrophage colony-stimulating factor antibody; Crohn's disease; Surgery

Core tip: This retrospective study assesses the risk of surgery for management of ileal Crohn's disease (CD) among patients with elevated granulocyte-macrophage colony-stimulating factor (GM-CSF) auto-antibodies (Ab). In this cohort, 396 subjects underwent initial ileocecal resection or ileal resection for management of disease. Subsequently 165 patients (41.7%) required a second ICR or IR. Factors predicting faster time to a second surgery were elevated GM-CSF Ab and elevated GM-CSF cytokine. Patients with low GM-CSF cytokine levels and the protective allele for ATG16L1 had longer intervals between a first and second surgery. To improve long term outcomes for patients with Ileal CD we need to optimize therapeutic options for patients with low GM-CSF bioavailability and genetic risk markers for ATG16L1.

Gathungu G, Zhang Y, Tian X, Bonkowski E, Rowehl L, Krumsiek J, Nix B, Chalk C, Trapnell B, Zhu W, Newberry R, Denson L, Li E. Impaired granulocyte-macrophage colony-stimulating factor bioactivity accelerates surgical recurrence in ileal Crohn's disease. *World J Gastroenterol* 2018; 24(5): 623-630 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i5/623.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i5.623

INTRODUCTION

The incidence and prevalence of Crohn's disease (CD) continues to rise with the highest rates reported to be 20.2 per 100000 person-years and 319 per 100000 persons in North America[1]. This chronic inflammatory disease is characterized by a transmural inflammation and can involve any region of the gastrointestinal tract. The transmural involvement particularly involving the ileum, often leads to fibrosis, luminal narrowing, and fistulas. In one population based cohort study of adult patients with CD, the cumulative probability of major abdominal surgery was 38%, 48%, and 58% at 5, 10, and 20 years after diagnosis, respectively[2]. Endoscopic recurrence after the initial ileocolic resection for CD may occur in up to 73% of patients within the first year after surgery[3]. Further, disease recurrence resulting in a second surgery for CD may occur in 31% to 50% of patients within 10 years of the initial surgery[4,5].

A growing body of literature supports the use of serological and genetic markers to determine the prognosis for patients with increased risk for surgical recurrence. Granulocyte macrophage colony-stimulating factor (GM-CSF) is a cytokine that promotes myeloid cell development and maturation. In mice, deficiency of this important hematopoietic growth factor can contribute to mucosal inflammation and immunodeficiency[6,7]. Clinical trials of recombinant GM-CSF in CD have demonstrated that it may benefit a subset of CD patients[8-10]. Conversely, as we and others have previously shown, neutralizing GM-CSF auto-antibodies (Ab) are associated with a reduced bioactivity of GM-CSF, impaired neutrophil bacterial killing, and increased rates of intestinal resection

for ileal CD[7,11,12].

In the present study, we examined the predictive capacity of GM-CSF Ab in surgical recurrence rates after initial ileocolic resection for ileal CD. We also evaluated other clinical, serologic and genetic prognostic factors that might define the subset of patients with shorter time to surgical recurrence.

MATERIALS AND METHODS

Study population

After obtaining institutional review board approval, patients with CD enrolled between January 2005 and October 2015 at Washington University Medical Center in St. Louis, MO, were identified from a prospectively maintained database of Inflammatory Bowel Disease patients at the Digestive Diseases Research Core Centers (DDRCC). A retrospective chart review was performed on all patients. Patients were excluded if the pathology report was not consistent with a diagnosis of CD. The retrospective chart review included surgical and gastroenterology office notes, surgical and endoscopic operative reports, hospital admission and discharge notes, pathology reports, and radiology studies.

Study design

Patients included in the study had ileal or ileocolonic CD and surgical resection of small bowel or ileocecal region for management of disease. The diagnosis of CD was based on established clinical, radiological, endoscopic and histopathological criteria. The characteristics recorded at baseline included, age, gender, race/ethnicity, duration of disease, location and behavior according to the Montreal classification[13], smoking history, number and type of surgery, and exposure to IBD therapies. We also extracted available data for infection with *Clostridium difficile* (*C. diff*).

DNA samples were obtained from peripheral whole blood samples collected at enrollment in the registry. Samples were genotyped using the Illumina Golden Gate custom Immunochip array. Genetic analysis focused on SNPs rs2066843, rs2066844, rs2066845, rs2076756 and rs2066847 in NOD2, rs2241880 in ATG16L1, and rs13361189 in IRGM. These polymorphisms have a role in bacteria sensing and autophagy.

Serum concentrations of GM-CSF cytokine and Ab were quantified by enzyme-linked immunosorbent assay (ELISA) as previously described[7]. Based upon prior studies elevated serum GM-CSF Ab was defined as $\geqslant$ 1.6 mcg/mL.

ELISA for detection of anti-Saccharomyces cerevisiae antibodies

QUANTA Lite (INOVA Diagnostics, Inc., San Diego, CA, United States) ASCA IgG and IgA enzyme-linked immunosorbent assays (ELISA) were used for determination of serum anti-*Saccharomyces cerevisiae antibodies* (ASCAs) levels as described by the manufacturer's instructions. Briefly, 100 microliters of patient's serum at a dilution of 1:100 were added to 96-well polystyrene microwell plates adhered with partially purified and disrupted Saccharomyces cerevisiae antigen. Bound ASCAs were detected by incubation with horseradish peroxidase IgG or IgA conjugate (goat anti-human). The absorbance (optical density, OD) was read at 450 nm using a SpectraMax MiniMax Imaging Cytometer. On each plate a high and a low positive, as well as a negative control were included. ASCA reactivity was determined by the formula: sample OD/low positive OD $\times$ 25. The positive cut off values for both ASCA IgA and IgG as set by the manufacturer were 25 Units (U)/mL.

Statistical analysis

The statistical methods of this study were reviewed by Wei Zhu, PhD at Stony Brook University. Descriptive statistics of demographic variables were generated using Graph Pad Prism 5.04 for Windows, GraphPad Software, San Diego, CA, United States.

In order to detect risk factors associated with early surgical recurrence, a Cox proportional hazard model[14] was fitted with main effects and first-order interactions of clinical and genotype variables. A stepwise variable selection based on Bayesian information criteria (BIC) was performed to select the relevant subset of variables. Within the model, p value was adjusted as previously described by Li *et al*[15], with the cutoff at FDR < 0.05. Model fitting and selection were generated using R 3.1.1 (http://cran.r-project.org).

RESULTS

Patient demographic and clinical characteristics

The study group included 412 adult patients with CD and a prior history of surgery for management of disease (Table 1). The mean age of CD patients at the time of this study was 49.9 ± 14.5 years. The mean disease duration was 22.5 ± 12.5 years. The majority of patients (50.26 %) were smokers.

The first surgical procedure for IBD management occurred at a median of 3 years post diagnosis. Most patients underwent intestinal resection with ileocolonic anastomosis. Ileocecal resection (ICR) was performed in 370/412 (90%) patients and ileal resection (IR) was performed in 26/412 patients (6.3%). The remaining 3.2% of patients underwent other intestinal surgeries including total colectomy, ileostomy creation, upper small bowel surgery, partial colectomy, proctectomy, diverting colostomy, and enterofistula repair. Two hundred and fifty eight patients (62.62%) underwent the first surgery for management of CD prior to enrollment in the registry in 2005. Table 2 outlines the patient characteristics by Montreal Classification[13]. The mean age at diagnosis of CD patients was 27.8 ± 11.7 years. Chart review revealed that 105 patients

Table 1 Demographic characteristics of the subjects

Patient characteristics (n = 412)	n (%)
Gender, Female	242 (58.74)
Race, White	368 (89.3)
Age, mean ± SD	49.88 ± 14.49
Smoking	
Current smoker	180 (43.69)
Ex-smoker	27 (6.57)
Never smoker	203 (49.27)
Surgery for CD[1]	
First Ileocolic resection	370 (89.80)
First Ileal resection	26 (6.31)
Second Ileocolic resection	152 (36.89)
Second Ileal resection	13 (3.15)
Exposure to medication	
Immunomodulators pre-T1	105 (25.48)
Anti-TNF agent pre-T1	80 (19.41)
Immunomodulators pre-T2	187 (45.39)
Anti-TNF agent pre-T2	120 (29.13)

[1]3.2% of patients underwent other intestinal surgeries as noted in the results section of the text. CD: Crohn's disease.

Table 2 Phenotypes by Montreal classification

	n (%)
Age at diagnosis	
A1 (⩽ 16)	56 (13.63)
A2 (17-40)	296 (72.57)
A3 (> 40)	60 (14.6)
Disease location	
L1 ileal	279 (67.71)
L2 colonic	2 (0.485)
L3 ileocolonic	131 (31.87)
L4 upper GI disease	50 (12.17)
p perianal disease	125 (30.41)
Disease behavior	
B1 non-stricturing, non-penetrating	35 (08.49)
B2 stricturing	167 (40.63)
B3 penetrating	197 (47.93)

(25.48%) had exposure to Immunomodulator therapy (Azathioprine, 6-mercaptopurine or Methotrexate) prior to the first surgery. In addition, 80 patients (19.41%) had exposure to Infliximab or Adalimumab prior to the first surgery.

Prevalence of GM-CSF Ab and ASCA

Serum samples were drawn from 254 and 150 patients before and after the first ICR/IR surgery for management of CD, respectively. Similarly serum samples were drawn from 98 and 67 patients before and after the second ICR/IR surgery, respectively. The serum GM-CSF Ab level was elevated at a median (interquartile range, IQR) of 3.81 (0.93-11.01) mcg/mL and consistent with previous studies. There were 273 (66.4%) CD cases with GM-CSF Ab level ⩾ 1.6 mcg/mL. When we applied the positive cut off values for both ASCA IgA and IgG as set by the manufacturer, 25 U/mL, 313 / 412 (76%) of patients were positive for ASCA IgA and 334 / 412 (81%) were positive for ASCA IgG. Two hundred and eighty seven (70%) of patients were concordantly positive, 35 (8%) patients were concordantly negative, whereas 16 (4%) of patients were positive only for ASCA IgA and 28 (7%) only for ASCA IgG. The mean, median and range values of all four serological assays, ASCA IgA, IgG, GM-CSF cytokine and GM-CSF Ab are shown in Table 3.

Surgical recurrence after initial ICR or small bowel resection and associated risk or protective factors

A total of 224 patients (54.5%) required two or more IBD related surgical procedures. One hundred and twenty one patients (29.37%) underwent the second surgery for management of CD prior to enrollment in the registry in 2005. Chart review revealed that 187 patients (45.39%) had exposure to Immunomodulator therapy (Azathioprine, 6-mercaptopurine or Methotrexate) prior to a second surgery. In addition, 80 patients (29.13%) had exposure to Infliximab or Adalimumab prior to a second surgery. Time to the second surgery was significantly shorter in patients with structuring (B2) or penetrating (B3) disease behavior as shown in Figure 1.

Factors predicting a faster time to a second surgery were assessed by survival analysis using the Cox proportional hazard model to test for association with gender, disease phenotype, smoking status, serological assays, IBD polymorphisms and use of medications (Immunomodulators or anti-TNF) between the first and second surgery (Table 4). All serological assays (GM-CSF cytokine, GM-CSF Ab, ASCA IgA and ASCA IgG) were examined as continuous, log transformed variables while the remaining variables were included as categorical variables.

A total of 333 subjects were included in this analysis after removal of subjects who did not have ICR/IR and those with missing data. Patients with high GM-CSF cytokine or Ab levels had a significantly shorter time to a second surgery compared to those with low levels. Patients with exposure to immunomodulators between first and second surgery had significantly longer intervals to the second surgery.

When examining the interaction of GM-CSF with all the other variables patients with protective alleles for ATG16L1 and low GM-CSF cytokine levels had significantly longer intervals to the second surgery. Patients with the protective allele for ATG16L1 and low GM-CSF Ab levels also experienced a longer interval to the second surgery but this was not statistically significant. The other polymorphisms for the genes NOD2 and IRGM did not have significant correlation with time to the second surgery.

A positive smoking history did not correlate with a faster time to second surgery. The median GM-CSF Ab levels (Figure 2) was significantly lower in smokers when compared to non-smokers ($P < 0.0001$) and significantly lower when compared to ex-smokers ($P < 0.01$). In addition smokers who expressed low GM-CSF Ab has significantly longer intervals to the second

Table 3 Values for serological assays

Serological assays	Mean ± SD	Median (IQRs)	Range
GM-CSF cytokine, pg/mL	3.18 ± 8.61	1.28 (0.43-3.05)	0.0052-136.5
GM-CSF Ab, mcg/mL	10.64 ± 19.76	3.81 (0.93-11.01)	0.08-241.2
ASCA IgA, U/mL	95.21 ± 77.38	67.51 (27.86-174.4)	1.92-242.8
ASCA IgG, U/mL	69.79 ± 41.09	66.86 (31.76-104.2)	3.17-158.1

GM-CSF Ab: Granulocyte-macrophage colony-stimulating factor auto-antibodies; ASCA: Anti-*Saccharomyces cerevisiae* antibodies; CD: Crohn's disease.

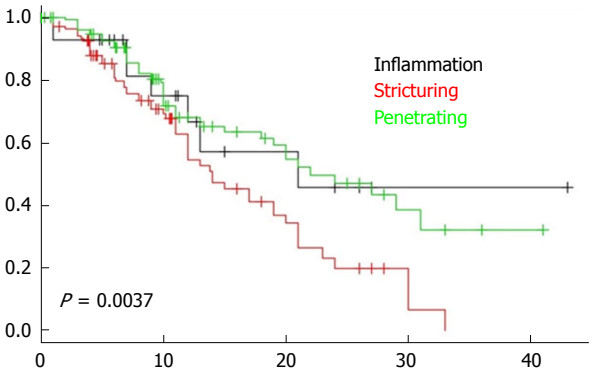

Figure 1 Comparison of survival Kaplan-Meier curves of patients according to disease behavior. Log-rank test, $P = 0.0037$. CD: Crohn's disease.

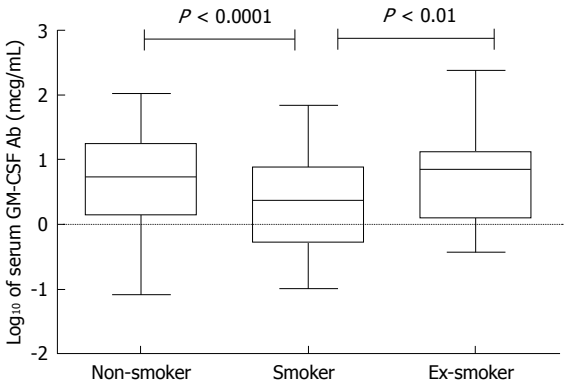

Figure 2 Serum granulocyte-macrophage colony-stimulating factor auto-antibodies levels in patients with Crohn's disease stratified by smoking status. The $\log_{10}$ transformation of serum GM-CSF Ab (in micrograms per milliliter) is shown. The middle line represents the median, and the lower edge and the upper edge of the box represent the 25% and 75% quartiles, respectively. The bottom and top lines represent the minimum and maximum values, respectively. There were 201 non-smokers, 180 smokers and 27 ex-smokers with CD. Kruskal-Wallis test with Dunn's post-test revealed significant differences between non-smokers and smokers and between smokers and ex-smokers. GM-CSF Ab: Granulocyte-macrophage colony-stimulating factor auto-antibodies; CD: Crohn's disease.

surgery.

DISCUSSION

In this cohort of 412 patients with ileal CD, 396 subjects underwent at least one ileocolic resection or ileal resection for management of their disease. Subsequently 165 of them (41.7%) required a second ICR or IR. Within the cohort of 412 patients, 410 had isolated Ileal or ileocolonic disease, 2 patients had colonic disease but evolved to ileocolonic location by the conclusion of the study. Ileal disease location is associated with stricturing and penetrating disease behavior (B2/B3) and increased risk of surgery. In fact, we demonstrated a faster time to the second surgery in those with B2 and B3 disease behavior compared to non-stricturing, non-penetrating disease (B1). In addition, patients with structuring disease had significantly decreased rates of survival (Figure 1) to the second surgery ($P = 0.0037$). However, we sought to determine additional variables that are potentially modifiable in this large cohort of CD patients with aggressive ileal CD.

We and others have reported previously, that elevated GM-CSF Ab is associated with increased rates of stricturing behavior and surgery in adult and pediatric CD[7,11,12]. Further, Däbritz *et al*[16], showed that longitudinal measurements of serum GM-CSF Ab levels in adult and pediatric patients with CD or ulcerative colitis reliably predicted disease recurrence 2 to 7 mo before a relapse as defined by clinical assessment using the Crohn's or ulcerative colitis disease activity indices (CDAI, PCDAI, UCDAI, PUCAI) in Adults and Pediatric patients with CD or ulcerative colitis. In our cohort of ileal CD patients, the time to a second surgery was significantly accelerated in those with elevated GM-CSF Ab level even after accounting for all other variables in our analysis. This study confirms the prior association of elevated GM-CSF and aggressive CD and here we report the link between elevated GM-CSF Ab and a shorter time to surgical recurrence.

GM-CSF cytokine has a central role in intestinal homeostasis by enhancing innate immune responses to microbial pathogens[17]. Within the gastrointestinal tract, expression of GM-CSF cytokine is found on Paneth cells of the intestine and the GM-CSF receptor beta chain is present along epithelial cells of the small intestine[18]. While the underlying pathogenesis that drives over-expression of GM-CSF Ab is not fully defined, in vitro studies demonstrate a neutralizing effect on GM-CSF cytokine activity by reducing the bioactivity of free GM-CSF[7]. GM-CSF autoantibodies are produced by lamina propria mononuclear cells isolated from resection specimen from patients with stricturing CD[19]. In addition patients with elevated GM-CSF have reduced neutrophil bacterial killing[19]. The addition of GM-CSF cytokine increases neutrophil bacterial killing in CD only on washed neutrophils in which GM-CSF antibodies are depleted[19]. We examined GM-CSF cytokine level in this

Table 4 Risk factors at or after the initial Crohn's disease surgery associated with time to the second Crohn's disease surgery

Main effects	HR	95%CI	*P* value	FDR
Immunomodulators[1]	0.486	0.306-0.773	0.002	0.026
GM-CSF Ab level	3.493	1.430-8.538	0.005	0.030
GM-CSF cytokine level	2.489	1.292-4.796	0.005	0.030
B2 disease behavior[2]	1.415	0.517-3.874	0.491	0.624
B3 disease behavior[3]	0.415	0.146-1.176	0.091	0.145
Smoking[4]	1.286	0.277-5.981	0.743	0.800
ASCA IgG, U/mL	1.318	0.799-2.174	0.270	0.378
Age at diagnosis	0.983	0.963-1.003	0.094	0.145
Clostridium difficile infection	0.035	0.001-1.092	0.051	0.113
antiTNF-α[5]	1.272	0.662-2.447	0.461	0.615
ATG16L1 polymorphism	1.793	0.982-3.273	0.052	0.113
IRGM polymorphism	0.092	0.004-2.262	0.136	0.201
ASCA IgA, U/mL	0.976	0.755-1.262	0.852	0.852
NOD2 polymorphisms	1.123	0.741-1.702	0.577	0.673
Interactions				
GM-CSF Ab level and smoking	0.612	0.454-0.825	0.001	0.026
GM-CSF cytokine level and ATG16L1	0.652	0.480-0.885	0.005	0.030
GM-CSF Ab level and ASCA IgG, U/mL	0.808	0.674-0.968	0.018	0.081
GM-CSF Ab level and ATG16L1	0.719	0.538-0.962	0.023	0.081
B2 disease behavior and *Clostridium difficile*	21.57	1.456-319.271	0.023	0.081
B2 disease behavior and smoking	1.601	0.318-8.059	0.560	0.673
B3 disease behavior and smoking	5.117	0.966-27.141	0.050	0.113
GM-CSF Ab level and *Clostridium difficile*	2.865	1.065-7.715	0.033	0.104
GM-CSF cytokine level and *Clostridium difficile*	0.902	0.325-2.501	0.840	0.852
Smoking and IRGM	0.406	0.145-1.134	0.079	0.145
ASCA IgG, U/mL and IRGM	1.867	0.908-3.835	0.083	0.145
GMCSF cytokine level and antiTNF-α	0.765	0.562-1.041	0.082	0.145
GM-CSF Ab level and antiTNF-α	0.941	0.703-1.260	0.676	0.757
GM-CSF cytokine level and ASCA IgA, U/mL	0.865	0.750-0.997	0.041	0.113

[1]Use of Immunomodulators between first and second CD surgery including: Azathioprine, 6-Mercaptopurine, Methotrexate; [2]Stricturing behavior (B2) compared to Inflammatory (B1) disease behavior; [3]Penetrating behavior (B3) compared to Inflammatory (B1) disease behavior; [4]Active smoker before the first CD surgery; [5]Use of Anti-tumor Necrosis Factor-alpha therapy between first and second CD surgery including: Infliximab, Adalimumab. Cox proportional hazard model; A Cox proportional hazard model was fitted with main effects and first-order interactions of clinical and genotype variables. A stepwise variable selection was performed to select the relevant subset of variables. Within the model, *P* value was adjusted with the cutoff at FDR < 0.05. Model fitting and selection were generated using R 3.1.1 (http://cran.r-project.org). Table 4 displays the *P*-values for all fitted terms in the final model. GM-CSF Ab: Granulocyte-macrophage colony-stimulating factor auto-antibodies; ASCA: Anti-*Saccharomyces cerevisiae* antibodies; CD: Crohn's disease.

study and found that patients with elevated levels had significantly shorter intervals to the second surgery. We also evaluated the interaction between GM-CSF cytokine and its neutralizing antibody and found no interaction effect. In healthy individuals, GM-CSF in the circulation is tightly regulated at low or even undetectable levels but can rise to high levels in response to immune stimuli such as lipopolysaccharide. Chronic overexpression leads to pathological changes that can result in severe damage to affected organs[20]. This may be explained by the pleiotropic effects that result from GM-CSF cytokine function[21].

We confirmed that use of imunomodulators is associated with a longer duration between the first and second surgical resection for CD. This finding first reported by Unkart *et al*[22] highlights the importance of step up therapy in a subset of patients with aggressive CD behavior. It is reported that early use of TNF antagonists is associated with reduced risk of developing strictures in adults[23] and in children early monotherapy with TNF antagonists results in better overall clinical and growth outcomes at 1 year[24]. In this study, 70.2% of patients exposed to TNF antagonists after the initial ICR/IR did not require a second surgery. In addition, only 27.8% of the 165 patients that required a second ICR/IR had exposure to TNF antagonists prior to the second surgery. Nonetheless we did not find that exposure to TNF antagonists was associated with a longer duration between the first and second surgery. It is worthwhile mentioning that the earliest use of TNF antagonists for this cohort was the year 1994 but 31 subjects (18.8%) required 2 or more ICR/IR surgical events prior to this year. In addition 144 subjects (87.2%) who required a second surgery had long standing disease of 3 or more years prior to the second surgery.

In this study a large subset of patients were current or ex-smokers (50.1%) and practically all subjects except one had ileal disease location. In a prior study we found that smokers with Ileal CD had lower GM-CSF Ab levels[12]. Similarly, in the present cohort the median GM-CSF Ab level was 2.5 in smokers and 5.4 in non-smokers (Figure 2). This resulted in a complex interaction effect of smoking such that smokers who expressed low GM-CSF Ab level had a longer interval

between the first and second surgery.

A limitation of the study was the retrospective nature of the clinical data collection. In addition we were not able to clearly delineate precise duration of exposure to immunomodulators or biologics, the response and tolerance of these medications. The serum collection was cross sectional and so future studies should examine serial measurements that correlate with time of diagnosis and surgical events.

The strengths of our study are that we were able to distinguish between modifiable and non-modifiable factors that impact disease behavior in CD. Modifiable factors like preventing exposure to smoking and early use of immunomodulators and TNF antagonists are well recognized but it is important to highlight their role in prevention of aggressive disease behavior. The non-modifiable factors like GM-CSF cytokine bioavailability and carriage of risk alleles for ATG16 L1 can be used to profile patients that may benefit from top-down therapy or alternatively newer more targeted therapy.

ARTICLE HIGHLIGHTS

Research background

Crohn's disease (CD) is a chronic inflammatory disease is characterized by a transmural inflammation that targets any region of the gastrointestinal tract. The transmural involvement particularly involving the ileum, often leads to fibrosis, luminal narrowing, and fistulas. In one population based cohort study of adult patients with CD, the cumulative probability of major abdominal surgery was 38% to 58% within 5 to 20 years after diagnosis. Further, disease recurrence resulting in a second surgery for management of CD may occur in 31% to 50% of patients within 10 years of the initial surgery. There remains a need for serological and genetic markers that can reliably identify patients with increased risk for surgical recurrence.

Research motivation

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a cytokine that promotes myeloid cell development and maturation. Deficiency of this important hematopoietic growth factor can contribute to mucosal inflammation and immunodeficiency. We and others have previously shown, neutralizing GM-CSF auto-antibodies (Ab) are associated with a reduced bioactivity of GM-CSF, impaired neutrophil bacterial killing, and increased rates of intestinal resection for ileal CD. In the present study, we examined the predictive capacity of GM-CSF Ab in surgical recurrence rates after initial ileocolic resection for ileal CD. We also evaluated other clinical, serologic and genetic prognostic factors that might define the subset of patients with shorter time to surgical recurrence.

Research objectives

The development of biomarkers for diagnosis and prognosis of CD is evolving and serological assays like ASCA, ANCA, Anti-glycan antibodies and GM-CSF Ab are important discoveries. Some assays are reliable predictors for diagnosis and for staging severity. Here we demonstrate that GM-CSF Ab is a reliable biomarker for characterizing severity and reliably predicts patients at greater risk for aggressive disease behavior and the need for surgery.

Research methods

We reviewed 412 patients which included in the study had ileal or ileocolonic CD and surgical resection of small bowel or ileocecal region for management of disease from a clinical database at tertiary academic hospital. Serum samples were analyzed for serological assays, which included GM-CSF cytokine, GM-CSF Ab, ASCA IgG and IgA, and genetic markers included SNPs rs2066843, rs2066844, rs2066845, rs2076756 and rs2066847 in NOD2, rs2241880 in ATG16L1, and rs13361189 in IRGM. The predictors of surgical recurrence were assessed by the Cox proportional-hazards models.

Research results

Of 96% patients underwent initial Ileocecal resection (ICR) or Ileal resection (IR) and subsequently 40% of patients required a second ICR/IR for CD. GM-CSF Ab level was elevated at a median of 3.81 mcg/mL. Factors predicting faster time to a second surgery included elevated GM-CSF Ab and elevated GM-CSF cytokine. Factors predicting longer duration between first and second surgery included use of Immunomodulators, the interaction effect of low GM-CSF Ab levels and smoking, and the interaction effect of low GM-CSF cytokine levels and ATG16L1.

Research conclusions

In this study patients with elevated GM-CSF Ab had shorter intervals between the first and second surgery for management of CD. This is the first study to describe this. Therefore routine surveillance of this serological assay may facilitate the identification of patients at risk for disease complications and allow clinicians to optimize medical therapy.

Research perspectives

Elevated GM-CSF Ab is a reliable assay for defining patients who are at a greater risk for surgery. A clinical assay is needed.

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ORIGINAL ARTICLE

Retrospective Study

PIK3CA and *TP53* mutations predict overall survival of stage Ⅱ/Ⅲ colorectal cancer patients

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Author contributions: Li AJ, Lin MB and Yin L designed the research; Li AJ, Li HG, Chen Y and Jiang HH performed the research; Li AJ, Wu W, Tang EJ and Li HG contributed to the analysis; Lin MB, Chen Y and Yin L supervised the study; Li AJ and Li HG wrote the paper.

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Abstract

AIM

To investigate the predictive value of *PIK3CA* and *TP53* mutation status in colorectal cancer (CRC) patients treated with 5-fluorouracil-based chemotherapy.

METHODS

In this study, a total of 315 patients with histologically proven CRC were enrolled from Yangpu Hospital affiliated to Shanghai Tongji University between 2007 and 2011. Of these patients, 241 with stage Ⅱ/Ⅲ CRC received 5-fluorouracil-based adjuvant chemotherapy. Formalin-fixed paraffin-embedded lesion samples of the patients with curatively resected CRC were collected.

Next-generation sequencing was performed to identify somatic gene mutations. The correlation of *PIK3CA* and *TP53* mutation status with overall survival (OS) was analyzed using a Cox proportional hazard model and the Kaplan-Meier method.

RESULTS

Among the 241 patients with stage Ⅱ/Ⅲ in this cohort, the *PIK3CA* and/or *TP53* mutation was detected in 177 patients, among which 54 patients had *PIK3CA* and *TP53* double mutations. The *PIK3CA* or *TP53* mutation was not significantly correlated with OS in univariate and multivariate analyses. Compared with patients without *PIK3CA* and *TP53* mutations, those with double *PIK3CA*-*TP53* mutations showed a significantly worse survival (univariate HR = 2.21; 95%CI: 1.15-4.24; multivariate HR = 2.02; 95%CI: 1.04-3.91). The *PIK3CA* mutation located in the kinase domain showed a trend toward a shorter OS compared with wild-type tumors (multivariate HR = 1.56; 95%CI: 1.00-2.44; P = 0.052). The Kaplan-Meier curve showed that patients harboring the *PIK3CA* mutation located in the kinase domain had a worse clinical outcome than those with wild-type status (Log-rank P = 0.041)

CONCLUSION

Double mutation of *PIK3CA* and *TP53* is correlated with a shorter OS in stage Ⅱ/Ⅲ CRC patients treated with 5-fluorouracil-based therapy.

Key words: Overall survival; Colorectal cancer; *PIK3CA*; *TP53*; 5-fluorouracil; Double mutation

Core tip: Targeted next-generation sequencing was used to detect gene mutations rather than mutational hotspots in the present study. This manuscript is by far the first to report the predictive value of the combined mutation status of *PIK3CA* and *TP53* in colorectal cancer patients receiving 5-FU-based adjuvant chemotherapy. Our data revealed that the double mutation of *PIK3CA* and *TP53* is an independent predictive factor for overall survival in stage Ⅱ/Ⅲ patients receiving 5-FU-based chemotherapy.

Li AJ, Li HG, Tang EJ, Wu W, Chen Y, Jiang HH, Lin MB, Yin L. *PIK3CA* and *TP53* mutations predict overall survival of stage Ⅱ/Ⅲ colorectal cancer patients. *World J Gastroenterol* 2018; 24(5): 631-640 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i5/631.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i5.631

INTRODUCTION

Colorectal cancer (CRC) is now the third most common cancer worldwide[1][Ferlay, 2010 #165]. The genesis of colorectal cancer is a multi-step, multi-gene process, receiving a great deal of interactive influence from genetic and environmental factors. Attributed to advancements in surgical techniques and the popularization of chemotherapy and targeted therapy, the treatment of CRC has rapidly evolved over several decades. 5-fluorouracil (5-FU)-based adjuvant chemotherapy is regarded as a first-line chemotherapy in both adjuvant and palliative settings for advanced CRC. Regrettably, however, a considerable number of patients not only fail to show an objective response to first-line chemotherapy treatment but also suffer from side effects[2,3]. Hence, studies of the biomarkers related to clinical outcome are in urgent demand as a way to identify whether patients benefit from adjuvant chemotherapy.

At present, the response rate of CRC patients receiving 5-FU-based adjuvant thermotherapy is approximately 40%-50%[3-7], and despite much research on the prediction of response, it has not been possible to identify a predictive biomarker as an indicator of the likely benefit in patients receiving 5-FU-based adjuvant chemotherapy. Thus, an early, quick, efficient, and accurate method is of great clinical significance and value to identify new predictive markers.

It is widely known that the multi-drug resistance (MDR) to anticancer drugs is the main cause of chemotherapy failure. Recent studies have indicated that activation of the PI3K/AKT signaling pathway can result in disturbance of cellular growth, proliferation, and survival in a variety of solid tumors[8] and can cause MDR of cancer cells through multiple mechanisms[9]. It is well documented that the effect of the aberrant regulation of the PI3K/AKT signaling pathway on cell growth and apoptosis induced by anti-cancer drugs was observed *in vitro* and *in vivo*[10-14]. Given the role of the PI3K/AKT signaling pathway in the development of resistance to anticancer drugs, it is conceivable that genetic mutations (such as *PIK3CA*) in the molecules of the PI3K/AKT signaling pathway could be a promising predictive biomarker for chemotherapy efficacy. *TP53*, the widely studied tumor suppressor gene which has an intimate connection with the occurrence and progression of many tumors in humans, mainly regulates several cellular processes, including cell cycle regulation, DNA repair, and apoptosis[15,16]. Multiple studies have bolstered the notion that *TP53* is correlated with the drug resistance of tumor cells and could emerge as a biomarker with predictive value and potential clinical utility[17-20]. Thus, we proposed that *PIK3CA* and *TP53* mutation status is likely well associated with clinical outcome in patients undergoing chemotherapy.

In the present study, we evaluated the predictive value of *PIK3CA* and *TP53* mutation status in CRC patients undergoing 5-FU-based chemotherapy after curative surgery and identified subgroups of patients

who greatly benefited from specific treatment regimens.

MATERIALS AND METHODS

Study population

In this study, a total of 315 patients with histologically proven CRC were enrolled from Yangpu Hospital affiliated to Shanghai Tongji University between 2007 and 2011. Among these patients, 241 with stage Ⅱ/Ⅲ CRC received 5-FU-based adjuvant chemotherapy as a first-line treatment for at least six cycles. Tumor staging was strictly abided by the TNM classification of the American Joint Committee on Cancer. Formalin-fixed paraffin-embedded (FFPE) lesion samples of the patients with curatively resected CRC were collected from the pathology department where the primary colorectal tumors from the patients in this study were preserved. Histopathological diagnosis by an experienced pathologist was performed for all tissue samples, and at least 70% of tumor cells could be observed in the whole section through light microscopy. Any case that did not meet the experimental standard was excluded from this study. The clinicopathological features, including pathologic stage, tumor location, and date of diagnosis and death, were collected. The definition of proximal colon cancer included the cecum and ascending and transverse colon, while tumors located in the splenic flexure and descending and sigmoid colon were characterized as distal colon cancer, and the rectum was defined from the rectosigmoid junction (the end of the sigmoid colon) to the dentate line. Written informed consent was obtained from all participants at the time of study enrollment. This study was approved by the Medical Ethics Committee of Yangpu Hospital.

DNA extraction and targeted next-generation sequencing

Genomic DNA was extracted from FFPE sections of the 315 tumors. The 10-μm-thick sections were subjected to standard deparaffinization procedures and proteinase K digestion overnight. Genomic DNA was isolated using a QIAamp DNA FFPE Tissue kit (Qiagen). All extracted DNA was quantified with a Qubit 3.0 fluorometer (Thermo Fisher Scientific) and Bioanalyzer 2100 (Agilent) before targeted next-generation sequencing[21,22].

Next-generation mutational analysis was performed using an Ion Torrent platform (Thermo Fisher Scientific) in CRC and matched normal tissues of 315 patients to identify somatic gene mutation profile. DNA library was generated using an Ion AmpliSeq DNA library kit (Thermo Fisher Scientific). According to the manufacturer's protocols, 10 ng of DNA was used as the template to amplify targets with sequencing primer panel (Thermo Fisher Scientific). Amplified targets were digested with Fupa enzyme and subsequently ligated with adapters. The library was quantified with a quantitative PCR kit and loaded into chips in Ion Chef (Thermo Fisher Scientific). The chips were loaded into an Ion S5 XL sequencer (Thermo Fisher Scientific) for sequencing. Sequencing data were processed and analyzed on a bioinformatics analysis server termed as Ion Reporter (Thermo Fisher Scientific)[23].

Statistical analysis

The study endpoint was overall survival (OS), which was calculated from pathologic diagnosis to death, regardless of cause. All statistical analyses were performed using Stata software (version 14.2). We used Cox proportional hazards models to estimate the hazard ratios (HRs) of survival adjusted for baseline patient variables. The Kaplan-Meier method was performed to generate a survival curve, with significance evaluated using a log-rank test. Where appropriate, categorical and continuous variables were estimated by the χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Altogether, 315 CRC patients were enrolled in this study; 14.6% (n = 46) of them had stage I CRC, while 40.6% (n = 128), 35.9% (n = 113), and 8.9% (n = 28) had stages Ⅱ, Ⅲ, and Ⅳ CRC, respectively. The clinicopathological features of the study population are summarized in Table 1. Genomic DNA from FFPE lesion samples of the patients with curatively resected CRC was screened for somatic mutations in the *PIK3CA* and *TP53* genes. Among the 315 patients, the incidence of *PIK3CA* and *TP53* mutations was 38.4% (n = 121) and 65.1% (n = 205), respectively, while wild type was detected in 61.6% (n = 194) and 34.9% (n = 110), respectively. In the study cohort, *PIK3CA*-mutated tumors were significantly correlated with proximal location (P = 0.036), while *TP53*-mutated tumors were not significantly associated with any examined clinical features. The frequency of *PIK3CA* mutations in the proximal, distal, and rectum location was 50% (39/78), 38.7% (29/75), and 32.7% (53/162), respectively.

Among the 241 patients with stage Ⅱ/Ⅲ disease in this cohort, the *PIK3CA* and/or *TP53* mutation was detected in 177 patients, of whom 54 had *PIK3CA* and *TP53* double mutations. As reported in Table 1, mutation status of the *PIK3CA* and *TP53* genes was comparable according to baseline characteristics. No significant difference was observed between these variables and baseline characteristics (Table 1).

For individual sites of *PIK3CA* mutation, the most frequent mutation in CRC was the mutation of Glu545Lys (5.1%), followed by Glu542Lys (2.2%). The most frequent *TP53* mutations included: Arg175His (5.1%), Arg282Trp (3.5%), Gly245Ser (2.9%), and Arg248Gln (2.9%) (Table 2).

Predictive value of PIK3CA or TP53 mutation in stage Ⅱ/Ⅲ CRC patients

All stage Ⅱ/Ⅲ CRC patients (n = 241) received 5-FU-

Table 1 Patient demographics and disease characteristics *n* (%)

Clinicopathological feature	*PIK3CA*		*P* value	*TP53*		*P* value	Both *PIK3CA* and *TP53* wild-type	Both *PIK3CA* and *TP53* mutation	Others	*P* value
	Wild-type	Mutation		Wild-type	Mutation					
Age (yr)										
< 60	52 (26.80)	29 (23.97)		31 (28.18)	50 (24.39)		26 (31.33)	22 (28.57)	33 (21.29)	
60-70	47 (24.23)	33 (27.27)		28 (25.45)	52 (25.37)		20 (24.10)	18 (23.38)	42 (27.10)	
⩾ 70	95 (48.97)	59 (48.76)	0.776	51 (46.36)	103 (50.24)	0.733	37 (44.58)	37 (48.05)	80 (51.61)	0.500
Sex										
Male	107 (55.15)	73 (60.33)		67 (60.91)	113 (55.12)		47 (56.63)	45 (58.44)	88 (56.77)	
Female	87 (44.85)	48 (39.67)	0.367	43 (39.09)	92 (44.88)	0.322	36 (43.37)	32 (41.56)	67 (43.23)	0.965
Tumor location										
Rectum	109 (56.19)	53 (43.80)		53 (48.18)	109 (53.17)		48 (57.83)	41 (53.25)	73 (47.10)	
Proximal	39 (20.10)	39 (32.23)		32 (29.09)	46 (22.44)		17 (20.48)	19 (24.68)	42 (27.10)	
Distal	46 (23.71)	29 (23.97)	0.036	25 (22.73)	50 (24.39)	0.426	18 (21.69)	17 (22.08)	40 (25.81)	0.601
Stage T										
T1-T2	34 (17.53)	26 (21.49)		20 (18.18)	40 (19.51)		15 (18.07)	16 (20.78)	29 (18.71)	
T3	38 (19.59)	19 (15.70)		21 (19.09)	36 (17.56)		17 (20.48)	11 (14.29)	29 (18.71)	
T4	122 (62.89)	76 (62.81)	0.539	69 (62.73)	129 (62.93)	0.923	51 (61.45)	50 (64.94)	97 (62.58)	0.884
Stage N										
N0	118 (60.82)	70 (57.85)		68 (61.82)	120 (58.54)		53 (63.86)	45 (58.44)	90 (58.06)	
N1	55 (28.35)	35 (28.93)		28 (25.45)	62 (30.24)		20 (24.10)	23 (29.87)	47 (30.32)	
N2	21 (10.82)	16 (13.22)	0.785	14 (12.73)	23 (11.22)	0.656	10 (12.05)	9 (11.69)	18 (11.61)	0.889
Stage										
Ⅰ	23 (11.86)	23 (19.01)		16 (14.55)	30 (14.63)		12 (14.46)	14 (18.18)	20 (12.90)	
Ⅱ	86 (44.33)	42 (34.71)		47 (42.73)	81 (39.51)		37 (44.58)	28 (36.36)	63 (40.65)	
Ⅲ	70 (36.08)	43 (35.54)		38 (34.55)	75 (36.59)		27 (32.53)	26 (33.77)	60 (38.71)	
Ⅳ	15 (7.73)	13 (10.74)	0.166	9 (8.18)	19 (9.27)	0.948	7 (8.43)	9 (11.69)	12 (7.74)	0.774

Table 2 Top ten mutations of *PIK3CA* and *TP53* in this study

PIK3CA	*n* (%)	*TP53*	*n* (%)
Glu545Lys	16 (5.1)	Arg175His	16 (5.1)
Glu542Lys	7 (2.2)	Arg282Trp	11 (3.5)
Val105Ile	6 (1.9)	Gly245Ser	9 (2.9)
Met1004Ile	6 (1.9)	Arg248Gln	9 (2.9)
His1047Arg	6 (1.9)	Arg273His	8 (2.5)
Glu218Lys	6 (1.9)	Arg273Cys	7 (2.2)
Trp552Ter	5 (1.6)	Arg248Trp	7 (2.2)
Ser438Phe	5 (1.6)	Ser260Phe	6 (1.9)
Pro835Leu	5 (1.6)	Glu358Lys	6 (1.9)
Asp1029Asn	5 (1.6)	Pro153Ser	5 (1.6)

based adjuvant chemotherapy for at least six cycles as first-line treatment after operation. In univariate and multivariate analyses, neither *PIK3CA* nor *TP53* mutation was significantly correlated with patient survival (Table 3). The Kaplan-Meier curve showed that patients harboring the *TP53* mutation had a worse clinical outcome than patients with wild-type status (Log-rank *P* = 0.046; Figure 1A), and no association was found between *PIK3CA* and clinical outcome (Log-rank *P* = 0.150; Figure 1B).

Predictive value of double PIK3CA-TP53 mutations in stage Ⅱ/Ⅲ CRC patients

We assessed the predictive value of double *PIK3CA*-*TP53* mutations for survival in stage Ⅱ/Ⅲ CRC patients treated with 5-FU-based chemotherapy according to the statistical results of Cox proportional hazards and Kaplan-Meier analyses. Compared with concomitant *PIK3CA* and *TP53* wild-type tumors, double *PIK3CA*-*TP53* mutations were a significantly poor predictive factor for OS (univariate HR = 2.21; 95%CI: 1.15-4.24; multivariate HR = 2.02; 95%CI: 1.04-3.91) (Table 3). In contrast, no association was found between the mutational status of a single gene, either *PIK3CA* or *TP53*, and OS (*P* = 0.629; Table 3). The Kaplan-Meier curve showed a shorter OS in patients harboring double *PIK3CA* and *TP53* mutations compared with concomitant *PIK3CA* and *TP53* wild-type patients (Log-rank *P* = 0.034; Figure 1C).

Association of PIK3CA mutation in a functional domain with clinical outcome in stage Ⅱ/Ⅲ CRC patients

In the multivariable analysis of the association of *PIK3CA* functional domain mutation with OS, no significant difference was observed. The results of multivariable analysis are shown in Table 4. As suggested by the results, the *PIK3CA* mutation located in the kinase domain showed a trend toward a shorter OS compared with wild-type tumors (multivariate HR = 1.56; 95%CI: 1.00-2.44; *P* = 0.052). The Kaplan-Meier curve showed that patients harboring the *PIK3CA* mutation located in the kinase domain had a worse clinical outcome than those with wild-type status (Log-rank *P* = 0.041; Figure 2).

DISCUSSION

CRC is one of the most common malignancies. Despite much research on biomarkers in patients with cancer, the number of biomarkers with predictive value and potential clinical utility is pitifully small[24-27]. Moreover, co-occurring genetic alterations have been detected

Table 3 Univariate and multivariate analyses (Cox proportional hazards model) of OS for patients with stage Ⅱ/Ⅲ CRC treated with 5-FU-based chemotherapy according to *PIK3CA* and/or *TP53* mutation status *n* (%)

	Alive	Dead	Univariate HR (95%CI)	*P* value	Multivariate HR (95%CI)	*P* value
PIK3CA						
Wild-type	114 (73.08)	42 (26.92)	1 (Ref.)		1 (Ref.)	
Occasional	5 (55.56)	4 (44.44)	1.93 (0.69-5.39)	0.208	1.40 (0.49-4.05)	0.530
Recurrent	48 (63.16)	28 (36.84)	1.50 (0.93-2.42)	0.096	1.29 (0.79-2.11)	0.314
TP53						
Wild-type	63 (74.12)	22 (25.88)	1 (Ref.)		1 (Ref.)	
Occasional	32 (80.00)	8 (20.00)	0.80 (0.35-1.79)	0.583	0.84 (0.37-1.94)	0.687
Recurrent	72 (62.07)	44 (37.93)	1.65 (0.99-2.76)	0.055	1.68 (0.98-2.87)	0.057
PIK3CA and *TP53*						
Both *PIK3CA* and *TP53* Wild-type	49 (76.56)	15 (23.44)	1 (Ref.)		1 (Ref.)	
Both *PIK3CA* and *TP53* Mutation	31 (57.41)	23 (42.59)	2.21 (1.15-4.24)	0.017	2.02 (1.04-3.91)	0.037
Others	87 (70.73)	36 (29.27)	1.31 (0.72-2.40)	0.376	1.16 (0.63-2.16)	0.629

Occasional mutation was defined as a single tumor with mutation of *PIK3CA* or *TP53*, while recurrent was mutations detected in two or more tumors. HR: Hazard ratio; 95%CI: 95% confidence interval; OS: Overall survival; CRC: Colorectal cancer.

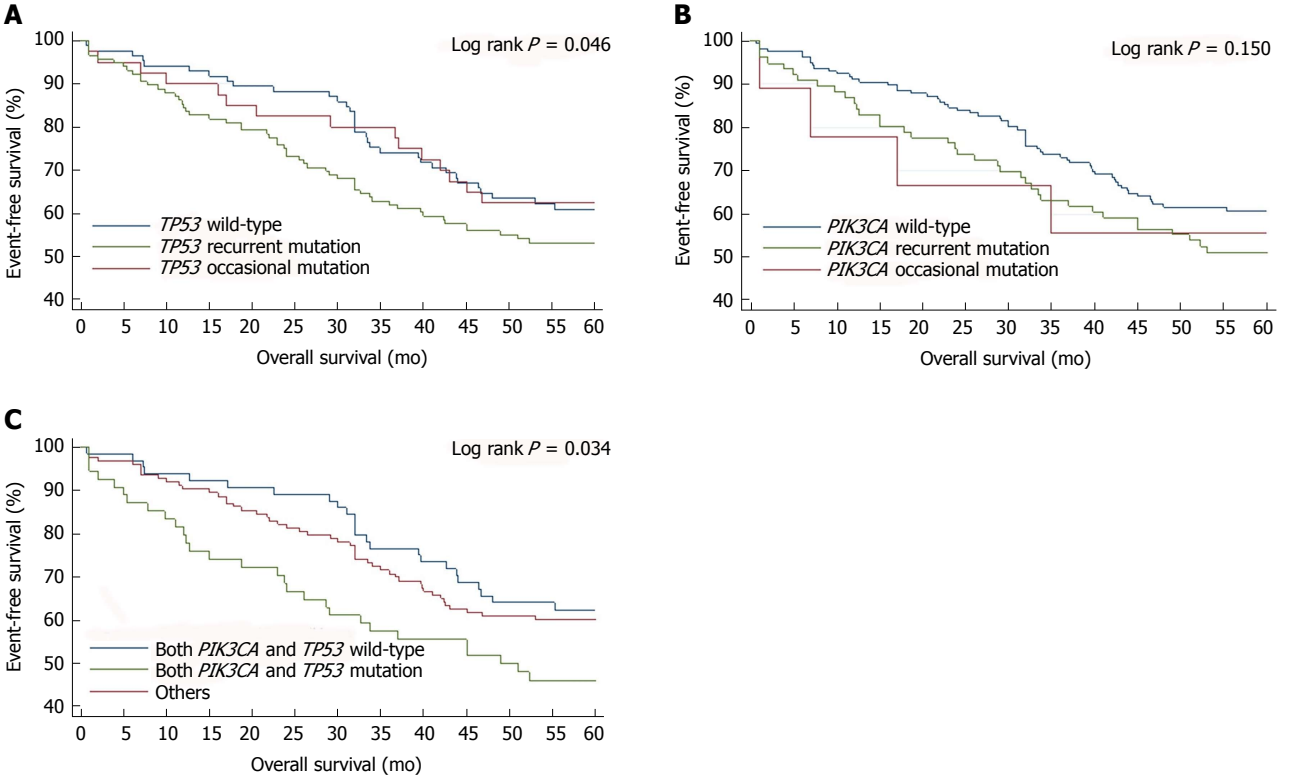

Figure 1 Kaplan-Meier survival analysis for overall survival in patients with stage Ⅱ/Ⅲ colorectal cancer according to *TP53* mutation status (A); *PIK3CA* mutation status (B); and *PIK3CA* and *TP53* mutation status (C).

in multiple malignancies[28]. In the present study, we evaluated two biomarkers with predictive value to identify subgroups of patients who would greatly benefit from 5-FU-based chemotherapy. We found that the double mutation of *PIK3CA* and *TP53* was greatly associated with worse clinical outcomes in 241 stage Ⅱ/Ⅲ CRC patients receiving 5-FU-based adjuvant chemotherapy. In contrast, mutations in *PIK3CA* or *TP53* alone had no effect on the OS of CRC patients.

Although mutations in these two genes have been widely researched, the present study is by far the first to report the predictive role of the combined mutation status of *PIK3CA* and *TP53* in CRC patients. Targeted next-generation sequencing was used to detect gene mutations rather than mutational hotspots in the present study. The frequencies of *PIK3CA* and *TP53* mutations were 35% (85/241) and 65% (156/241), respectively, which are consistent with previously published studies reporting *PIK3CA* and *TP53* mutations in 10%-32% and 40%-60% of CRC patients, respectively, in Western studies[29-32]. The present results are consistent with those of two previous studies, showing that the proximal

Table 4 Association of *PIK3CA* functional domain mutations with overall survival in stage Ⅱ/Ⅲ CRC patients *n* (%)

Domain	Alive	Dead	Multivariate HR (95%CI)	*P* value
Kinase domain				
Wild-type	116 (60.42)	76 (39.58)	1 (Ref.)	
Mutation	23 (46.94)	26 (53.06)	1.56 (1.00-2.44)	0.052
C2 domain				
Wild-type	118 (58.71)	83 (41.29)	1 (Ref.)	
Mutation	21 (52.50)	19 (47.50)	1.13 (0.68-1.86)	0.638
Helical domain				
Wild-type	110 (59.14)	76 (40.86)	1 (Ref.)	
Mutation	29 (52.73)	26 (47.27)	1.30 (0.83-2.05)	0.248
p85 binding domain				
Wild-type	127 (57.73)	93 (42.27)	1 (Ref.)	
Mutation	12 (57.14)	9 (42.86)	1.05 (0.53-2.08)	0.894
Ras binding domain				
Wild-type	123 (58.57)	87 (41.43)	1 (Ref.)	
Mutation	16 (51.61)	15 (48.39)	1.27 (0.73-2.20)	0.404

HR: Hazard ratio; 95%CI: 95% confidence interval.

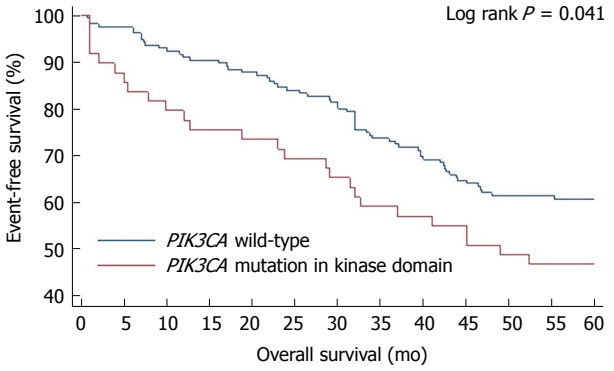

Figure 2 Kaplan-Meier survival analysis for overall survival according to the *PIK3CA* kinase domain mutation status in stage Ⅱ/Ⅲ patients.

colon showed a higher frequency of *PIK3CA* mutations than any other sites[33,34].

Several studies on the predictive roles of the *PIK3CA* mutation for response have been published[35-37]. A meta-analysis showed that the *PIK3CA* mutation in *KRAS* wild-type patients with metastatic CRC could predict responses to anti-EGFR monoclonal antibody therapy[38], while another study identified the *PIK3CA* mutation in exon 20[39]. Moreover, recent studies have suggested that the *PIK3CA* mutation might serve as a predictive biomarker for adjuvant aspirin therapy in CRC[40,41]. Thus, the *PIK3CA* mutation has a plausible role as a predictive marker for response to drugs. In clinical studies, there was no evidence that a mutation in *PIK3CA* was associated with 5-FU-based treatment benefits in colorectal cancer[42]. The present results are consistent with those of a previous study, showing that *PIK3CA* was limited as a marker for predicting responses to 5-FU-based treatment in CRC.

The inactivation of tumor suppressor genes plays a key role in tumorigenesis[43]. Obviously, the detection of *TP53* status in view of one of the most common tumor suppressor genes is important in the research of cancer[44]. *TP53* could serve as a potential biomarker for prognosis with potential predictive value and clinical utility. However, the controversial results of the available literature make it difficult to achieve a chorus of approval[45-49]. Several studies have shown that the *TP53* status was an independent predictive factor for responses to 5-FU[47,50,51], although other studies showed null association[46]. Many reasons account for these discrepant results, such as lack of reproducibility, underpowered robust statistical analysis, poor study design, and general methodological differences. There is no consensus on whether or not *TP53* emerges as a critical selection criterion to predict chemotherapy efficacy in CRC patients. Interestingly, our results showed that no association was found between clinical outcome and *TP53* status in univariate and multivariate analyses. Based on the results of the Kaplan-Meier curve, patients with wild-type *TP53* had a significantly prolonged survival compared to those harboring the *TP53* mutation.

The effect of a single gene variation as a predictive biomarker is often modest, but sometimes an additive or powerful predictive effect can be achieved by combining multiple gene alterations with the same function. Indeed, gene alteration of *PIK3CA* or *TP53* can result in cell apoptosis and drug-resistance of tumor cells through activating specific signaling pathways. Recent studies have reported that *PIK3CA* expression was correlated with the expression of *MDR-1* (encoding the MDR-associated protein P-glycoprotein)[52,53]. Additionally, the up-regulation of *MRP-1* (encoding the MDR-associated protein) induced by the activation of PI3K could cause the chemoresistance of cells in prostatic carcinomas[54]. This same regulatory mechanism also occurred in *TP53*, as studies have shown that wild-type *TP53* could serve as a negative regulator of both *MDR-1* and *MRP-1*[18,55], implicating potential associations of combined *PIK3CA*

and *TP53* with clinical outcomes to comprehend the value of their combination in predicting the benefit of patients receiving 5-FU-based chemotherapy. Interestingly, the double mutation of *PIK3CA* and *TP53* has previously been correlated with a shorter OS. Remarkably, multivariate analysis showed that this correlation was independent of age, gender, stage, and tumor location, thereby confirming that the combined analysis of *PIK3CA* and *TP53* mutation status could become a marker to identify subgroups of patients who have a poor prognosis and provide valuable information for more clinical therapy projects.

The *PIK3CA* gene is divided into five functional domains: p85 binding domain, Ras binding domain, C2 domain, helical domain, and kinase domain[30]. The main mutation of *PIK3CA* occurs in exons 9 and 20, corresponding to the helical and kinase domains, respectively[56]. Recent studies have shown that patients treated with anti-EGFR monoclonal antibodies (MoAbs) and harboring *PIK3CA* mutations in exon 20 were significantly associated with worsening outcomes in KRAS wild-type mCRC[39,57]. In the present study, the Cox proportional hazard model analysis of the effect of the *PIK3CA* mutation occurring in the kinase domain on clinical outcome reached marginally statistical significance (P = 0.052), whereas the Kaplan-Meier curve achieved statistical significance (Log-rank P = 0.041; Figure 2). The trend for statistical significance was evident for worsening clinical outcomes with mutations occurring in the kinase domain.

Thus far, with regard to research on *PIK3CA* or *TP53* mutations, obviously, the tumor samples of patients with CRC were collected from a single hospital in most studies, and even for many multi-centered clinical trials, patients were enrolled on the basis of epidemiological settings. Similarly, in the present study, we used tumor samples from a single hospital to reduce selection bias. Additionally, genetic heterogeneity is a reality of all tumors and is decreased by limiting the study to stage Ⅱ/Ⅲ patients receiving the same chemotherapy regimens. In addition, considering that a small number of total samples could lead to a less robust statistical analysis, a large sample size (n = 241) warranted adequate statistical power in the present study.

In conclusion, the present study suggests that the double mutation of *PIK3CA* and *TP53* is correlated with a shorter OS of stage Ⅱ/Ⅲ CRC patients receiving 5-FU-based therapy and hence serves as a novel biomarker to identify subgroups of patients who have poor clinical outcome, with potential clinical utility.

ARTICLE HIGHLIGHTS

Research background

5-fluorouracil (5-FU) remains one of the most effective and commonly used chemotherapeutic agents in both adjuvant and palliative settings for advanced colorectal cancer (CRC). However, many CRC patients treated with 5-FU-based adjuvant chemotherapy not only fail to show an objective response to chemotherapy treatment but also suffer from side effects. Therefore, predictive markers are in urgent demand to identify whether patients can benefit from adjuvant chemotherapy.

Research motivation

Multiple studies have indicated that *PIK3CA* and *TP53* mutation status was correlated with drug resistance of tumor cells. By analyzing the associations between mutation status of these two genes and overall survival (OS), we may be able to identify subgroups of patients who have a poor prognosis, which can help clinicians make suitable treatment of patients.

Research objectives

The objectives of this study were to detect gene mutations of *PIK3CA* and *TP53* by using targeted next-generation sequencing (NGS) in a large cohort of CRC patients, and to investigate the predictive value of the mutational status of *PIK3CA* and *TP53*, alone or in combination.

Research methods

A total of 315 patients with histologically proven CRC between 2007 and 2011 were retrospectively analyzed. Formalin-fixed paraffin-embedded lesion samples of the patients with curatively resected CRC were collected from the pathology department. Ten-μm-thick sections from FFPE tumor samples were used for DNA extraction with a QIAamp DNA FFPE Tissue kit (Qiagen). Targeted NGS was performed using the Ion Torrent platform to characterize the mutational spectrum of *PIK3CA* and *TP53* genes. The distribution of gene mutation according to clinicopathologic variables was analyzed using Chi-square tests. The associations between mutation status of *PIK3CA* and *TP53* and OS were evaluated using Cox proportional hazards models adjusted for clinicopathologic variables. The Kaplan-Meier method was performed to generate a survival curve, with significance evaluated using a long-rank test.

Research results

Among the 315 patients, the incidence of *PIK3CA* and *TP53* mutations was 38.4% (n = 121) and 65.1% (n = 205), respectively. A significant difference was observed in the distribution of *PIK3CA* mutations according to tumor location (P = 0.036). The frequency of *PIK3CA* mutations in the proximal, distal, and rectum location was 50% (39/78), 38.7% (29/75), and 32.7% (53/162), respectively. The *PIK3CA* and/or *TP53* mutation was detected in 177 out of 241 patients with stage Ⅱ/Ⅲ CRC receiving 5-FU-based adjuvant chemotherapy, of whom 54 had *PIK3CA* and *TP53* double mutations. In both univariate and multivariate analyses, neither *PIK3CA* nor *TP53* mutation was significantly correlated with OS. In Kaplan-Meier survival curve, patients with *TP53* mutation had a worse clinical outcome than patients with wild-type *TP53* (Log-rank P = 0.046). Compared with patients without *PIK3CA* and *TP53* mutations, those with double *PIK3CA*-*TP53* mutations had a significantly poorer OS (univariate HR = 2.21; 95%CI: 1.15-4.24; multivariate HR = 2.02; 95%CI: 1.04-3.91). In contrast, the presence of a single gene mutation, either *PIK3CA* or *TP53*, was not significantly associated with OS. The Kaplan-Meier curve showed that shorter OS was detected in patients harboring double *PIK3CA* and *TP53* mutations (Log-rank P = 0.034). In Kaplan-Meier survival curve, patients harboring the *PIK3CA* mutation located in the kinase domain experienced a significantly shorter OS when compared with wild-type status (Log-rank P = 0.041).

Research conclusions

This study is by far the first to report the predictive role of the combined mutation status of *PIK3CA* and *TP53* in CRC patients receiving 5-FU-based adjuvant chemotherapy. Our data revealed that the double mutation of *PIK3CA* and *TP53* is correlated with a shorter OS of stage Ⅱ/Ⅲ CRC patients receiving 5-FU-based therapy and hence serves as a novel biomarker to identify subgroups of patients who have poor clinical outcome, with potential clinical utility.

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Observational Study

Faecal calprotectin and magnetic resonance imaging in detecting Crohn's disease endoscopic postoperative recurrence

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Abstract

AIM

To assess magnetic resonance imaging (MRI) and faecal calprotectin to detect endoscopic postoperative recurrence in patients with Crohn's disease (CD).

METHODS

From two tertiary centers, all patients with CD who underwent ileocolonic resection were consecutively and prospectively included. All the patients underwent MRI and endoscopy within the first year after surgery or after the restoration of intestinal continuity [median = 6 mo (5.0-9.3)]. The stools were collected the day before the colonoscopy to evaluate faecal calprotectin level. Endoscopic postoperative recurrence (POR) was defined as Rutgeerts' index $\geqslant$ i2b. The MRI was analyzed independently by two radiologists blinded from clinical data.

RESULTS

Apparent diffusion coefficient (ADC) was lower in patients with endoscopic POR compared to those with no recurrence (2.03 ± 0.32 *vs* 2.27 ± 0.38 × 10^{-3} mm²/s, P = 0.032). Clermont score (10.4 ± 5.8 *vs* 7.4 ± 4.5, P = 0.038) and relative contrast enhancement (RCE) (129.4% ± 62.8% *vs* 76.4% ± 32.6%, P = 0.007) were significantly associated with endoscopic POR contrary to the magnetic resonance index of activity (MaRIA) (7.3 ± 4.5 *vs* 4.8 ± 3.7; P = 0.15) and MR scoring system (P = 0.056). ADC < 2.35 × 10^{-3} mm²/s [sensitivity = 0.85, specificity = 0.65, positive predictive value (PPV) = 0.85, negative predictive value (NPV) = 0.65] and RCE > 100% (sensitivity = 0.75, specificity = 0.81, PPV = 0.75, NPV = 0.81) were the best cut-off values to identify endoscopic POR. Clermont score > 6.4 (sensitivity = 0.61, specificity = 0.82, PPV = 0.73, NPV = 0.74), MaRIA > 3.76 (sensitivity = 0.61, specificity = 0.82, PPV = 0.73, NPV = 0.74) and a MR scoring system $\geqslant$ MR1 (sensitivity = 0.54, specificity = 0.82, PPV = 0.70, and NPV = 0.70) demonstrated interesting performances to detect endoscopic POR. Faecal calprotectin values were significantly higher in patients with endoscopic POR (114 ± 54.5 μg/g *vs* 354.8 ± 432.5 μg/g; P = 0.0075). Faecal calprotectin > 100 μg/g demonstrated high performances to detect endoscopic POR (sensitivity = 0.67, specificity = 0.93, PPV = 0.89 and NPV = 0.77).

CONCLUSION

Faecal calprotectin and MRI are two reliable tools to detect endoscopic POR in patients with CD.

Key words: Faecal calprotectin; Magnetic resonance imaging; Postoperative recurrence; Crohn's disease; Clermont score; Magnetic resonance index of activity

Core tip: Performing a colonoscopy within the first year after surgery is now recommended in the management of postoperative Crohn's disease (CD) to decrease the risk of symptomatic recurrence. However, endoscopy is felt as a burdensome procedure by the patients highlighting the need for more convenient tools. In our prospective study from two referral centers, we showed that faecal calprotectin measurement and magnetic resonance imaging with Clermont score or magnetic resonance index of activity calculation are two reliable tools to detect early endoscopic postoperative recurrence in CD and could then be an alternative to colonoscopy.

Baillet P, Cadiot G, Goutte M, Goutorbe F, Brixi H, Hoeffel C, Allimant C, Reymond M, Obritin-Guilhen H, Magnin B, Bommelaer G, Pereira B, Hordonneau C, Buisson A. Faecal calprotectin and magnetic resonance imaging in detecting Crohn's disease endoscopic postoperative recurrence. *World J Gastroenterol* 2018; 24(5): 641-650 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i5/641.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i5.641

INTRODUCTION

Crohn's disease (CD) is a chronic, progressive and disabling inflammatory disorder, which can highly impacts the patients' quality of life[1-3]. The natural course of CD can lead to bowel damages such as strictures or fistulas requiring surgical management[1-3]. Despite an increased use of biologics, surgery is still warranted in half of the patients within ten years after diagnosis[1]. As intestinal resection is not curative, postoperative recurrence (POR) remains a key issue in the management of patients with CD. Up to 75% of the patients experienced endoscopic POR within the first year after surgery in referral centers[4]. More than 25 years ago, Rutgeerts and colleagues demonstrated that the postoperative course of CD is very heterogeneous[5]. They proposed a stratification of the patients according to the early endoscopic findings within the first year after the surgery, namely the Rutgeerts' index, to predict the risk of clinical postoperative recurrence[5]. Recently, the postoperative Crohn's endoscopic recurrence (POCER) trial confirmed previous retrospective data suggesting that an endoscopy-based strategy with a therapeutic step-up according to the

Rutgeerts' index decreased the risk of clinical POR in CD patients[6-10]. Even though the best threshold to define endoscopic POR using this index is still debated[11-13], performing an endoscopy is now recommended for all the patients with CD within the first year after intestinal resection[14]. However, colonoscopy remains a burdensome procedure for the patients owing to the bowel cleansing, the general anesthesia and the risk of complications[15,16] highlighting the need to develop more convenient tools.

In this context, magnetic resonance imaging (MRI) is more accepted than endoscopy and has shown a reliable accuracy to detect endoscopic activity in CD patients[17-21]. The magnetic resonance index of activity (MaRIA)[17,18,22] and the Clermont score[20,21,23-25] are the two main MRI scores that have been validated compared to endoscopy in CD. These two scores demonstrated high performances to grade CD severity and to evaluate mucosal healing[19,26]. However, the MaRIA and the Clermont score have not been investigated so far in the early postoperative course of patients with CD. Only one Austrian group has hitherto proposed an index, so-called the MR scoring system, to detect endoscopic POR. The authors observed promising results compared to the Rutgeerts' index in predicting the risk of clinical POR in patients with CD[27,28].

Another alternative could be the use of faecal calprotectin measurement to predict the risk of clinical POR. In the last decade, faecal calprotectin demonstrated very reliable performances to diagnose CD, to assess disease activity and to predict clinical relapse[29-40]. Recently, a few works reported convincing results on the use of faecal calprotectin in the early postoperative phase in CD patients[41-44].

In the present study, we aimed to assess the performances of MRI and faecal calprotectin to detect endoscopic POR within the first year following surgery in patients with CD.

MATERIALS AND METHODS

Ethical consideration

The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements. Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a priori approval by the institution's human research committee. The study was approved by local Ethics Committee (#2014/CE 42).

Patients

All the patients with CD older than 18 years-old, who underwent a CD-related ileocolonic resection, were consecutively enrolled in this prospective study. They were included from two tertiary centers between January 2014 and December 2016. An endoscopic evaluation was performed for each patient within the first year after surgery (or after restoration of intestinal continuity for the patients with temporary ostomy). The anastomosis was reached by the colonoscope for all the patients. Patients' demographics and clinical activity were collected the same day. We used the Crohn's disease activity index (CDAI)[45] to grade disease activity. Blood samples were taken prior to the endoscopy to measure high sensitive serum C-reactive protein (CRP) level by immuno-nephelometric method (Vista, Siemens, Berlin, Germany). Stools were collected in the morning the day before the endoscopy. All the patients were also evaluated by magnetic resonance imaging with diffusion-weighted sequences. Colonoscopy and MRI had to be performed within one month. The choice of medication to prevent postoperative recurrence was free and depended on the physician's decision.

Faecal calprotectin testing

Stools were collected in the morning the day before the endoscopy to reduce intra-individual variation, and immediately stored at 4 ℃. The bowel cleansing was started in all patients after stool collection. Patients were instructed to transport the stool samples in a dedicated container at 4 ℃. Faecal samples were immediately transferred, upon patient arrival, to the local Biochemistry Laboratory. Stool cultures were performed on all inflammatory bowel disease (IBD) samples to exclude gastrointestinal infection. Calprotectin was measured using quantitative immunochromatographic test Quantum Blue High Range (Bühlmann Laboratories AG, Schönenbuch, Switzerland), according to the manufacturer's instructions. All the biochemistry tests were done by individuals blinded from clinical, endoscopic and radiological data.

Endoscopy

After bowel cleansing, endoscopy was performed under anaesthesia with propofol (PROPOFOL DAKOTA PHARM; Sanofi-Aventis, Paris, France). All colonoscopies were performed by experienced IBD endoscopists in each center using column video colonoscopy (QFC L 140; Olympus, Tokyo, Japan). The endoscopists were blinded from biochemistry and MRI data. The endoscopic lesions were graded using the Rutgeerts' index[5] as routinely used in the two IBD units during the postoperative phase. We defined endoscopic POR as Rutgeerts' index $\geqslant$ i2b. However, we performed also sensitivity analyses using different cut-off values such as Rutgeerts' index $\geqslant$ i2 or $\geqslant$ i3.

MRI examination

On the day of MRI, patients had to have been fasting for at least four h before the examination. An oral ingestion of 500 mL to 1000 mL of PEG (Fortrans®, Ipsen Pharma, Paris, France) was used to achieve an adequate intestinal distension.The MRI imaging examinations with no bowel cleansing the day before the examination and

with no colonic distension (no enema) were performed as previously described[20,21,23,24] with a 1.5 Tesla GE Optima MR 450w (General Electric HealthCare, Fairfield, CT) in Clermont-Ferrand, France, and with a 1.5 Tesla Avanto MRC1 (Siemens, Erlangen, Germany) in Reims, France.

MRI analysis

Each examination was interpreted independently by two radiologists: one experienced IBD radiologist (CH) and one junior (PB)[20,21,23,24] who were blinded from endoscopy and biochemistry data. The analyses were focused on the perianastomotic area. The following characteristics were collected: oedema, ulcers, bowel wall thickening (mm), adjacent enlarged lymph nodes (> 8 mm in shortest diameter), comb sign, penetrating complications (fistula, abscess, phlegmon), and Relative Contrast Enhancement (RCE)[17]. For quantitative assessment, the apparent diffusion coefficient (ADC) was calculated on the ADC map independently in separate sessions by the two radiologists in the peri-anastomotic area or in the area of highest signal intensity in the bowel wall. As previously published, the definition of this area was based on the judgment of the radiologist [20,21,23-25]. MR scoring system was defined as MR0 (no abnormal features), MR1 (minimal mucosal changes), MR2 (diffuse aphtoid ileitis, moderate recurrence), and MR3 (severe recurrence with trans- and extramural changes)[27,28]. The Clermont score[20,21,23-25] was calculated using the following formula: 1.646 × bowel thickness - 1.321 × ADC + 5.613 × oedema + 8.306 × ulcers + 5.039. The MaRIA was calculated using the following formula for each of the five segments: MaRIA = 1.5 × wall thickening (mm) + 0.02 × RCE + 5 × edema + 10 × ulceration[17,18,22].

Data managing and statistical analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at Clermont-Ferrand University Hospital[46]. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing (1) an intuitive interface for validated data entry; (2) audit trails for tracking data manipulation and export procedures; (3) automated export procedures for seamless data downloads to common statistical packages; and (4) procedures for importing data from external sources.

Statistical analyses were performed using Stata software (version 13, StataCorp, College Station, United States). The tests were two-sided, with a type I error set at $\alpha = 0.05$. Baseline characteristics were presented as mean ± SD or median (interquartile range) according to statistical distribution (assumption of normality assessed using the Shapiro-Wilk test) for continuous data and as the number of patients and associated percentages for categorical parameters. Comparisons of patients' characteristics between the independent groups (*i.e.,* Rutgeerts' index) were performed using chi-squared or Fisher's exact tests for categorical variables, and Student's t-test or Mann-Whitney test if assumptions of t-test were not met (1) normality, and (2) assumption of homoscedasticity studied using Fisher-Snedecor test) for quantitative parameters. Receiver operating characteristic (ROC) curves were used to determine the best biomarker to predict Rutgeerts' index. The best thresholds were determined according to biological relevance and to usually recommended indices reported in literature (Youden, Liu and efficiency). Sensitivity (se), specificity (spe) and negative (NPV) and positive predictive values (PPV) were presented with 95% confidence intervals. Concordance has been studied using kappa coefficient and accuracy for categorical parameters. Kappa values were studied in relation to usual recommendations: < 0.2 (negligible), 0.2-0.4 (low/weak consistency), 0.4-0.6 (moderate agreement), 0.6-0.8 (substantial/good agreement) and > 0.8 (excellent agreement). For quantitative parameters, the concordance was studied using correlation coefficient, Lin's concordance coefficient and Bland and Altman graph[47].

RESULTS

Baseline characteristics of the patients

Overall, 30 CD patients were enrolled in this study. The main characteristics of these patients are provided in Table 1. Among them, half of the patients (15/30) were female and 7 (23.3%) were active smokers. Mean age and mean disease duration at the time of inclusion were 34.9 ± 14.1 years and 9.0 ± 9.5 years, respectively. Fifteen patients presented with pure ileal involvement (L1 according to Montreal classification), only one patient (3.3%) had pure colonic location (L2 according to Montreal classification) and 14 patients had ileocolonic CD (46.7%). Six patients experienced perianal lesions (21.4%). In the current study, the patients were treated with no medication (20.0%), 5-ASA (6.7%), thiopurines (56.7%), anti-TNF agents (20.0%) or several of these drugs for preventing endoscopic POR. The distribution of the endoscopic findings according to the Rutgeerts' index within the first year of surgery is provided in Figure 1.

MRI parameters in detecting endoscopic postoperative recurrence

The median interval between surgery and endoscopy was 6 mo (5.0-9.3) The median interval between MRI and endoscopy was 14 d (6.5-31). We compared all the MRI parameters in patients with or without endoscopic POR. We did not observe any ulceration among the 30 MRI. The bowel wall was not significantly thickened in the patients with endoscopic POR (3.59 ± 1.69 mm *vs* 2.83 ± 1.55 mm, $P = 0.26$). The detection of oedema was not significantly associated with the occurrence of endoscopic POR (38.5% *vs*

Table 1 Characteristics at the time of endoscopy of the 30 patients with Crohn's disease enrolled in this study *n* (%)

Patients' characteristics	Value
Age at diagnosis, (yr), mean ± SD	34.9 ± 14.1
Disease duration, (yr), mean ± SD	9.0 ± 9.5
Female gender	15 (50.0)
Active smokers	7 (23.3)
Montreal classification	
CD location	
L1	15 (50.0)
L2	1 (3.3)
L3	14 (46.7)
CD behaviour	
B1	1 (3.3)
B2	16 (53.3)
B3	13(43.3)
Perianal lesions	7 (23.3)
Therapy to prevent endoscopic POR[1]	
None	6 (20.0)
5-ASA	2 (6.7)
Thiopurines	17 (56.7)
Anti-TNF agent	6 (20.0)
CDAI, median (interquartile range)	78 (26-86)
CRP, median (interquartile range)	2.9 (2.9-3.9)
Faecal Calprotectin (μg/g), median (IQR)	100 (100-136)

[1]The patients were treated with one or several of these medications. CD: Crohn's disease; POR: Postoperative recurrence; TNF: Tumor Necrosis Factor; CDAI: Crohn's disease activity index; CRP: C-reactive protein.

11.8%, P = 0.19). The value of ADC was lower in the patients with endoscopic POR compared to those with no recurrence (2.03 ± 0.32 *vs* 2.27 ± 0.38 × 10^{-3} mm²/s, P = 0.032). The Clermont score (10.4 ± 5.8 *vs* 7.4 ± 4.5, P = 0.038) and the RCE (129.4% ± 62.8% *vs* 76.4% ± 32.6%, P = 0.007) were increased in the patients with endoscopic POR. In contrast, MaRIA 7.3 ± 4.5 *vs* 4.8 ± 3.7; P = 0.15) was not significantly higher in the patients with endoscopic POR. The proportion of patients with endoscopic POR was 30.0%, 71.4% and 66.7% in the patients with MR scoring system MR0, MR1 and MR2, respectively (P = 0.11). However, the proportion of patients with endoscopic POR seemed to be higher in the patients with MR scoring system MR1 and MR2 *vs* MR0 (53.8% *vs* 17.6%, P = 0.056).

Using ROC curves, we determined that a value of ADC < 2.35 × 10^{-3} mm²/s [Se = 0.85 (0.38-0.86), Spe = 0.65 (0.55-0.98), PPV = 0.85 (0.55-0.98), NPV = 0.65 (0.38-0.86)] and a value of RCE > 100% [Se = 0.75 (0.46-0.91), Spe = 0.81 (0.56-0.91), PPV = 0.75 (0.46-0.91), NPV = 0.81 (0.56-0.91)] were the best cut-off values to identify endoscopic POR (Figure 2). We showed also that a Clermont score > 6.4 [Se = 0.61 (0.35-0.82), Spe = 0.82 (0.58-0.94), PPV = 0.73 (0.47-0.95), NPV = 0.74 (0.46-0.95)] and a MaRIA > 3.76 [Se = 0.61 (0.35-0.82), Spe = 0.82 (0.58-0.94), PPV = 0.73 (0.46-0.98), NPV = 0.74 (0.46-0.96)] demonstrated interesting performances to detect endoscopic POR (Figure 2). In addition, a MR scoring system ⩾ MR1 showed the following performances to detect endoscopic POR: Se = 0.54 (0.29-0.77), Spe = 0.82 (0.58-0.94), PPV = 0.70 (0.42-0.98), and NPV = 0.70 (0.50-0.90) (Figure 2).

Clinical and biological markers in detecting endoscopic POR

The CDAI was not significantly higher in patients with endoscopic POR [median values = 80 (51-86) *vs* 62 (10-85); P = 0.17] and the performances of CDAI > 150 to detect endoscopic POR were: Se = 0.08 (0.02-0.36), Spe = 0.94 (0.70-1.00), PPV 0.50 (0.01-0.99) and NPV = 0.56 (0.35-0.75) (Figure 2).

The CRP value was not significantly higher in patients with endoscopic POR (6.2 ± 7.8 *vs* 3.2 ± 1.6; P = 0.43). Using a ROC curve, we observed the following performances of CRP level above 5 g/L: Se = 0.31 (0.09-0.61), Spe = 0.88 (0.62-0.98), PPV = 0.67 (0.22-0.96), NPV = 0.61 (0.39-0.80).

Faecal calprotectin values were significantly higher in patients with endoscopic POR (354.8 ± 432.5 μg/g *vs* 114 ± 54.5 μg/g; P = 0.0075) (Figure 2). Using a ROC curve, we found that the cut-off value of faecal calprotectin > 100 μg/g demonstrated high performances to detect endoscopic POR [Se = 0.67 (0.39-0.86), Spe = 0.93 (0.66-1.00), PPV = 0.89 (0.68-1.00) and NPV = 0.77 (0.56-0.97) and accuracy 0.80 (0.66-0.96)] (Figure 2). The exclusive measure of faecal calprotectin level in the postoperative setting would have been able to adequately stratify patients as having no sign of endoscopic POR in most of the patients and therefore might allow in our cohort avoiding 13 colonoscopies *i.e.* 43.3% of the total number (13 true negative patients). However, it would have missed 4 false negative patients (13.3%).

We did not observe any improvement of performances to detect endoscopic POR with concomitant or successive use of MRI and faecal calprotectin (data not shown).

Sensitivity analysis

We performed the same investigations in using different cut-off value of Rutgeerts' index ⩾ i2 or ⩾ i3. These results are detailed in Supplementary Tables 1 and 2.

Inter-observer variation study

The inter-observer agreement between the two radiologists was 96.7% for the MR scoring system with a κ-value of 0.933 ± 0.139. We observed substantial concordances (Lin's concordance coefficient with 95% confidence interval) between the two readers regarding bowel thickness [0.97 (0.94-0.99)], ADC [0.71 (0.53-0.89)], RCE [0.73 (0.55-0.91)], Clermont score [0.990 (0.98-1.00)], and MaRIA [0.99 (0.98-1.00)]. We calculated the median relative variation of ADC [6.5% (5.1-11.0)], RCE [15.0% (8.8-25.5)], Clermont score [9.5% (4.5-9.5)], and MaRIA [9.6% (5.3-13.6)]. The inter-reader agreement was 96.7% using a cut-off value of MaRIA of 3.76 (κ = 0.92 ± 0.18) and 93.3% using a

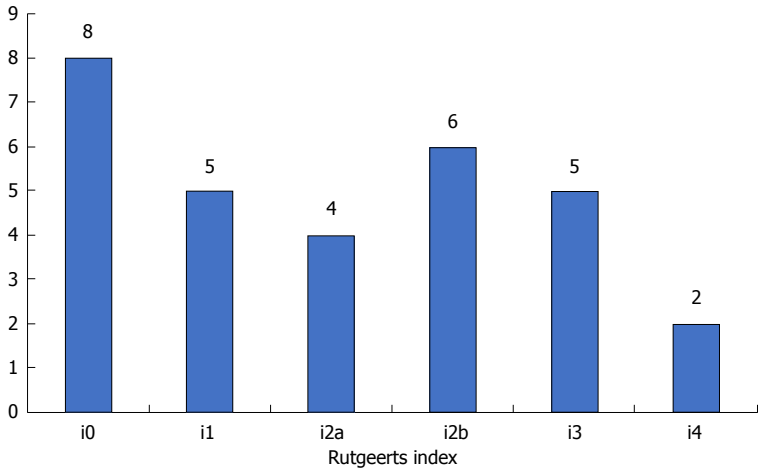

Figure 1 Distribution of the endoscopic findings according to the Rutgeerts' index within the first year of surgery in 30 patients with Crohn's disease.

threshold of Clemont score above 6.4 (κ = 0.86 ± 0.18) (no significant difference between the two scores).

DISCUSSION

In this prospective study, we showed, for the first time in the same cohort that faecal calprotectin and MRI are two reliable alternative tools to detect endoscopic POR with CD. In addition, MRI parameters including Clermont score, MaRIA and MR scoring system demonstrated substantial inter-reader agreement.

As surgery is not curative in CD and the rate of endoscopic POR can reach more than 75% within the first year after the surgery in some referral centers[4], the prevention of POR remains a major concern for the IBD physicians. Recently, the data retrieved from the POCER trial confirmed the positive impact of a tailored therapeutic management based on the early endoscopic findings (within the first year following the intestinal resection) compared to a monitoring based on clinical activity[6]. A lower rate of endoscopic POR at 18 mo was observed in the active care arm compared to the control group (49% *vs* 67%, P = 0.03)[6]. Then, performing an endoscopic evaluation within the first week following the surgery (or the restoration of intestinal continuity) is recommended in patients with CD. However, the colonoscopy is felt as a burdensome procedure by most of the patients leading some of them to deny performing this examination. This observation highlights the need to develop more convenient tools for these patients. A nationwide survey including 916 IBD patients recently reported that stools collection for faecal biomarkers and MRI were considered as more acceptable than colonoscopy by CD patients[15]. Accordingly, investigating the performances of faecal calprotectin and MRI to detect endoscopic POR seemed highly relevant.

The early endoscopic evaluation is performed using the Rutgeerts' index[5]. The usual definition of endoscopic POR is a Rutgeerts' index $\geqslant$ i2 as the likelihood of reappearance of symptoms in the five years following the surgery was 40% in i2-patients and more than 75% in i3- or i4-patients compared to less than 15% in patients with Rutgeerts' index $\leqslant$ i1[5]. However, the debate is currently growing regarding this definition owing to the heterogeneity of the i2-subgroup encompassing several conditions such as more than five aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions or ulcers up to 1 cm confined to ileocolonic anastomosis. The characterization of the lesions confined to the anastomosis is a problem in daily practice as it is sometimes difficult to exclude alternative diagnosis such as post-surgical or ischemic consequences. Then, the i2-group is now divided into two subgroups *i.e*. i2a (lesions confined to the anastomosis) and i2b (more than 5 aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions). Two teams attempted recently to show a different course of the disease between these two subgroups (i2a *vs* i2b) but failed to do so[11,12]. Unfortunately, this question should be very difficult to figure out as a step-up therapeutic strategy is performed in almost all the patients with endoscopic lesions classified as i2a and i2b. Consequently, investigating the natural history of this subgroup of patients will be probably no longer possible. Although it is still matter of debate, we decided to define endoscopic POR as a Rutgeerts' index $\geqslant$ i2b but we performed a sensitivity analysis using other thresholds including Rutgeerts' index $\geqslant$ i2.

To date, only one team studied the potential role of MRI to replace early endoscopic evaluation in CD patients who underwent ileocolonic resection[27,28]. They arbitrarily created the MR scoring system dedicated to the postoperative phase as: MR0 (no abnormal features), MR1 (minimal mucosal changes), MR2 (diffuse aphtoid ileitis, moderate recurrence), and MR3 (severe recurrence with trans- and extramural changes)[28]. The authors observed a good interobserver agreement between the MR scoring system and the Rutgeerts'

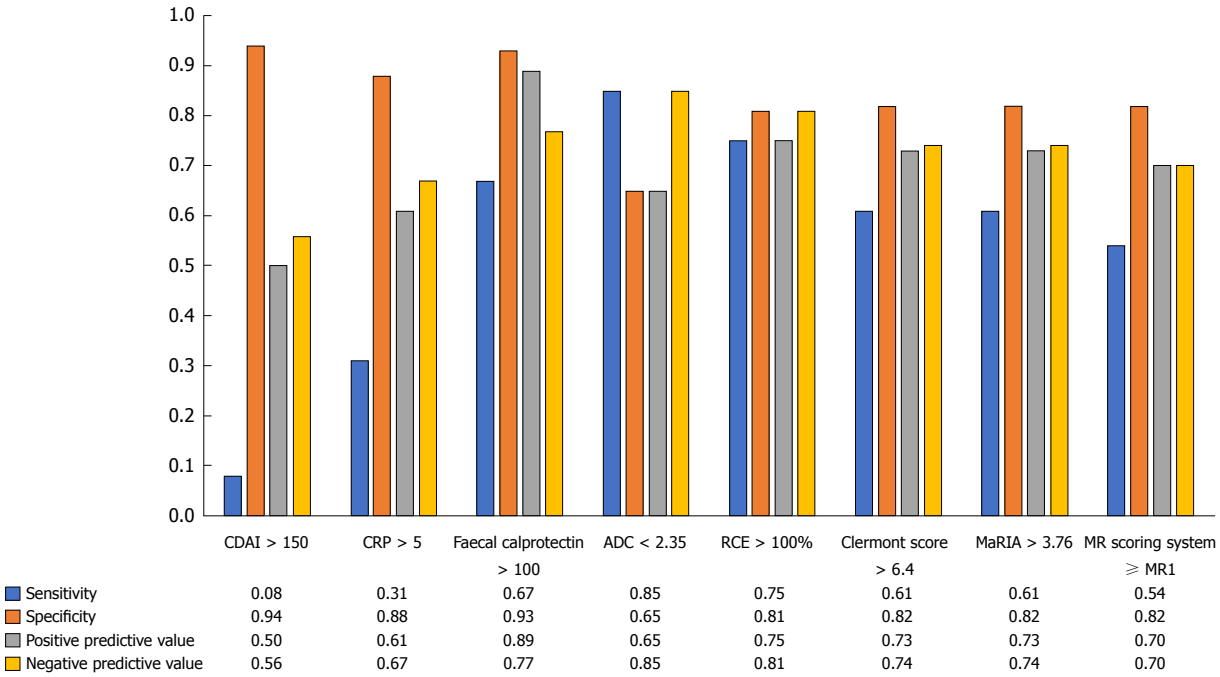

Figure 2 Performances of clinical, biological and magnetic resonance imaging parameters to detect endoscopic postoperative recurrence within the first year after surgery in patients with Crohn's disease.

index (agreement rate = 77.8% and κ = 0.67). AMR scoring system ⩾ MR2 showed a trend to be predictive of a higher risk of clinical POR (P = 0.09)[27]. In our cohort, the MR scoring system demonstrated a clear trend to be able to detect the occurrence of endoscopic POR (53.8% *vs* 17.6%, P = 0.056). However, we did not find the same threshold (⩾ MR1 rather than ⩾ MR2). It could be partly explained by the very high level of severe endoscopic POR in the Austrian cohort *i.e.* 5 patients with i3 (16.7%) and 14 patients with i4 (46.7%) contrary to ours: 5 patients with i3 (16.7%) and 2 patients with i4 (6.7%). In our study, we investigated, for the first time, the potential role of each individual MRI item to detect endoscopic POR. We observed that the bowel thickness was not a reliable parameter to distinguish patients with or without endoscopic POR, which is not surprising, as a mildly thickened bowel wall can be seen in CD patients after surgery even in the absence of endoscopic recurrence. We also observed that the presence of oedema was not significantly different between the two groups (38.5% *vs* 11.8%, P = 0.19). Finally, we did not observe any severe lesions on MRI such as ulcerations, fistula or stenosis, which is in line with the early stage of the disease within the first year following the surgery.

The MaRIA[17-19,22] and the Clermont score[19-21,23,24,48] are the two available MRI scores validated against endoscopy to assess ileocolonic activity in patients with CD. We investigated the performances of these two validated scores to detect endoscopic POR. We observed substantial positive (0.73 for both) and negative predictive values (0.74 for both) of these two scores to identify POR. The cut-off values were lower than those usually used to detect inflammatory activity in CD[18,21]. It is consistent with the fact that the early postoperative lesions are mostly mild and limited to the mucosa. The most sensitive MRI features to detect these minimal shifts are the quantitative parameters assessing the degree of inflammation such as the RCE (for the injected sequences) and the ADC (for the diffusion-weighted sequences), which showed substantial accuracy to detect endoscopic POR in our cohort. However, the impact of these quantitative parameters among the calculation of the MaRIA and the Clermont score remains limited. Even though RCE and ADC demonstrated high inter-reader agreement[49], these items could be two equipment-dependent metric values, which could be difficult to use alone despite their substantial performances to detect endoscopic POR.

Faecal calprotectin is hitherto the most effective faecal biomarker to assess endoscopic activity in patients with CD[29-40]. Recently, a French prospective study and a post-hoc analysis of the POCER trial reported that a level of faecal calprotectin > 100 μg/g was the best threshold to detect endoscopic POR (Rutgeerts' index ⩾ i2) with high negative predictive value between 91 and 93%[42,43]. Then, the authors calculated that they could avoid from 30% to 47% of unnecessary colonoscopies[42,43]. In our cohort, we also identified a level of faecal calprotectin > 100 μg/g as the best cut-off value to show an endoscopic POR (Rutgeerts' index

⩾ i2b). Contrary to the previous study, we observed a lower negative predictive value (77%) but a higher positive predictive value (89%). The lower level of NPV is consistent with the data from a secondary analysis of the TOPPIC trial[50] (88 patients) reporting a NPV of 75% even though they observed in the same time a low PPV (58%). Concerning the high PPV, it could be partly explained by the different assays used across the studies and should be confirmed in other larger independent cohorts. In our cohort, the use of faecal calprotectin would have avoided 43.3% of unnecessary colonoscopy (13 true negative patients/30) but would have missed 13.3% of recurrences (4 false negative patients /30). However, we did not observe any severe endoscopic POR (i3 or i4) among these 4 patients. Finally, we confirmed that the CDAI and the CRP level are not accurate enough to monitor patients with CD within the first months after the surgery.

In the same cohort, we investigated the performances of faecal calprotectin and MRI. Our study was not powered to directly compare these two potential alternatives to colonoscopy and then we did not observe any significant difference between these two tools. However, we observed numerically a mild trend favoring faecal calprotectin. Of course, this result has to be confirmed in independent larger cohorts before drawing any conclusion.

The main strengths of our study included its prospective design with concomitant evaluation of faecal biomarker and MRI. In addition, we investigated the potential impact of each MRI parameter including the two most validated scores *i.e.,* the MaRIA and the Clermont score. The main limitation of this study is the sample size even though our sample size calculation was based on the two prior Austrian studies[27,28] and then remains one of the larger published so far on the role of MRI in the postoperative CD.

Faecal calprotectin and MRI are two reliable options to detect endoscopic POR within the first year after ileocolonic resection in patients with CD and could be used as a more convenient tool than colonoscopy.

ARTICLE HIGHLIGHTS

Research background

Surgical resection is unfortunately not curative in Crohn's disease (CD), and postoperative recurrence (POR) remains a crucial issue in these patients. Performing an endoscopy within the first year after surgery is recommended in clinical practice. However, colonoscopy remains a burdensome procedure for the patients highlighting the need to develop more convenient tools. In this context, MRI and faecal calprotectin are more accepted than endoscopy and have shown a reliable accuracy to detect endoscopic activity in patients with CD. MRI scores such as the magnetic resonance index of activity (MaRIA) and the Clermont score and faecal calprotectin could then be used as an alternative to detect endoscopic POR but their performances remains poorly investigated.

Research motivation

Developping more convenient tools to detect early postoperative recurrence, is a key point in patients with Crohn's disease.

Research objectives

In this study, we assessed the performances of MRI and faecal calprotectin to detect endoscopic postoperative recurrence in patients with Crohn's disease.

Research methods

It was a multicentre prospective observational study.

Research results

ADC < 2.35 × 10^{-3} mm²/s (sensitivity = 0.85, specificity = 0.65, positive predictive value (PPV) = 0.85, negative predictive value (NPV) = 0.65) and RCE > 100% (sensitivity = 0.75, specificity = 0.81, PPV = 0.75, NPV = 0.81) were the best cut-off values to identify endoscopic POR. Clermont score > 6.4 (sensitivity = 0.61, specificity = 0.82, PPV = 0.73, NPV = 0.74), MaRIA > 3.76 (sensitivity = 0.61, specificity = 0.82, PPV = 0.73, NPV = 0.74) and a MR scoring system ⩾ MR1 (sensitivity = 0.54, specificity = 0.82, PPV = 0.70, and NPV = 0.70) demonstrated interesting performances to detect endoscopic POR. Faecal calprotectin > 100 μg/g demonstrated high performances to detect endoscopic POR (sensitivity = 0.67, specificity = 0.93, PPV = 0.89 and NPV = 0.77).

Research conclusions

Faecal calprotectin and MRI are two reliable options to detect endoscopic POR within the first year after ileocolonic resection in patients with CD and could be used as a more convenient tool than colonoscopy.

Research perspectives

Additional studies from independent cohorts should be conducted to confirm these data.

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CASE REPORT

Sequential spinal and intracranial dural metastases in gastric adenocarcinoma: A case report

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Author contributions: Kim H and Han HS designed the report; Kim H, Yi KS, Kim WD, Son SM and Han HS collected the patient's clinical data; Kim H, Yang Y, Kwon J and Han HS contributed to data interpretation and drafting of the article; all authors read and approved the final version of this manuscript.

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Abstract

Dural metastasis from primary gastric adenocarcinoma has been rarely reported, and its prognosis is very poor because it frequently leads to acute subdural hematoma. Here, we describe a case with sequential spinal and cranial dural metastases from gastric adenocarcinoma without subdural hematoma. A 43-year-old woman with gastric adenocarcinoma and well-controlled peritoneal carcinomatosis presented with back pain, right radiating leg pain, left facial palsy, and hearing loss. Magnetic resonance imaging of the spine

and brain revealed dural masses at the lumbosacral junction with invasion to the L5 and S1 nerve roots and at the skull base with invasion to the internal auditory canal. She was treated with local radiotherapy, and her pain and neurologic symptoms improved after palliative radiotherapy. This is the first reported case of dural metastases of gastric adenocarcinoma of the spine and skull base but with a relatively indolent course and without subdural hematoma.

Key words: Adenocarcinoma; Gastric; Metastasis; Dura; Radiotherapy

Core tip: Although gastric adenocarcinoma can metastasize to almost any organ, metastasis to the dura mater is rarely reported. Here, we report a rare and clinically meaningful case of dural metastases to the spine and skull base from gastric adenocarcinoma that was treated with palliative local radiotherapy. This is the first reported case of dural metastases of gastric adenocarcinoma of the spine and skull base with involvement of the surrounding structures but with a relatively indolent course and without subdural hematoma. Local radiotherapy was effective for the relief of neurologic symptoms.

Kim H, Yi KS, Kim WD, Son SM, Yang Y, Kwon J, Han HS. Sequential spinal and intracranial dural metastases in gastric adenocarcinoma: A case report. *World J Gastroenterol* 2018; 24(5): 651-656 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i5/651.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i5.651

INTRODUCTION

Gastric cancer is the fourth most frequent malignancy and the second most common cause of cancer-related death in the world[1]; it is also the second most frequent malignancy in Korea[2]. Although patients with early gastric cancer can be cured by surgical resection, a large majority of patients relapse after surgical resection or are initially diagnosed with metastatic disease. The most common metastatic sites of gastric cancer are the liver, peritoneum, lymph nodes, lung, and bone[3]. Central nervous system metastasis is a very rare manifestation of gastric cancer, occurring in 0.16-0.69% of patients, and mostly consists of brain parenchymal metastasis or leptomeningeal carcinomatosis[4]. Dural metastasis of all central nervous system metastasis has been rarely reported in cases of gastric cancer[5-9]. Here, we report a rare and clinically meaningful case of dural metastases from gastric adenocarcinoma that was treated with palliative local radiotherapy.

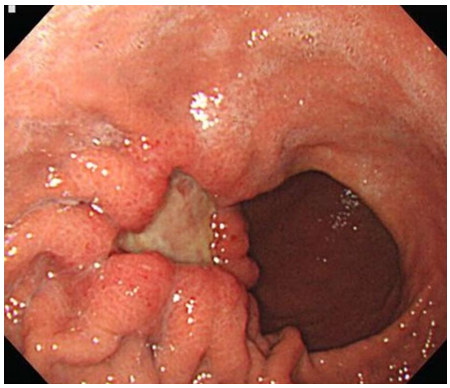

Figure 1 Esophagogastroduodenoscopy revealed a 3-cm sized ulcerofungating mass on the anterior wall of the greater curvature of the gastric body.

CASE REPORT

A 43-year-old woman with metastatic gastric cancer presented with a 1-wk history of progressively worsening lower back and radiating right leg pain in December 2016. Physical examination revealed mild numbness at the right inner thigh, but there were no abnormal findings, including sensory abnormalities or motor weakness in the lower extremities, on physical examination. She had a history of advanced gastric adenocarcinoma that was diagnosed in September 2014. Esophagogastroduodenoscopy revealed a 3-cm sized ulcerofungating mass on the anterior wall of the greater curvature of the gastric body (Figure 1), and endoscopic biopsy confirmed a histologic diagnosis of poorly cohesive carcinoma. A computed tomography (CT) scan of the abdomen revealed lymph node enlargement in the perigastric area and no evidence of metastasis, the clinical stage was determined as T3N1M0, cStageⅢ. She underwent a subtotal gastrectomy with D2 lymph node dissection, and the postoperative pathologic findings were poorly cohesive carcinoma (pT4aN2M0, pStageⅢB).

The patient was treated with adjuvant capecitabine and oxaliplatin chemotherapy for 6 mo. However, in September 2015, she was diagnosed with ovarian metastasis and peritoneal carcinomatosis. She was treated with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy followed by systemic chemotherapy (2nd line paclitaxel and 3rd line irinotecan) until December 2016. Magnetic resonance imaging (MRI) of the lumbosacral spine revealed an enhancing dural mass at the lumbosacral junction with invasion to the right L5 and S1 nerve roots (Figure 2). She was treated with 3D conformal radiotherapy of 36 Gy in 12 fractions to the lumbosacral junction under a diagnosis of spinal dural metastasis from gastric adenocarcinoma.

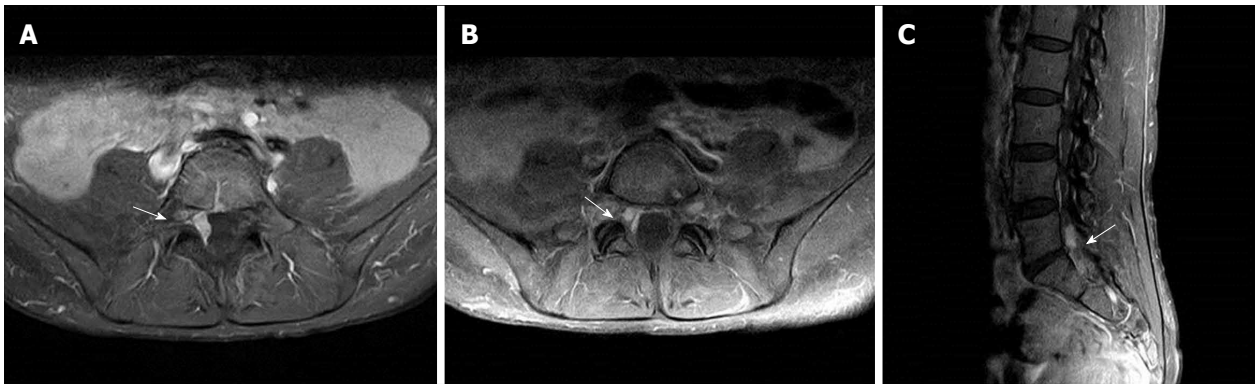

Figure 2 Magnetic resonance imaging of the lumbosacral spine. Axial (A, B) and sagittal (C) contrast-enhanced T1-weighted imaging of the spine MRI shows a focal dural mass with invasion to the right L5 and S1 nerve roots at the L5-S1 level. MRI: Magnetic resonance imaging.

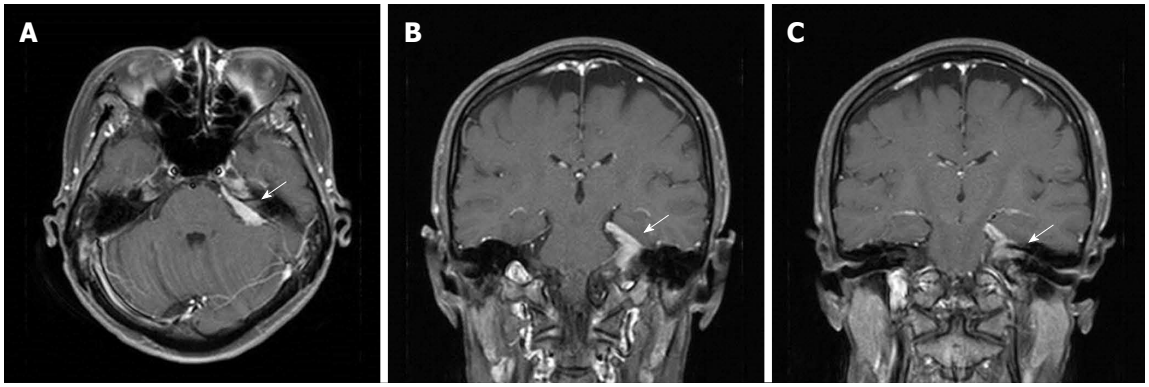

Figure 3 Magnetic resonance imaging of the brain. On axial (A) and coronal (B and C) contrast-enhanced T1-weighted imaging of the brain MRI, a homogenous, enhancing dural mass is visible at the left cerebellopontine angle cistern (A and B) invading the left trigeminal nerve and internal auditory canal (C). MRI: Magnetic resonance imaging.

Her lower back and radiating right leg pain abated after palliative radiotherapy.

At 3 mo later, in April 2017, she presented with a 2-wk history of a progressively worsening loss of sensation on the left side of the face and hearing loss in the left ear. On physical examination, she showed numbness of the left face and tongue, and motor weakness of the chewing muscles. Pure-tone audiometry showed left side deafness. MRI of the brain revealed an enhancing dural lesion at the left cerebellopontine angle with extension to the trigeminal nerve and internal auditory canal (Figure 3). There was no evidence of other distant metastases upon CT scan of the thorax, abdomen, and pelvis. She was treated with intensity modulated radiotherapy of 40 Gy in 10 fractions to the dural mass at the left skull base under a diagnosis of cranial dural metastasis from gastric adenocarcinoma. Her left hearing loss did not improve, but the left facial palsy improved after palliative radiotherapy.

However, in October 2017, she presented with a radiating right upper arm pain and headache 8 mo after the initial diagnosis of dural metastasis from gastric adenocarcinoma. MRI of the brain revealed multiple enhancing lesions at the cerebral and cerebellar hemispheres, and MRI of the spine revealed enhancing lesions at spinal cord at T1/2 and T10/11 (Figure 4). Moreover, metastatic adenocarcinoma cells were observed by cerebrospinal fluid cytology. She was treated with palliative radiotherapy to the whole brain and intramedullary lesion at T1/2, and received intrathecal methotrexate chemotherapy. However, she died of progressive disease 10 mo after the initial diagnosis of dural metastasis from gastric adenocarcinoma.

DISCUSSION

Dural metastases of extraneuronal malignancies are detected in approximately 10% of autopsy cases[10]. The relatively common neoplasms associated with dural metastasis are breast, prostate, lung cancer, melanoma and hematologic malignancies[11,12]. However, dural metastasis in gastric adenocarcinoma has been rarely reported[5-9]. Direct extension of bone metastases and direct hematogenous spread have been proposed as mechanisms for the spread of extraneuronal malignancies to the dura[11,12]. Dural metastases are usually

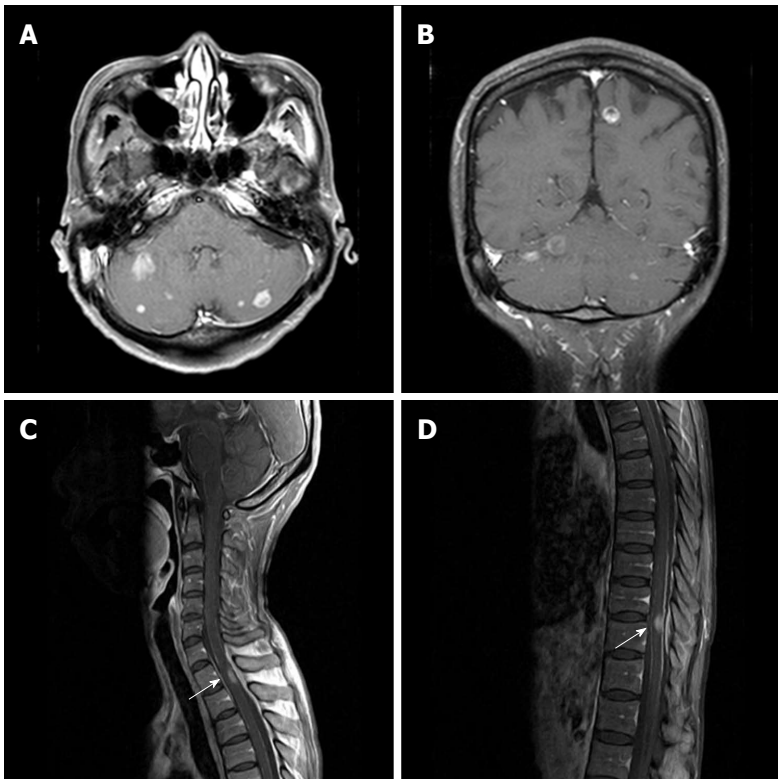

Figure 4 Magnetic resonance imaging of the brain and spine. On axial (A) and coronal (B) contrast-enhanced T1-weighted imaging of the brain MRI revealed multiple enhancing nodules at the cerebral and cerebellar hemispheres. On sagittal contrast-enhanced T1-weighted imaging of the spine MRI revealed eccentric nodular enhancement at spinal cord at T1/2 (C) and T10/11 (D). MRI: Magnetic resonance imaging.

a late manifestation of the malignant disease, which contributes greatly to the severity of the prognosis. The lesions often remain clinically asymptomatic, but they may become symptomatic due to mass effect or the accumulation of subdural fluids, including acute or chronic hematomas and malignant effusion.

The available literature suggests a poor prognosis for patients presenting with dural metastases, although this is most likely a reflection of the disseminated nature of the underlying disease rather than a direct consequence of dural involvement[11,12]. Survival varies considerably according to the primary tumor type and the extent of the systemic disease; favorable courses are generally observed in primary breast, prostate, renal cell carcinoma, and hematologic malignancy. Dural metastasis from primary gastric adenocarcinoma has been rarely reported, and its prognosis is very poor because the prognosis of metastatic gastric adenocarcinoma itself is poor and because it frequently leads to subdural hematoma[5-9]. The prognosis of subdural hematoma caused by dural metastasis is extremely poor, and in most reported cases, the patients died within a short period of time[9].

Several mechanisms have been proposed for the formation of subdural hematomas associated with dural metastasis. Obstruction of the dural veins by tumor cells can cause dilatation of the capillaries in the inner areolar layer, followed by rupture and subdural hemorrhage. Infiltration of the dura by malignant cells can also induce an angiodesmoplastic reaction, resulting in the formation of an abnormal neomembrane that is highly vascular, into which hemorrhage can occur more easily[8,9,11]. Kunii *et al*[13] analyzed 51 reported cases of subdural hematoma and found that the most common histological type in which subdural hematoma occurred was adenocarcinoma and the most common primary tumor site was the stomach. In our case, unlike in the previously reported cases of dural metastasis from gastric adenocarcinoma, acute or chronic subdural hematoma did not develop and the course of the disease was relatively indolent, as is more commonly seen in breast or prostate cancer.

No standard of treatment has been clearly defined for the management of dural metastasis. High-dose dexamethasone can produce symptom relief even in the absence of cerebral edema, and evacuation of symptomatic subdural hematoma should be considered. Surgical resection is rarely performed and is reserved for single metastatic lesions causing severe symptoms. Stereotactic radiosurgery or local radiotherapy either alone or in combination with whole brain radiotherapy seems to be a safe and effective treatment option[11,12]. Therefore, the therapeutic approach employed should be chosen based on the individual condition of each

patient. In the described case, local radiotherapy was performed because the systemic cancer was controlled and there was no subdural hematoma. As a result, cancer pain and neurologic deficits caused by the dural mass were effectively controlled.

This case differs in some ways from the previously reported cases of gastric adenocarcinoma with dural metastasis. First, this case was not associated with subdural hematoma or fluid collection. Second, dural metastases in the previous cases occurred in the dura mater around the cerebral cortex, but in this case, metastases occurred sequentially in the dura mater around the spinal canal and skull base. Therefore, while in the previously reported cases the main symptoms were associated with invasion to the underlying brain parenchyma, resulting in elevated intracranial pressure, in this case the patient presented with lower back pain and symptoms associated with trigeminal nerve palsy. Third, the lesions in this case were spread widely along the dura mater, and there was invasion of the surrounding structures. The initial, spinal dural metastasis caused invasion to the L5 and S1 nerve roots, resulting in radiating leg pain, and the second, cranial dural metastasis at the skull base caused invasion to the internal auditory canal, resulting in hearing loss.

This is the first reported case of gastric adenocarcinoma metastasizing sequentially to the dura mater in the spinal canal and skull base without subdural hematoma. Although these circumstances are rare, local radiotherapy may be effective for the relief of neurologic symptoms in cases of dural metastasis of gastric adenocarcinoma with an indolent course and controlled systemic disease.

ARTICLE HIGHLIGHTS

Case characteristics

A 43-year-old woman with gastric adenocarcinoma presented with back pain, right radiating leg pain, left facial palsy, and hearing loss.

Clinical diagnosis

Spinal and brain metastases were suspected.

Differential diagnosis

Spinal bone metastasis, brain parenchymal metastasis or leptomeningeal carcinomatosis.

Laboratory diagnosis

All laboratory tests were within normal limits.

Imaging diagnosis

Magnetic resonance imaging of the spine and brain revealed dural masses at the lumbosacral junction with invasion to the L5 and S1 nerve roots and at the skull base with invasion to the internal auditory canal.

Pathological diagnosis

The postoperative pathologic findings were poorly cohesive carcinoma and the patient was diagnosed with advanced gastric adenocarcinoma.

Treatment

The patient was treated with palliative local radiotherapy.

Related reports

Dural metastasis from primary gastric adenocarcinoma has been rarely reported, and its prognosis is very poor because it frequently leads to subdural hematoma. The prognosis of subdural hematoma caused by dural metastasis is extremely poor, and in most reported cases, the patients died within a short period of time.

Term explanation

Dural metastasis, also named pachymeningeal metastasis, refers to metastasis to the dura mater within the craniospinal axis.

Experiences and lessons

Although dural metastasis from primary gastric adenocarcinoma has been rarely reported, local radiotherapy may be effective for the relief of neurologic symptoms in cases of dural metastasis of gastric adenocarcinoma with an indolent course and controlled systemic disease.

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