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EDITORIAL

Future therapeutic implications of new molecular mechanism of colorectal cancer

Sen Lu, Cheng-You Jia, Jian-She Yang

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Sen Lu, Department of Surgical Anesthesiology, First Affiliated Hospital of Anhui University of Science and Technology, Huainan 232001, Anhui Province, China

Cheng-You Jia, Jian-She Yang, Department of Nuclear Medicine and Oncology Research, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, China

Corresponding author: Jian-She Yang, MD, MM, MSc, PhD, Academic Editor, Academic Fellow, Academic Research, Full Professor, Professor, Department of Nuclear Medicine and Oncology Research, Shanghai Tenth People's Hospital, Tongji University School of Medicine, No. 301 Yanchang Road (Middle), Shanghai 200072, China. yangjs@impcas.ac.cn

Abstract

High incidence (10.2%) and mortality (9.2%) rates led to the ranking of colorectal cancer (CRC) as the second most malignant tumor spectrum worldwide in 2020. Treatment strategies are becoming highly dependent on the molecular characteristics of CRC. The classical theories accept two models depicting the origin of CRC: The progression of adenoma to cancer and transformation from serrated polyps to cancer. However, the molecular mechanism of CRC development is very complex. For instance, CRCs originating from laterally spreading tumors (LST) do not adhere to any of these models and exhibit extremely serious progression and poor outcomes. In this article, we present another possible pathway involved in CRC development, particularly from LST, with important molecular characteristics, which would facilitate the design of a novel strategy for targeted therapy.

Key Words: Colorectal cancer; Laterally spreading tumors; Molecular mechanism; Truncated adenomatous polyposis coli mutation; Golgi fragmentation; Cancerous mechanism

Core Tip: Although laterally spreading tumors (LST) are considered vital precancerous lesions of colorectal cancer (CRC), the mechanism mediating their transition to CRC development is unclear. Adenomatous polyposis coli (APC)-truncating mutations driven by Golgi fragmentation are very important cellular events that can abrogate the microtubule binding properties of APC. This effect reduces the stability of microtubules, impacts cell proliferation and survival, causes chromosomal instability, and increases migration. Downstream characteristics of Golgi fragmentation indicate alterations in Ataxia-telangiectasia mutated and anoctamin 5 expression, whereas their gene expression changes are significant in LST. This implies a novel pathway for CRC development from LST.

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INTRODUCTION

In 2020, the World Health Organization International Agency for Research on Cancer officially released the latest cancer data, showing a 10.2% and 12.2% incidence of colorectal cancer (CRC)[1,2]. Consequently, CRC was ranked the third and second highest in malignant tumor spectrum and in China, respectively, while its mortality of 9.2% ranked it the second highest among all cancers[1,2]. Most incidences of CRC develop from benign polyps (adenomas and serrated polyps) through a series of genetic and epigenetic changes that occur over 10 to 15 years[3,4].

A laterally spreading tumor (LST) is a special digestive tract tumor that is an important precancerous lesion of CRC. Its morphological features are hidden and, consequently, it is easily misdiagnosed. LST can develop into progressive CRC within 3 years with a very poor prognosis[5]. Large-scale controlled studies have shown that LST patients have an 8.4%-52.5% possibility of developing CRC, and benign LST lesions can develop into advanced CRC within 3 years[5].

Pathologically, LST has certain similarities to colorectal polyps and adenoma, and the molecular mechanisms underlying their progression to carcinoma have been clearly elucidated. However, studies on how LST develops into CRC are rare, and the molecular mechanism of the associated carcinogenesis is unclear. Therefore, systematically and comprehensively exploring the molecular mechanisms underlying the malignant transformation of LST to CRC is critical. Specifically, the exploration would have potential theoretical significance and clinical value for early diagnosis and precise treatment of CRC. Here, we present a potential alternative pathway mediating the development of CRC, particularly from LST, with critical molecular characteristics, which would facilitate the design of a novel strategy for targeted therapy.

MECHANISM OF CRC DEVELOPMENT

Two classical models have been proposed for the development and progression of CRC from colon epithelial cells. The first model describes the process of transformation of adenoma to cancer, mainly initiated by mutations in the adenomatous polyposis coli (*APC*) gene. In this model, *APC* mutation is followed by mutations in Kirsten rat sarcoma viral oncogene homolog (*KRAS*)/neuroblastoma rat sarcoma viral oncogenes, mothers against decapentaplegic homolog 4, and finally, tumor protein p53 (*TP53*).

The other model describes the transformation from serrated polyps to cancer, with the driver mutation of catenin beta 1 secondary to mutations of *KRAS*/B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha[6]. These mutations eventually result in transforming growth factor-beta receptor type 2 (TGFRB2) overexpression[6]. The main signaling molecules involved are Wnt, mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and TP53, which are hyperactivated[7]. Several other signaling pathways, involving hedgehog, erb-b2 receptor tyrosine kinase, ras homolog family member A, Notch, bone morphogenetic protein, Hippo, AMP-activated protein kinase, nuclear factor kB, and Jun N terminal kinase, also participate in the occurrence and development of CRC[8].

TRUNCATED *APC* MUTATION

Several studies have indicated that *APC* mutations are extremely important in both the mutation

frequency and different stages of CRC tumor development[9-11]. APC is the "gatekeeper" gene of the colorectal mucosal epithelium and the key molecule regulating colon epithelial cell homeostasis, polarity, and movement. *APC* acts as a tumor suppressor gene of CRC and 80% of sporadic CRCs harbor mutations, which are widely considered an early event in colorectal malignancy. Somatic point mutations of *APC* mainly occur in the mutation cluster region (MCR); however, *APC* contains several other mutations in its protein-coding region[9,10].

The most important cytological events after mutation in the MCR are the structural truncation of APC (amino acid sequence from 1362 to 1540, namely, the APC-2,3 repeats) and lack of axin, catenin, microtubules, and other binding sites[11]. Mutations in truncated APC result in the loss of the microtubule-binding properties of APC and further reduce microtubule stability. Truncating mutations cause *APC* to lose the properties of normal tumor suppressor genes and show "acquired" proto-oncogene properties, including abnormal cell proliferation and survival, chromosomal instability, and increased migration.

Truncated APC binds to and activates APC-stimulated guanine nucleotide exchange factor (ASEF), which is closely related to actin remodeling and movement and causes significant changes in cell structure and function[12]. Knockdown of *ASEF* or *APC*-truncating mutations markedly reduces cell migration; however, overexpression of full-length APC does not increase ASEF-mediated cell migration. The Golgi complex is a dynamic organelle that is essential for sorting, processing, and transporting proteins by which the stability of cellular structures is maintained.

Fragmentation of the Golgi apparatus is observed in age-related diseases including Alzheimer's disease, Parkinson's disease, and cancer[13]. The Golgi apparatus may be closely involved in the development of human diseases; however, the mechanisms and significance of its fragmentation are poorly understood. *APC*-truncating mutations induce fragmentation of the Golgi apparatus and lead to structural reorganization of cytoskeletal proteins and actin[13]. This causes cells to exhibit abnormal biological behavior, such as loss of polarity and increased migration[13]. Therefore, *APC*-truncating mutations driven by Golgi fragmentation are an important event in normal cells.

Mutation-rich regions of *APC* in the normal mucosa, LST, colorectal adenocarcinoma, and colorectal adenoma specimens were detected using polymerase chain reaction-single-strand conformation polymorphism[14]. The results of that study showed that while no *APC* mutations were observed in the normal mucosa of the large intestine, the mutation rates were 25%, 30%, and 27.8% in LST, colorectal adenocarcinoma, and colorectal adenoma, respectively[14]. The difference in rates between the specimens was not statistically significant and the results were consistent with other reported *APC* mutation rates of 15.5%-42.4% in LST specimens. Two recent studies showed *APC* mutation frequencies of 80% (10 samples) and 57% (14 samples) in LST[15]. Therefore, *APC* mutation may act as an important initiator in LST development[16].

TP53 AND *CRC*

TP53 and *CRC* are important intracellular tumor suppressor genes, and the main biological function of *TP53* is the repair of cellular damage. Normal *TP53* can be used to monitor the integrity of genomic DNA in real time. During DNA damage, TP53 stops cell division at the G1/S phase to allow cells to have enough time to repair the damage. For irreparable DNA damage, TP53 induces programmed apoptotic cell death, thereby inhibiting the generation of possible mutant cancerous cells[17,18].

TP53 mutations primarily occur during the middle and late stages of carcinogenesis. Numerous *TP53* mutations reduce the proportion of wild-type *TP53* and weaken its function in monitoring genomic DNA integrity, thereby allowing tumorigenesis[19]. Studies on *TP53* and *LST* are scarce, and a recent comprehensive and unbiased screening of the genome, epigenome, and transcriptome was conducted based on the Cancer Genome Atlas (TCGA) database[14].

Bioinformatic data integrated from 11 LST samples and validated in an additional cohort of 84 benign colorectal injury samples, identified several high-frequency genetic, epigenetic, and transcriptional alterations[14]. Deletions occurred in chromosomes 1p, 5q, 14q, and 18, whereas doubling occurred in chromosomes 7, 8, 13, 19, and 20. Furthermore, these alterations were highly prevalent in the panel of the colorectal and rectal adenocarcinoma validation groups. The main signaling pathways associated with LST are axonal guidance, thyroid cancer, human embryonic stem cell pluripotency (Nanog homeobox), and Wnt/β-catenin.

Cohort validation studies compared 10 LSTs with 212 CRC samples with a focus on five major altered signaling pathways, Wnt, TGF β, PI3K, MAPK, and P53[20,21]. The results showed that the differences in the TGFβ and TP53 signaling pathways were significant[20,21]. The results suggested that ataxia-telangiectasia mutated (ATM), a very important molecule in the TP53 pathway, was significantly increased, which stabilized TP53 molecules. The expression of another very important gene, anoctamin 5 (*ANO5*), was significantly reduced, leading to mitochondrial fragmentation.

Our results based on TCGA data analysis also confirmed a significantly low expression level of *ANO5* in LST. The expression of the Golgi fragmentation-related genes *ATM* and *ANO5* was significantly different. This differential expression weakened the expression of the wild-type TP53 transcription

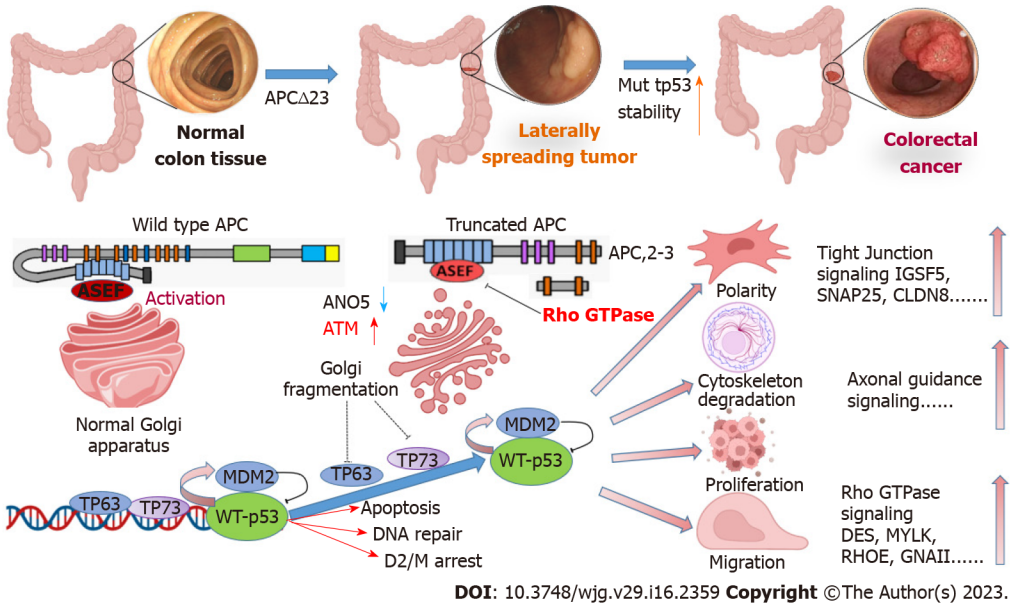

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Figure 1 Schematic diagram of mechanism by which adenomatous polyposis coli-truncating mutations regulate P53 to promote laterally spreading tumor transition to colorectal cancer. APC: Adenomatous polyposis coli; ASEF: Adenomatous polyposis coli-stimulated guanine nucleotide exchange factor; ANO5: Anoctamin 5; ATM: Ataxia-telangiectasia mutation; TP: Tumor protein; IGSF5: Immunoglobulin superfamily member 5; SNAP25: Synaptosome associated protein 25; DES: Desmin; MYLK: Myosin light chain kinase; RHOE: Ras homolog gene family, member E; GNALL: G protein subunit alpha i1.

factors MDM2 proto-oncogene (MDM2) and TP73, and subsequently modulated TP53 stability in the TP53 signaling pathway. These two important signaling molecules are closely related to the stability of TP53 mutants. These data suggest that TP53 mutation has a unique molecular mechanism that differs from that in polyps and adenomas in CRC development from LST.

In our opinion, the progression of LST to CRC is distinct from the development of common polyps or adenoma carcinogenesis. Therefore, we proposed a two-stage cascade mutation hypothesis from LST to CRC (Figure 1): The driver stage and cancerous stage. In the driver stage, APC-truncating mutations drive Golgi fragmentation, resulting in reorganization of cellular structural proteins, thereby leading to abnormal polarity and lateral growth. In the cancerous stage, Golgi fragmentation further affects the repair mechanism of TP53 base mismatch mediated by the heat shock proteins MDM2, TP63, and TP73, resulting in increased stability of mutant TP53 and promotion of LST progression to carcinoma.

SPECIAL GENE EXPRESSION ANALYSIS

Based on the mRNA data of 25711 samples from 980 healthy donors in the genotype-tissue expression (GTEx) v8 database, pan-cancer mRNA-sequencing (mRNA-seq, n = 11057) and survival (n = 10121) data were downloaded from the TCGA database. We then performed a differential expression analysis of *ATM*, *ANO5*, *APC*, and *TP53* in pan-cancer and adjacent normal samples. Survival analysis of these genes was also performed in pan-cancers.

We analyzed the expression profiles of *ATM*, *ANO5*, *APC*, and *TP53* in human normal and pan-cancer samples and found that *ATM*, *ANO5*, and *APC* were significantly downregulated in rectal adenocarcinoma (READ), colon adenocarcinoma (COAD), and READ-COAD samples. In contrast, TP53 showed an obviously higher level in READ, COAD, and READ-COAD samples than in normal samples.

Furthermore, the survival analysis results indicated that *ATM*, *ANO5*, *APC*, and *TP53* expression were correlated with the overall survival (OS) of lung squamous cell carcinoma (LUSC), thyroid carcinoma (THCA), mesothelioma (MESO), pancreatic adenocarcinoma (PAAD), COAD, brain lower grade glioma (LGG), and breast invasive carcinoma (BRCA) patients.

Analysis of ATM, ANO5, APC, and TP53 expression in normal and pan-cancer samples

We analyzed the expression profiles of *ATM*, *ANO5*, *APC*, and *TP53* in normal human tissues based on transcripts per million (TPM) values from the GTEx v8 database. The results indicated that *ATM*, *ANO5*, *APC*, and *TP53* were highly or moderately expressed in most organs or tissues (1 < average TPM < 32), except that *ANO5* expression was low in the whole blood, vagina, and breast (average TPM < 1, Figure 2A).

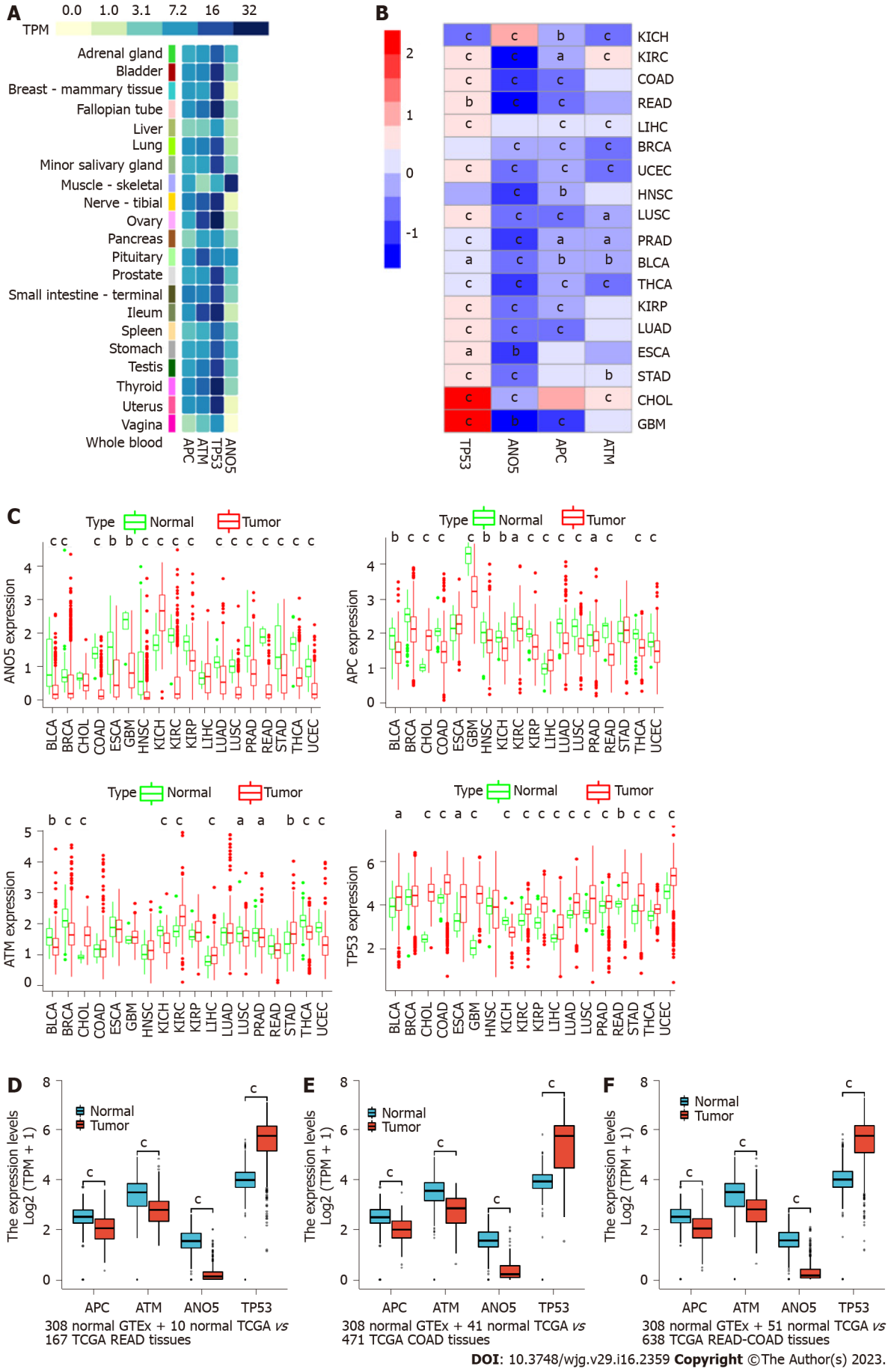

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Figure 2 Analysis of ataxia-telangiectasia mutated gene, anoctamin 5, adenomatous polyposis coli, and tumor protein 53 expression in

normal and pan-cancer samples. A: Expression profiles of ataxia-telangiectasia mutated (ATM), anoctamin 5 (ANO5), adenomatous polyposis coli (APC), and tumor protein 53 (TP53) in normal human organs or tissues; B: Heatmap of log2[fragments per kilobase of exon per million mapped fragments (FPKM) + 1] expression status of *ATM, ANO5, APC*, and *TP53* between cancer and cancer-adjacent samples. Red and blue represent upregulation and downregulation of gene expression, respectively; C: Log2(FPKM + 1) expression status of *ATM, ANO5, APC*, and *TP53* between cancer and cancer-adjacent samples; D: Rectal adenocarcinoma (READ) and normal samples; E: Colon adenocarcinoma (COAD) and normal samples; F: READ-COAD and normal samples. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. KICH: Kidney chromophobe; KIRC: Kidney renal clear cell carcinoma; COAD: Colon adenocarcinoma; READ: Rectal adenocarcinoma; LIHC: Liver hepatocellular carcinoma; BRCA: Breast invasive carcinoma; UCEC: Uterine corpus endometrial carcinoma; HNSC: Head and neck squamous cell carcinoma; LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma; BLCA: Breast invasive carcinoma; THCA: Thyroid carcinoma; KIRP: Kidney renal papillary cell carcinoma; LUAD: Lung adenocarcinoma; ESCA: Esophageal carcinoma; STAD: Stomach adenocarcinoma; CHOL: Cholangiocarcinoma; GBM: Glioblatoma; APC: Adenomatous polyposis coli; ANO5: Anoctamin 5; ATM: Ataxia-telangiectasia mutation; TP: Tumor protein.

Then, we performed a differential expression analysis of mRNAs for these genes across 18 cancer types that had over five pairs of cancer-adjacent samples based on the $\log_2$[fragments per kilobase of exon per million mapped fragments (FPKM) + 1] data from TCGA. Figure 2B shows the heatmap of $\log_2$(FPKM + 1) expression status of *ATM, ANO5, APC*, and *TP53* between cancer and cancer-adjacent samples where red and blue represent upregulation and downregulation, respectively]. Figure 2C shows the $\log_2$(FPKM + 1) expression status of *ATM, ANO5, APC*, and *TP53* between cancer and cancer-adjacent samples.

Table 1 shows that the expression of *TP53* was significantly upregulated in bladder cancer (BLCA), cholangiocarcinoma (CHOL), COAD, esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), LUSC, prostate adenocarcinoma (PRAD), READ, stomach adenocarcinoma (STAD), THCA, and uterine corpus endometrial carcinoma (UCEC) and downregulated in kidney chromophobe (KICH), compared with the expression levels in cancer-adjacent samples ($|\log_2 FC| > 0.19$, 4.99E-12 < P < 0.03). The *ANO5* level was significantly higher in KICH and obviously lower in BLCA, BRCA, COAD, ESCA, GBM, HNSC, KIRC, KIRP, LUAD, LUSC, PRAD, READ, STAD, THCA, and UCEC than in cancer-adjacent samples ($|\log_2 FC| > 0.46$, 2.36E-26 < P < 0.003).

APC expression was significantly upregulated in CHOL and LIHC relative to that in cancer-adjacent samples and an obvious downregulation was noticed in BLCA, BRCA, COAD, GBM, HNSC, KICH, KIRC, KIRP, LUAD, LUSC, PRAD, READ, THCA, and UCEC ($|\log_2 FC| > 0.25$, 1.46E-15 < P < 0.03). In addition, the *ATM* expression was obviously upregulated in CHOL, KIRC, LIHC, and STAD compared to that in cancer-adjacent samples. In contrast, BLCA, BRCA, KICH, LUSC, PRAD, THCA, and UCEC ($|\log_2 FC| > 0.12$, 2.14E-16 < P < 0.049) were obviously downregulated.

We also analyzed the difference in expression among COAD, READ, and their cancer-adjacent samples based on the merged and batch-normalized TPM expression data of the GTEx and TCGA. The results showed that *ATM, ANO5*, and *APC* were significantly downregulated, whereas TP53 showed an obviously higher expression in READ (Figure 2D), COAD (Figure 2E), and READ-COAD (Figure 2F) samples than in normal samples (P < 0.001).

Association of ATM, ANO5, APC, and TP53 with cancer prognosis

To explore the role of *ATM, ANO5, APC*, and *TP53* in pan-cancer prognosis, we conducted a survival analysis in pan-cancers based on the $\log_2$(FPKM + 1) data and clinical survival data of 33 cancer types. The survival map of *ATM, ANO5, APC*, and *TP53* expression in pan-cancers indicated that the expression of these four genes was correlated with OS in LUSC, THCA, MESO, PAAD, COAD, LGG, and BRCA (Figure 3A). The relationship between *ATM, ANO5, APC*, and *TP53* and the OS of cancer patients ($^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$) was identified.

Briefly, LUSC or THCA patients with *ANO5* expression had a poor OS [1.40 < hazard ratio (HR) < 3.80, P = 0.014], whereas MESO or PAAD patients with *ANO5* expression showed a good OS (0.53 < HR < 0.57, 0.0031 < P < 0.018). *ATM* expression was indicative of a poor OS in COAD (HR = 1.70, P = 0.038). *TP53* expression was positively associated with a poor OS in BRCA (HR = 1.40, P = 0.038), LGG (HR = 1.60, P = 0.0067), and PAAD (HR = 12, P = 0.0033). In contrast, *ATM* expression exhibited a positive association with good OS in COAD (HR = 0.54, P = 0.012). Moreover, *APC* expression showed a good co-relation with OS in BRCA (HR = 0.49, P = 9E-06, Figure 3B).

CONCLUSION

APC-truncating mutations driven by Golgi fragmentation are a very important cellular event, which can cause loss of the microtubule binding properties of APC. This effect further reduces microtubule stability, resulting in abnormal cell proliferation and survival, chromosomal instability, and increased migration. Downstream characteristics of Golgi fragmentation include gene expression alterations with *ATM* upregulation and *ANO5* downregulation, which are significant in LST. These observations suggest

Table 1 Identification of ataxia-telangiectasia mutated, anoctamin 5, adenomatous polyposis coli, and tumor protein 53 expression in pan-cancer samples

Cancer type	APC		TP53		ATM		ANO5	
	LogFC	*P* value	LogFC	*P* value	LogFC	*P* value	LogFC	*P* value
BLCA	-0.28889	0.005445	0.314908	0.032102	-0.27112	0.002207	-0.75984	5.46E-07
BRCA	-0.37268	1.46E-15	0.070558	0.261271	-0.41783	2.30E-16	-0.26734	2.03E-17
CHOL	0.798977	4.07E-06	2.043449	1.51E-07	0.718097	8.17E-06	-0.08998	0.123426
COAD	-0.57688	2.42E-15	0.505369	4.40E-09	0.086761	0.429393	-1.16109	3.06E-24
ESCA	0.134442	0.480134	0.792589	0.011648	-0.0696	0.629714	-0.81744	0.003288
GBM	-1.01588	0.000957	2.388451	0.000173	0.016551	0.761476	-1.26367	0.001745
HNSC	-0.18476	0.004628	-0.08774	0.958273	0.116251	0.114312	-0.82404	7.30E-09
KICH	-0.32313	0.003139	-0.63636	1.15E-07	-0.42688	1.83E-05	0.889727	7.05E-07
KIRC	-0.12313	0.033897	0.452895	2.19E-16	0.476935	2.14E-16	-1.43405	4.66E-34
KIRP	-0.34452	1.85E-05	0.784867	3.85E-12	0.110608	0.113455	-0.50628	2.70E-09
LIHC	0.253395	8.34E-06	0.41186	5.80E-06	0.187905	0.000776	0.070468	0.537737
LUAD	-0.4475	1.24E-13	0.430958	3.79E-09	0.14328	0.135749	-0.46868	9.13E-13
LUSC	-0.51847	1.39E-15	0.467462	0.000189	-0.14078	0.016441	-0.73565	5.71E-25
PRAD	-0.17991	0.011276	0.193039	0.000387	-0.12553	0.049659	-0.89276	6.11E-19
READ	-0.71729	3.89E-05	0.638392	0.007547	-0.18753	0.144797	-1.5861	1.99E-07
STAD	0.025202	0.628002	0.660337	1.23E-06	0.321073	0.001535	-0.63734	2.77E-05
THCA	-0.36112	1.91E-13	0.320993	4.99E-12	-0.40106	1.15E-13	-0.89401	2.36E-26
UCEC	-0.34584	3.78E-06	0.552104	1.66E-08	-0.56028	9.35E-14	-0.63913	2.84E-15

BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; ESCA: Esophageal carcinoma; GBM: Glioblatoma; HNSC: Head and neck squamous cell carcinoma; KICH: Kidney chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma; READ: Rectal adenocarcinoma esophageal; STAD: Stomach adenocarcinoma; THCA: Thyroid carcinoma; UCEC: Uterine corpus endometrial carcinoma; APC: Adenomatous polyposis coli; ANO5: Anoctamin 5; ATM: Ataxia-telangiectasia mutation; TP: Tumor protein.

the existence of a unique and novel pathway for the development of CRC from LST, and future studies of potential CRC treatment should focus on this newly identified mechanism.

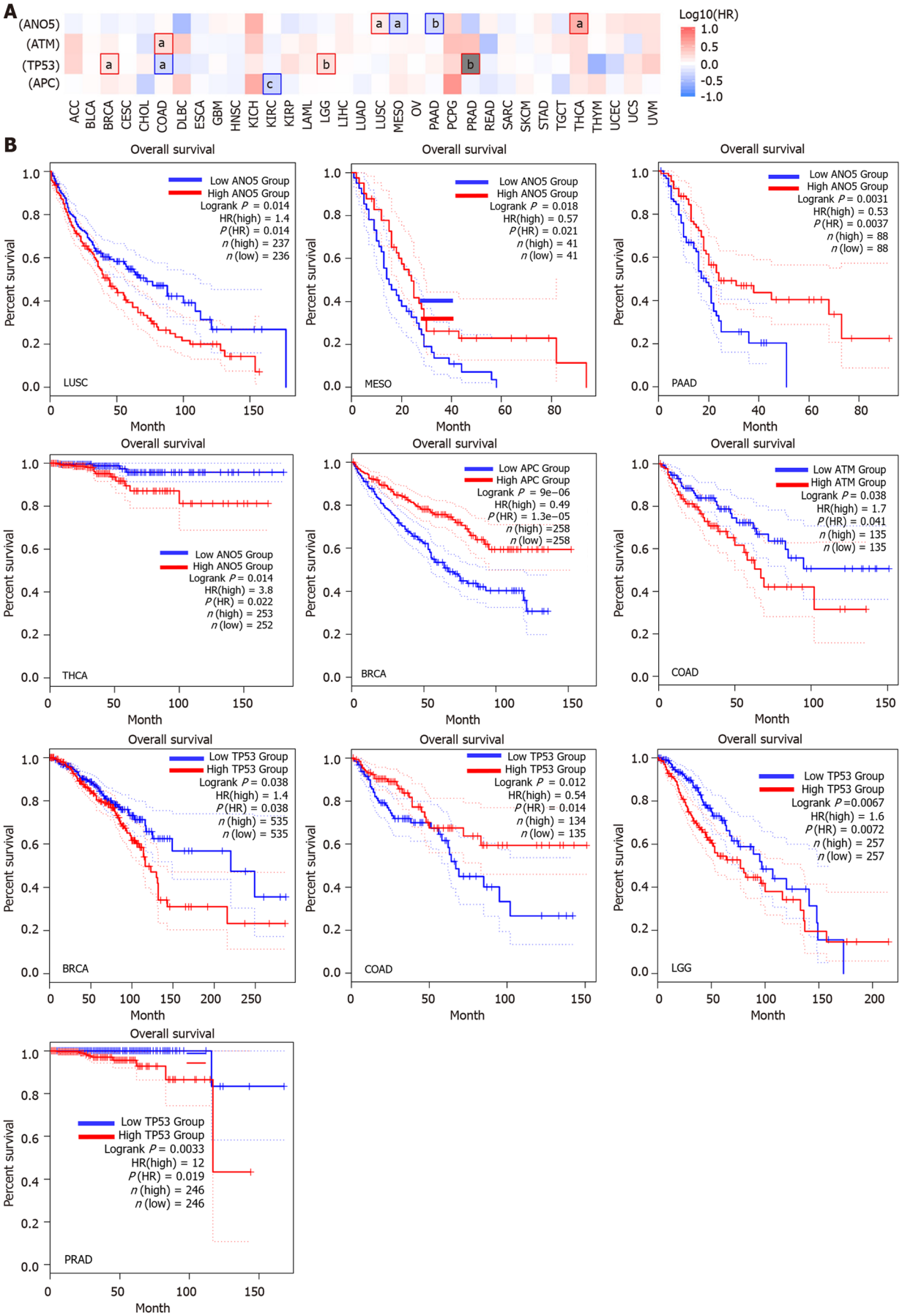

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Figure 3 Association of ataxia-telangiectasia mutated gene, anoctamin 5, adenomatous polyposis coli, and tumor protein 53 with

prognosis of cancer in patients. A: Survival map of ataxia-telangiectasia mutated (*ATM*) gene, anoctamin 5 (*ANO5*), adenomatous polyposis coli (*APC*), and tumor protein 53 (*TP53*) expression in pan-cancers; B: Relationship of *ATM, ANO5, APC*, and *TP53* expression with overall survival of cancer patients. [a]*P* < 0.05, [b]*P* < 0.01, [c]*P* < 0.001. ACC: Adrenocortical carcinoma; BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; DLBC: Lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: Esophageal carcinoma; GBM: Glioblatoma; HNSC: Head and neck squamous cell carcinoma; KICH: Kidney chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LAML: Acute myeloid leukemia; LGG: Brain lower grade glioma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PCPG: Pheochromocytoma and paraganglioma; PRAD: Prostate adenocarcinoma; READ: Rectal adenocarcinoma esophageal; SARC: Sarcomav; SKCM: Skin cutaneous melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular germ cell tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC: Uterine corpus endometrial carcinoma; UCS: Uterine carcinosarcoma; UVM: Uveal melanoma; HR: Hazard ratio; APC: Adenomatous polyposis coli; ANO5: Anoctamin 5; ATM: Ataxia-telangiectasia mutation; TP: Tumor protein.

FOOTNOTES

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Country/Territory of origin: China

ORCID number: Jian-She Yang 0000-0001-7069-6072.

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OPINION REVIEW

Current status and progress in laparoscopic surgery for gallbladder carcinoma

Jia Sun, Tian-Ge Xie, Zu-Yi Ma, Xin Wu, Bing-Lu Li

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Jia Sun, Tian-Ge Xie, Zu-Yi Ma, Xin Wu, Bing-Lu Li, Department of General Surgery, State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100730, China

Corresponding author: Bing-Lu Li, MD, PhD, Doctor, Professor, Department of General Surgery, State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, No. 1 Shuaifuyuan, Wangfujing, Dongcheng District, Beijing 100730, China.
pumchlbl@163.com

Abstract

Gallbladder carcinoma (GBC) is the most common biliary tract malignancy associated with a concealed onset, high invasiveness and poor prognosis. Radical surgery remains the only curative treatment for GBC, and the optimal extent of surgery depends on the tumor stage. Radical resection can be achieved by simple cholecystectomy for Tis and T1a GBC. However, whether simple cholecystectomy or extended cholecystectomy, including regional lymph node dissection and hepatectomy, is the standard surgical extent for T1b GBC remains controversial. Extended cholecystectomy should be performed for T2 and some T3 GBC without distant metastasis. Secondary radical surgery is essential for incidental gallbladder cancer diagnosed after cholecystectomy. For locally advanced GBC, hepatopancreatoduodenectomy may achieve R0 resection and improve long-term survival outcomes, but the extremely high risk of the surgery limits its implementation. Laparoscopic surgery has been widely used in the treatment of gastrointestinal malignancies. GBC was once regarded as a contraindication of laparoscopic surgery. However, with improvements in surgical instruments and skills, studies have shown that laparoscopic surgery will not result in a poorer prognosis for selected patients with GBC compared with open surgery. Moreover, laparoscopic surgery is associated with enhanced recovery after surgery since it is minimally invasive.

Key Words: Gallbladder carcinoma; Laparoscopic surgery; Simple cholecystectomy; Extended cholecystectomy; Hepatopancreatoduodenectomy; Incidental gallbladder cancer

Core Tip: Gallbladder carcinoma (GBC) is the most common biliary tract malignancy with a poor prognosis. Radical surgery is the mainstay of treatment, and the surgical extent depends on the tumor stage. Meanwhile, laparoscopic surgery has the advantage of enhanced recovery after surgery because it is minimally invasive, and has been widely used to treat gastrointestinal malignancies. Although GBC was once regarded as a contraindication for laparoscopic surgery, with improved surgical instruments and skills, recent studies have shown that laparoscopic surgery will not lead to a poorer prognosis compared with open surgery among selected patients with GBC in specialized centers.

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INTRODUCTION

Gallbladder carcinoma (GBC) has the highest incidence among malignant tumors of the biliary system, accounting for 80%-95% of all biliary tract cancers. GBC is more common in Chile, Japan and northern India[1]. Although GBC has a relatively low incidence of about 1.2% of all malignant tumors of the digestive system[2,3], its invasiveness is extremely high. The median survival time is six months, and the 5-year survival rate is less than 5%[4,5]. The prognosis is closely related to the tumor stage[5]. The high degree of malignancy of GBC is mainly due to the lack of submucosa and the relatively thin muscular layer of the gallbladder, and tumor cells are more likely to invade surrounding tissues and organs like the liver quickly[6]. Because of the frequent absence of typical symptoms, over 1/3 of patients are diagnosed with advanced GBC without the opportunity of radical operation. Only 15%-47% of patients with GBC diagnosed preoperatively will meet the indication for surgical resection. However, radical surgery remains the cornerstone of treatment because the effect of adjuvant therapy for GBC is very limited, and the surgical approach depends on the tumor stage (Table 1)[3]. With improvements in diagnostic and surgical techniques, the prognosis of patients with GBC who underwent radical surgery has been significantly improved in recent years[7].

Laparoscopic surgery has been widely used with the advent of "Enhanced Recovery After Surgery (ERAS)". With the advantages such as reducing the incision and magnifying the view, this surgical method can reduce intraoperative bleeding, alleviate postoperative pain, promote earlier oral intake, reduce complications like wound infection, and shorten the duration of hospitalization, achieving the goal of ERAS[8]. Since the rise of laparoscopic cholecystectomy in the early 1990s[9], laparoscopic surgery has been widely used to treat typical gastrointestinal malignant tumors. However, GBC was once regarded as a contraindication for laparoscopic surgery for the following main reasons[10,11]: (1) Bile spillage associated with intraoperative gallbladder perforation and repeated manipulation through the trocars could increase the incidence of peritoneal dissemination or port site metastasis (PSM); (2) The oncologic adequacy and safety of laparoscopic radical surgery for GBC still need to be verified by high-quality prospective studies; and (3) There were technical difficulties related to the procedure, such as lymph node dissection of hepatoduodenal ligament and around the hepatic artery, liver resection and bile duct resection in laparoscopic approaches. However, with the improvement of preoperative diagnosis of GBC, the progress of surgical skills and laparoscopic equipment, and the avoidance of bile spillage by careful manipulation and extensive implementation of retrieval bags, the incidence of peritoneal dissemination or PSM associated with laparoscopic surgery for GBC has been significantly reduced, with no significant difference in survival outcomes compared with open surgery in recent literature[10]. According to a systematic review by Berger-Richardson *et al*[12], the incidence of PSM after laparoscopic cholecystectomy for incidental GBC (IGBC) in the historic era (1991-1999) was about 18.6% and decreased to 10.3% in the modern era (2000-2014). Since the incidence of incision recurrence after open cholecystectomy is approximately 7%[12], the gap between the two approaches is gradually narrowing. Several studies have shown that there is no difference in the number of harvested lymph nodes by laparoscopy or laparotomy in radical resection of rectal cancer[13]. In addition, laparoscopy has been widely used in hepatectomy, which shows that the feasibility and safety of laparoscopic lymph node dissection and hepatectomy have been gradually proved by surgical experts[14]. Moreover, the development of laparoscopic suturing skills makes laparoscopic bile duct reconstruction possible[15]. The surgical extent of GBC varies greatly in different tumor stages. In order to ensure the safety and oncological adequacy of resection, surgeons should strictly select patients undergoing laparoscopic surgery for GBC[16]. This review will discuss the application of laparoscopic surgery in GBC according to the surgical approach and whether the cancer is IGBC.

Table 1 Summary of gallbladder carcinoma T staging according to the American Joint Committee on Cancer 8th edition and the corresponding surgical approach

	AJCC 8th T staging classification	Surgical approach
Tis	Carcinoma *in situ*	Simple cholecystectomy
T1a	Tumor invades the lamina propria	Simple cholecystectomy
T1b	Tumor invades the muscular layer	Extended cholecystectomy including cholecystectomy + lymphadenectomy ± hepatectomy (current consensus)/simple cholecystectomy (under debate)
T2a	Tumor invades the perimuscular connective tissue on the peritoneal side, without involvement of the serosa	Extended cholecystectomy including cholecystectomy + lymphadenectomy ± hepatectomy ± bile duct resection and reconstruction
T2b	Tumor invades the perimuscular connective tissue on the hepatic side, with no extension into the liver	Extended cholecystectomy including cholecystectomy + lymphadenectomy + hepatectomy ± bile duct resection and reconstruction
T3	Tumor perforates the serosa (visceral peritoneum) and/or directly invades the liver and/or one other adjacent organ or structure, such as the stomach, duodenum, colon, pancreas, omentum or extrahepatic bile ducts	Extended cholecystectomy including cholecystectomy + lymphadenectomy + hepatectomy ± bile duct resection and reconstruction (some T3 without distant metastasis)/hepatopancreatoduodenectomy (under debate)
T4	Tumor invades the main portal vein or hepatic artery or invades two or more extrahepatic organs or structures	No significant benefit from surgery

AJCC: American Joint Committee on Cancer.

SIMPLE CHOLECYSTECTOMY

For Tis and T1a GBC, R0 resection can be achieved through simple cholecystectomy, which meets the standard of oncological safety, and both laparoscopic and open surgery can reach the postoperative overall survival rate of 95%-100%[10]. However, it is controversial whether for T1b GBC, simple cholecystectomy or extended cholecystectomy, which includes lymph node dissection and hepatectomy, is oncologically safe[17], although the latter is recommended by the current guidelines[17]. A study of 536 T1b GBC subjects from Surveillance, Epidemiology, and End Results database showed that extended cholecystectomy could improve disease-specific and overall survival of those patients[18]. In addition, Butte *et al*[19] found that 35% of patients with T1b GBC had residual disease after simple cholecystectomy, which supports the necessity of extended cholecystectomy for patients with T1b GBC.

However, more studies in recent years have shown that simple cholecystectomy does not adversely affect the long-term prognosis of patients with T1b GBC[20-22], and the choice of surgical extent mainly depends on the experience of the surgeon[23]. In a meta-analysis in 2017, including 22 publications published in MEDLINE since 1994, Lee *et al*[17] compared the relationship between surgical extents *via* laparoscopic or open surgery and the prognosis of T1 GBC among patients with T1b GBC. They found that the risk difference between simple cholecystectomy and extended cholecystectomy was 0.03, while the risk ratio was 1.06, indicating no significant difference in overall survival outcomes between the two surgical extents. Recent studies have reported that long-term outcomes of patients with early GBC after laparoscopic cholecystectomy, which is now widely adopted, are comparable to laparotomy[24,25]. Therefore, the laparoscopic approach is safe and feasible for patients with early GBC undergoing simple cholecystectomy, and its minimally invasive characteristics can accelerate the postoperative rehabilitation process[26,27]. However, more large cohort studies are needed to confirm the long-term prognosis of this approach, given the low diagnostic rate and staging accuracy of T1 GBC. It should be taken into special consideration that gallbladder perforation caused by forceps during the operation will cause tumor dissemination. For suspected GBC, the resected gallbladder should be removed completely and extracted with a retrieval bag during laparoscopic cholecystectomy to avoid tumor dissemination caused by bile spillage into the abdominal cavity or port sites[24].

EXTENDED CHOLECYSTECTOMY

Extended cholecystectomy for GBC is now mainly recommended for T1b, T2 and some T3 patients without distant metastasis, which includes cholecystectomy + regional lymph node dissection ± adjacent hepatectomy ± bile duct resection and reconstruction[16]. A number of studies have shown that it is safe and effective to perform laparoscopic extended cholecystectomy for patients who meet criteria such as no surgical contraindications, no severe abdominal adhesion and tolerance of pneumoperitoneum. The postoperative recurrence and survival outcomes are comparable to and even better than those of laparotomy[28,29]. According to the single-center retrospective study of patients with T2 GBC treated from 2004 to 2017 by Jang *et al*[11], there was no significant difference between laparoscopic and open

extended cholecystectomy in terms of the number of retrieved lymph nodes and 5-year survival rate, and postoperative hospital stay was significantly shorter in the laparoscopic group. A recent meta-analysis including 14 studies comparing laparoscopic and open extended cholecystectomy for GBC published in several databases up to April 6, 2021, found that laparoscopic surgery had a lower risk of death than open surgery for T3 GBC, while there was no significant difference in death between the two methods for T1 and T2 GBC. In addition, the survival rate after laparoscopic surgery was higher than that after open surgery for the first two years for T2 and T3 GBC, but the three-year and five-year survival rates were similar between the two groups regardless of the tumor stage. Lastly, no significant difference in the overall recurrence was found between the two surgical approaches. The above results also confirm the feasibility and safety of laparoscopic extended cholecystectomy[30].

Lymph node dissection

Lymph node dissection during extended cholecystectomy for GBC is mainly used to stage the patient [31], but the optimal extent of regional lymphadenectomy is still under debate. In the published studies, the extent of lymph node dissection for GBC mainly includes lymph nodes around the hepatoduodenal ligament, and some centers also emphasize the necessity to dissect lymph nodes in the posterior superior pancreaticoduodenal area and along the common hepatic artery at the same time because of the high frequency of metastasis in this area and the possibly improved survival rate after complete resection[27,32,33]. More extensive dissection of the aorto-caval, celiac and superior mesenteric artery nodes has limited effect and insignificant survival benefit. However, some centers advocate aorto-caval node sampling at the beginning of the operation to estimate the presence of distant metastasis. Patients with positive lymph node biopsies in this area can hardly benefit from extended cholecystectomy for GBC[34]. Recent studies have shown that in selected patients with GBC, the extent of laparoscopic lymph node dissection and the number of retrieved lymph nodes are similar to open surgery with few intraoperative and postoperative complications[15,28,29,35,36]. A prospective cohort study by Yoon *et al* [36] showed that the median number of harvested lymph nodes was seven in the 32 patients with T1b-T2 GBC who underwent laparoscopic extended cholecystectomy in their center from 2004 to 2014, exceeding the minimum number of six recommended by the 8th AJCC[21,37], and there was no local recurrence within the extent of lymphadenectomy within 10 years after surgery[36], indicating the feasibility and oncological adequacy of laparoscopic lymph node dissection in patients with GBC. Vega *et al*[34] compared laparoscopic extended cholecystectomy with open surgery in 35 cases of GBC treated in their center, and found that the median number of lymph nodes harvested by the two methods was both six, and there was no significant difference in residual disease, recurrence rate, postoperative complications and 90-d mortality between the two surgical approaches.

Liver resection

Negative margins should be achieved in hepatectomy for patients with GBC in order to reduce tumor recurrence caused by liver micrometastasis. If the gallbladder is severely adherent to the liver, the attached thin layer of liver tissue is often removed in conjunction with the gallbladder to avoid bile spillage caused by gallbladder damage. The most common surgical extent is wedge resection for at least 2 cm of the liver bed, and IVb/V resection is also performed in some centers[16,34]. For some patients with T3 GBC, (extended) right hepatectomy can be performed to achieve R0 resection according to the patient's tolerance, but its clinical benefits need to be further confirmed as extensive hepatectomy will increase the rate of postoperative complications such as liver failure[1]. Current studies have reported the feasibility and safety of laparoscopic wedge resection or IVb/V resection[28,35,38], but there is no consensus on the best extent of hepatectomy, and no clinical data have confirmed the theoretical advantage of IVb/V resection over wedge resection[16]. A multicenter retrospective study by Lee *et al* [39] found that there was no significant difference in the 5-year survival rate or recurrence-free survival rate after wedge resection or IVb/V resection of the liver in patients with T2 GBC who underwent extended cholecystectomy, and some other researches also reported similar results[40,41].

It is worth noting that the hepatic-side and peritoneal-side GBC may have different prognoses. According to a multicenter retrospective study of patients with T2 GBC, the rates of nodal involvement, liver metastasis, postoperative intrahepatic recurrence and vascular and nerve invasion were higher in hepatic-side GBC, and the prognosis was worse than that of peritoneal-side GBC; however, there were no such differences in those with T1 and T3 GBC[42]. Some studies reported that the density of large vessels increased significantly in the deep layer of the gallbladder wall. The hepatic side of the gallbladder was drained by short veins directly connected to the intrahepatic portal veins, with the peritoneal side drained by 1 or 2 cystic veins terminating in the hepatic parenchyma or at the hepatic hilum[43]. The retrospective study suggested that the density of vessels and length of the drainage path caused the difference in the incidence of hepatic, vascular and lymphatic metastasis between hepatic-side and peritoneal-side GBC[42]. Another multicenter retrospective study showed that for patients with hepatic-side T2 GBC, the 5-year survival rate was higher in patients who underwent extended cholecystectomy, including both regional lymphadenectomy and hepatectomy, than in patients who underwent extended cholecystectomy without hepatectomy. In addition, the extent of hepatectomy did not affect the prognosis. Furthermore, for patients with peritoneal-side T2 GBC who underwent lymph node dissection, the 5-year survival rate was not affected by hepatectomy or the extent of lymphaden-

ectomy. Therefore, it is considered that extended cholecystectomy, including lymphadenectomy and R0 hepatectomy, is essential for patients with hepatic-side T2 GBC, while patients with peritoneal-side T2 GBC can only undergo cholecystectomy and lymph node dissection without hepatectomy[39].

Bile duct resection and reconstruction

Indications for bile duct resection in patients with GBC include a positive cystic duct margin, direct tumor invasion of the bile duct and inflammation or scarring around the hepatoduodenal ligament that compromise lymphadenectomy[16]. It is not recommended to perform routine bile duct resection for patients with GBC because it increases the risk of complications without improving the survival rate[44-47]. The present literature has proved the feasibility of laparoscopic bile duct resection in patients with GBC[48]. With the accumulation of experience in laparoscopic surgery in choledochal cysts and pancreatoduodenectomy, the need for bile duct resection and reconstruction is no longer a contraindication of laparoscopic extended cholecystectomy for GBC[16].

HEPATOPANCREATODUODENECTOMY

The gallbladder is adjacent to the liver, duodenum and colon. For patients with locally advanced GBC, it is feasible to achieve R0 resection with hepatopancreatoduodenectomy (HPD) and improve the long-term survival rate. However, only about 10% of the patients can meet the conditions for HPD[1], which include: Tumor at the body or bottom of the gallbladder (hepatic bed type); tumor invading the hepatic hilum (hilar type); massive mass (hepatic bed + hilar type); extensive regional lymph node metastasis (lymph node type); tumor invading the distal bile duct or duodenum; and lymph node metastasis around the head of the pancreas. The contraindications include chronic hepatic diseases, severe comorbidities, R2 resection, paraaortic lymph node metastasis, peritoneal dissemination and distant metastasis. Postoperative mortality and the risk of complications such as liver failure, pancreatic fistula and biliary leakage are extremely high[1]. It is reported that the in-hospital morbidity rate after HPD is more than 10%[49]. Less than 1000 cases of this surgical approach have been reported in the past 50 years[49,50]. Dr Kasumi of Japan performed the first HPD for a patient with GBC invading the duodenum in 1974[51,52]. Takasaki *et al*[53] performed HPD on five patients with GBC invading the duodenum and pancreatic head in 1980. The 30-d mortality was 60%, and the survival time of the other two patients was five months and 16 mo, respectively[1,53]. However, with the improvement in surgical and anesthetic techniques and perioperative management, the prognosis of HPD has been improved. It is reported that the 3-year and 5-year survival rates after HPD are 48% and 37% respectively[54], and more surgeons choose to try this procedure because it has a better prognosis than unresectable tumors [55].

Because of the technical difficulty and high risks of laparoscopic HPD, only four cases of locally advanced GBC or extrahepatic cholangiocarcinoma have been reported to undergo this surgical approach so far (Table 2)[56-59]. Despite the postoperative complications such as bile leakage and delayed gastric emptying[56], the successful implementation of laparoscopic HPD in the four cases has proven its safety and feasibility. This surgery should be performed in large volume centers[1], and the surgeons should have sufficient experience in laparoscopic pancreaticoduodenectomy and laparoscopic hepatectomy[60,61]. Patients who only need a small extent of hepatectomy should be selected as far as possible to reduce postoperative complications. If the patients need major hepatectomy, portal vein embolization should be performed before the operation to increase the remnant volume and avoid postoperative liver failure[49,62]. For patients with obstructive jaundice and cholangitis, bile drainage should be performed preoperatively to improve hepatic function and promote postoperative remnant liver regeneration[63]. The risk of pancreatic fistula after pancreatoduodenectomy in patients with GBC is usually greater than that in patients with adenocarcinoma of the pancreatic head attributed to the soft texture of the pancreatic gland and small pancreatic duct, which could be reduced by two-stage pancreatojejunostomy, external drainage of pancreatic fluid and wrapping omental flap[64].

INCIDENTAL GALLBLADDER CANCER

According to the literature, the incidence of IGBC after laparoscopic cholecystectomy ranges from 0.19% to 3.3% and has increased significantly with the widespread use of laparoscopic cholecystectomy[65]. About 47%-70% of GBC cases are incidentally found during or after cholecystectomy[34], and 45%-60% of patients with IGBC have residual disease after the initial cholecystectomy[45,66,67]. Patients with IGBC are usually at the early stage, and reresection can significantly improve oncological outcomes for patients with T1b-T3 GBC without distant metastasis[68-70]. For patients with bile spillage, positive margin, poorly differentiated tumor or high risks of tumor dissemination during the initial cholecystectomy, it is recommended to perform laparoscopy before secondary radical cholecystectomy to detect metastases that are difficult to be found by preoperative imaging and avoid ineffective reresection[71,72]. Inflammatory adhesion and fibrosis around the hepatoduodenal ligament and the

Table 2 Summary of the published cases of laparoscopic hepatopancreatoduodenectomy for locally advanced gallbladder carcinoma or extrahepatic cholangiocarcinoma

Ref.	Country	Age (yr)	Diagnosis	Operation	Operation duration (min)	Main complication	Hospital stay (d)
Zhang *et al*[57], 2014	China	61	ECC invading the duodenum	LPD + LRH	600	Bile leakage	16
Chong and Choi [58], 2019	South Korea	73	ECC	LPD + LLH	510	Cystitis	16
James *et al*[59], 2021	India	73	GBC infiltrating the CBD	LPD + segments IVb and V	610	Delayed gastric emptying	12
Yao[56], 2022	China	75	ECC + GBC	LPD + segments IVb and V	380	No	12

ECC: Extrahepatic cholangiocarcinoma; GBC: Gallbladder carcinoma; CBD: Common bile duct; LPD: Laparoscopic pancreatoduodenectomy; LRH: Laparoscopic right hemihepatectomy; LLH: Laparoscopic left hemihepatectomy.

gallbladder bed significantly increase the difficulty of radical reoperation for IGBC. However, a few studies have reported the feasibility of laparoscopic radical reresection for IGBC[15,28,73-75] and shown prognoses comparable to that of laparotomy in selected early GBC[70,76]. However, the effect of laparoscopic reresection in patients with IGBC after cholecystectomy for acute cholecystitis needs to be further studied[77-79]. The specimen of the previous operation should be assessed again by a specialized pathologist before reoperation for T stage[19,80], the tumor location (hepatic-side or peritoneal-side), a positive bile duct margin[42], peritoneal and lymphovascular invasion and the presence of Rokitansky-Aschoff sinuses[81], which will increase the rate of conversion to open surgery[34]. Although there is a risk of PSM after cholecystectomy for IGBC, routine port site resection is not recommended because it can't improve the oncological outcomes or reduce recurrence attributed to the high rate of combined peritoneal metastasis, and it can increase the risk of morbidities like incisional hernia[82].

DISCUSSION

Laparoscopic surgery has many advantages over open surgery. Firstly, for benign diseases, which can't be completely excluded from GBC before operation, such as xanthogranulomatous cholecystitis, laparoscopic surgery can retain the opportunity of minimally invasive treatment after frozen section analysis of the specimen is confirmed. Secondly, laparoscopy can provide a clearer surgical field, and laparoscopic exploration can detect liver or peritoneal metastases that are difficult to detect preoperatively, reducing the incidence of unnecessary laparotomy[10]. Thirdly, laparoscopic surgery can reduce postoperative complications such as ileus and infection by reducing contact between the viscera and external environment[83,84]. Finally, minimally invasive surgery can not only accelerate rehabilitation by reducing the incision, alleviating pain, reducing blood loss and promoting early mobilization and oral intake but also initiate postoperative adjuvant therapy earlier[85], which could improve quality of life and prolong long-time survival of the patients[86].

Recent studies have proven the short-term benefits of laparoscopic surgery compared to laparotomy for GBC. A single-center retrospective study by Dou *et al*[87], including 99 patients with T2 and T3 stage GBC who underwent radical resection, showed that compared with open surgery, the laparoscopic group had lower intraoperative bleeding volume (233.91 ± 26.35 mL *vs* 461.25 ± 53.15 mL, $P < 0.01$) and shorter postoperative hospital stay (10.32 ± 0.60 d *vs* 14.74 ± 0.91 d, $P < 0.01$); although it had longer operation time (292.35 ± 14.41 min *vs* 249.02 ± 13.30 min, $P = 0.033$). Lymph node yield (9.39 ± 0.68 *vs* 8.26 ± 0.52, $P = 0.208$) and incidence of postoperative morbidities, including bile leakage (0.11 *vs* 0.07, $P = 0.521$), postoperative bleeding (0.05 *vs* 0.02, $P = 0.448$) and abdominal abscess (0.05 *vs* 0.07, $P = 0.738$) were similar between the two groups[87]. Another retrospective analysis of 102 patients with GBC reported that the patients who underwent laparoscopic surgery experienced a shorter postoperative activity time (2 ± 1 d *vs* 4 ± 1 d, $P < 0.001$), eating time (2 ± 1 d *vs* 4 ± 2 d, $P < 0.001$) and drainage tube removal time (4 ± 3 d *vs* 6 ± 3 d, $P < 0.001$) compared with those who underwent open surgery[88]. Similarly, according to the 18 studies comparing laparoscopic and open radical cholecystectomy for GBC analyzed by Lv *et al*[89], the laparoscopic group had a significantly smaller volume of intraoperative blood loss, a shorter time of drainage tube extraction and diet recovery, a lower rate of postoperative complications such as pulmonary infection and thrombus formation (which was 10.1% compared with 15.8%) and a shorter length of postoperative hospital stay. The shorter hospital stay is theoretically because of reduced wound-related pain, early-period ambulation and earlier gastrointestinal peristalsis. Operative time, intraoperative gallbladder violation, R0 resection rate,

lymph node yield and overall recurrence rate were comparable in the two groups[89]. Predictive factors for conversion to open surgery may include a positive liver margin, massive intraoperative bleeding and an interval between surgeries of more than 60 d, which may cause severe abdominal adhesions[34]. In the prospective study of Cho *et al*[24], including 33 patients with early-stage GBC who underwent laparoscopic surgery, three patients with liver invasion noted by diagnostic laparoscopy had their procedure converted to laparotomy, and another conversion occurred owing to bleeding during locoregional laparoscopic lymphadenectomy. A retrospective study showed that 7 out of 30 patients undergoing laparoscopic extended cholecystectomy with bi-segmentectomy in their center required conversion to open surgery due to distortion of anatomical landmarks and suspected involvement of extrahepatic organs that caused technical difficulty[90]. The rate of conversion to open surgery decreases with the improvement of surgical experience and equipment.

Moreover, laparoscopic surgery will not worsen the survival outcomes compared with open surgery in selected early-stage GBC by experienced surgeons *via* improved diagnosis rate, staging accuracy and precision of operation to avoid bile spillage. A study by Yoon *et al*[36] showed that among the 45 patients with GBC who underwent laparoscopic extended cholecystectomy in their center, the 5-year survival rate of T1a and T1b GBC was 100%, and that of T2 GBC was more than 90%. Only four patients experienced recurrence postoperatively, which were all distant metastases. Itano *et al*[29] compared 16 patients with T2 GBC who underwent laparoscopic extended cholecystectomy with 14 patients who underwent open surgery and found no significant difference in disease-free or overall survival rate between the two groups. However, anatomical features such as thin gallbladder walls and the presence of Rokitansky-Aschoff sinuses make it difficult to evaluate the depth of tumor invasion, and the preoperative staging accuracy is only 40%[91]. Endoscopic or laparoscopic ultrasonography is superior to traditional abdominal ultrasonography and computed tomography in diagnosing T staging[92]. Only a few surgeons have rich experience in laparoscopic radical surgery for GBC, and no consensus has been reached on this operation. Steps such as laparoscopic lymph node dissection, hepatectomy and choledochojejunostomy demand high requirements on surgical instruments and techniques. For patients with a massive mass, duodenal or colonic invasion, jaundice or hilar involvement, more surgeons still prefer open surgery[34]. In addition, regarding the higher cost of laparoscopic surgery from the use of consumable materials and the possibility of conversion to laparotomy, some experts and patients still have concerns and disputes about laparoscopic surgery for GBC[10]. This surgical approach is still in the early stage of the adoption curve. More multicenter prospective studies are needed to confirm the safety and efficacy of laparoscopic surgery for GBC[16].

CONCLUSION

For strictly selected patients with early GBC, long-term survival outcomes of laparoscopic surgery are comparable to that of open surgery, and laparoscopic surgery has the advantage of accelerating rehabilitation because of its minimally invasive characteristics. However, as the progress of minimally invasive treatment for GBC is relatively slow, more studies are needed to further confirm its oncological safety and efficacy and improve the standardization of the procedures of laparoscopic surgery for GBC.

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Country/Territory of origin: China

ORCID number: Zu-Yi Ma 0000-0002-8517-7600; Xin Wu 0000-0002-3839-4768; Bing-Lu Li 0000-0002-9142-0793.

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REVIEW

Molecular regulation mechanism of intestinal stem cells in mucosal injury and repair in ulcerative colitis

Lie Zheng, Sheng-Lei Duan

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Lie Zheng, Sheng-Lei Duan, Department of Gastroenterology, Shaanxi Hospital of Traditional Chinese Medicine, Xi'an 730000, Shaanxi Province, China

Corresponding author: Lie Zheng, MD, Researcher, Department of Gastroenterology, Shaanxi Hospital of Traditional Chinese Medicine, No. 4 Xihuamen Street, Lianhu District, Xi'an 730000, Shaanxi Province, China. liezhenglie@163.com

Abstract

Ulcerative colitis (UC) is a chronic nonspecific inflammatory disease with complex causes. The main pathological changes were intestinal mucosal injury. Leucine-rich repeat-containing G protein coupled receptor 5 (LGR5)-labeled small intestine stem cells (ISCs) were located at the bottom of the small intestine recess and inlaid among Paneth cells. LGR5+ small ISCs are active proliferative adult stem cells, and their self-renewal, proliferation and differentiation disorders are closely related to the occurrence of intestinal inflammatory diseases. The Notch signaling pathway and Wnt/β-catenin signaling pathway are important regulators of LGR5-positive ISCs and together maintain the function of LGR5-positive ISCs. More importantly, the surviving stem cells after intestinal mucosal injury accelerate division, restore the number of stem cells, multiply and differentiate into mature intestinal epithelial cells, and repair the damaged intestinal mucosa. Therefore, in-depth study of multiple pathways and transplantation of LGR5-positive ISCs may become a new target for the treatment of UC.

Key Words: Molecular regulation; Mucosal injury; Regeneration; Ulcerative colitis

Core Tip: Intestinal mucosal injury is an important pathological change in ulcerative colitis (UC), and Leucine-rich repeat-containing G protein coupled receptor 5 (LGR5)-positive intestinal stem cells play an important role in the repair of intestinal mucosal injury. Through in-depth study of multiple signals, LGR5-positive intestine stem cell transplantation therapy may become an important means to treat UC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, relapsing inflammatory disease of the intestinal tract[1]. The course of the disease is prolonged and often brings heavy physiological, psychological and economic burdens to patients. Clinical remission based on symptom improvement does not alter the natural course of UC, and mucosal healing has been the primary therapeutic target of UC in recent years[2]. However, studies have shown that up to 40% of patients who achieve clinical and endoscopic remission still have persistent histological inflammation, which is associated with a higher risk of clinical recurrence of UC, receiving colectomy, and dysplasia[3].

Intestinal stem cells (ISCs) are important adult stem cells that drive the daily renewal of the intestinal epithelium through constant self-renewal, proliferation, and differentiation. ISCs are mainly located in intestinal recesses and play an important role in the repair of damaged intestinal mucosa[4]. In mammals, the gut consists of small villi that extend into the gut cavity and small intestine crypts that sink deep into the lining of the intestine. Leucine-rich repeat-containing G protein coupled receptor 5 (LGR5) is an important marker of ISCs[5]. Under the action of multiple signaling pathways in the body, LGR5-positive ISCs repair damaged intestinal mucosa and maintain intestinal homeostasis through self-renewal and differentiation potential. However, the internal mechanism of how multiple different signaling pathways interact with each other to synergistically regulate LGR5 cells with differentiation potential in UC remains unclear[6]. In this paper, the concept, location, quantity and cycle of ISCs, the repair mechanism of intestinal mucosa by ISCs, the renewal of colon epithelial cells and the regulation of nutritional molecules in damage repair were reviewed to further provide evidence-based medical evidence for the treatment objectives of UC.

CONCEPT, LOCATION, NUMBER AND CYCLE OF ISCS

Stem cells have the capacity for lifelong self-renewal. They are cells that can produce a variety of highly differentiated progeny and can respond differently to changes in their internal environment[7]. Morphologically, the cells at the bottom were counted as "one" in the longitudinal section of the lacunae. The ISCs were approximately located at the fourth layer of cells but fluctuated between the second layer and the seventh layer[8]. Stem cells have three levels of structure, each with different properties and functions. Stem cells have a long cycle. In general, stem cells undergo asymmetric division, but during development or after injury, they undergo symmetrical division and divide into two progeny stem cells to increase the number of stem cells. Normally, the excess stem cells produced by symmetrical division are eliminated by apoptosis or rapid differentiation[9]. After some lacunae die after toxic injury, such as radiation or chemotherapy, the remaining potential stem cells begin to exercise their stem cell potential and undergo symmetrical division to regenerate lacunae[10]. The lacunae also divide to produce more lacunae until the intestinal mucosa returns to normal.

ROLE OF ISCS IN INTESTINAL MUCOSA

Intestinal epithelial tissue is one of the most active self-renewing tissues in adult mammals. Intestinal epithelial cells renew every 5 d, and this process mainly depends on the continuous division and replenishment of ISCs. ISCs are a type of adult stem cell that are mainly distributed in the recesses of the intestine in mammals[11]. ISCs have asymmetric division, self-renewal, and pluripotency; that is, they proliferate and differentiate into a variety of cell types, including absorbent cells, goblet cells, intestinal endocrine cells, and Pan's cells. Each crypt of the intestinal mucosa contains 4 to 6 independent ISCs [12]. Morphologically, the count begins with cells at the base of the crypt, and the ISCs are located in the fourth layer of the crypt, where the stem cells have a very active cell cycle. ISCs first differentiate into transient extender cells, which are daughter cells with limited ability to divide and circulate[13]. The transient expansion cells settled at the base of the crypt for approximately 48 to 72 h, then gradually migrated upward, underwent approximately 6 rounds of cell division, and finally differentiated into terminal cells[14]. Studies have shown that small intestine recess stem cells can rapidly differentiate and repair damage in a small intestine radiation injury model under the action of insulin-like growth factor and hepatocyte growth factor[15]. Some scholars studied Drosophila intestinal mucosal damage induced by sodium glucan sulfate and found that the damaged intestine could secrete signaling proteins

to accelerate the division of ISCs to promote mucosal repair[16].

REPAIR MECHANISM OF ISCS ON INTESTINAL MUCOSA

Markers of ISCs

Each gut stem cell is coated with special protein receptors that selectively bind to or adhere to other "signaling" molecules. These cell surface receptors are known as stem cell markers. Currently, Musasi-1, telomerase reverse transcriptase (TERT) and ID14 are the main markers found in ISCs[17]. Musas-1 is a neural RNA-binding protein but has been shown to be a selective marker of ISCs in addition to the nervous system[18]. Some studies found that Musashi-1-positive cells were found in the small intestine of mice, and Musashi-1 was significantly increased in intestinal specimens of mice after reflex injury [19]. TERT is a ribonuclear protease complex. Studies have shown that immunohistochemical TERT-positive cells are mainly distributed in the base of the small intestine crypt, 4-7 cells away from the bottom of the crypt, and some cells are distributed in the interstitium surrounding the crypt[20]. ID14, a new gene found in Xenopus laevis, encodes a protein containing 315 amino acids[21]. Adult ID14 is mostly found in the intestine but is only weakly expressed in the stomach, lung and testis. Its expression in the intestine does not begin until the metamorphosis stage, which is closely related to the differentiation of adult intestinal epithelial cells[22].

Asymmetric division of ISCs

ISCs continuously increase the number of stem cells through asymmetric division to promote the self-renewal and repair of damaged intestinal tissues to maintain the dynamic balance of the intestinal mucosa[23]. Stem cells divide asymmetrically to form a daughter cell identical to the mother cell and a daughter cell capable of differentiation[24]. During this process of division, the stem cell DNA double strand tends to enter daughter cells that are identical to the mother cell so that the daughter cells that maintain the characteristics of the stem cell retain the mother strand DNA, thus maintaining the stability of the gene[25].

Neuroregulation of ISCs

Intestinal activity is innervated by the sympathetic, parasympathetic, and enteric nervous systems. The sympathetic and parasympathetic plexuses can promote the proliferation and regeneration of intestinal mucosal epithelial cells and accelerate the division of crypt cells through growth factors and inflammatory mediators[26]. The enteric nervous system consists of the intermuscular plexus and submucosal plexus, and most of its neurons are located in the intestinal wall[27]. It has been observed that chemical resection of the intestinal intermuscular nerve plexus can accelerate the proliferation of ISCs, indicating that the intermuscular nerve plexus has an inhibitory effect on intestinal mucosal cell renewal[28].

SELF-RENEWAL OF SMALL ISCS AND SMALL INTESTINAL EPITHELIUM

The intestinal epithelium is a single layer of cell epithelium covering the intestinal lining. As an important organ in mammals, the intestinal epithelium is responsible for digestion, absorption and resistance to intestinal pathogenic microorganisms[29]. Structurally, the epithelium of the small intestine is composed of a large number of repeating units called crypt villi[30]. The intestinal villi are composed of multiple differentiated cells that penetrate into the intestinal cavity to perform digestive and absorption functions, and the base of each villus encloses multiple intestinal recesses, each containing proliferative ISCs[31]. To avoid cytopathies caused by constant contact with external stimuli in the intestinal cavity, the small intestine epithelium is constantly renewing itself, and most cells renew themselves every 4-5 d on average. In line with this physiological function, small ISCs located in crypts have the ability of lifelong self-renewal, making the small intestinal epithelium an important model for adult stem cell research[32].

In the small intestine recess, small ISCs divide every 24 h on average, generating transient amplifying cells (TA cells) while renewing themselves[33]. Fast proliferating cells have a cell division cycle of approximately 12 h, migrating up the recess while performing several fast divisions[34]. In the process of upward migration, the descendant cells gradually differentiated into two types of cells, namely, the secretory lineage and the absorptive lineage. Secretory cells mainly include Paneth cells, goblet cells, and enteroendocrine cells, while absorptive cells mainly refer to intestinal epithelial cells. In contrast to the goblets, intestinal secretory cells and intestinal epithelial cells, which continue to migrate upward into the villi to perform their functions and reach the apex of the villi and undergo apoptosis within 3 to 5 d, Paneth cells migrate downward to the base of the crypt and survive for 3 to 6 wk[35].

Small ISCs and intestinal epithelial lesions

LGR5-labeled small ISCs not only mediate the normal self-renewal of the small intestine epithelium but

also act as the initiation cells of inflammatory cells in the case of mutation, seriously affecting life and health[36]. Since the self-renewal and repair rate of the small intestine epithelium is very fast, the imbalance of its renewal regulation easily leads to epithelial damage. LGR5-labeled small ISCs mediate the daily renewal of the small intestine epithelium, so the relationship between LGR5+ small ISCs and inflammation has received extensive attention[37].

Studies have shown that overactivation of the Wnt signaling pathway induces the release of inflammatory cytokines. Consistent with this, the vast majority of patients with UC carry the inactivated adenomatosis polyposis coli (*APC*) gene mutation. Using a mouse model, specific knockout of the APC gene in LGR5-labeled small ISCs resulted in a massive release of inflammatory cytokines in the short term[38]. Further studies showed that LGR5-positive cells in UC patients consistently produced all other cell types throughout the tumor tissue while self-renewing, demonstrating the tumor stem cell properties of LGR5-labeled cells[39].

Trophic molecular regulation of colon epithelial cell renewal and damage repair

The microenvironment refers to the surrounding environment where stem cells are located under physiological conditions and is usually composed of stem cells themselves, surrounding cells and the extracellular matrix[40]. Cell-to-cell contact in the microenvironment and the existence of various growth factors in the microenvironment coregulate the self-renewal and differentiation of stem cells [41]. LGR5+ small ISCs live in a specific environment, namely, at the bottom of the small intestine recess, mosaic among Pan's cells[42]. Paneth cells, TA cells, and peripheral mesenchymal cells together constitute a unique microenvironment for small ISCs, in which a variety of cell pathways, including the Wnt, Notch, epidermal growth factor (EGF), and bone morphogenetic protein (BMP) signaling pathways, cooperate to regulate the proliferation and differentiation of intestinal epithelial cells and repair after injury[43].

Wnt signaling pathway

The Wnt signaling pathway is a highly conserved signaling pathway that regulates cell proliferation, cell fate determination and cell differentiation and plays a crucial role in embryonic development and adult stem cell maintenance[44]. Mutations in the Wnt signaling pathway are closely related to the occurrence of many diseases, especially colorectal cancer.

The Wnt signaling pathway plays a key role in the dry maintenance of small ISCs[45]. The first event that prompted the link of Wnt signaling to small ISCs was the discovery of a large number of *APC* gene mutations in colorectal cancer. As an important inhibitory factor of Wnt signaling, *APC* plays an important role in regulating Wnt signal strength. Mutation of the *APC* gene leads to overactivation of Wnt signaling[46]. Therefore, the overactivation of Wnt signaling may be closely related to the occurrence of colorectal cancer. In mouse models, *APC* gene mutation or deletion leads to the development of colorectal cancer. Both T cell factor 4 (*TCF4*) gene knockout and beta-catenin gene knockout will result in rapid loss of proliferative stem cell regions in the crypt[47]. All of this evidence suggests that activation of Wnt signaling promotes the dryness of small ISCs[48]. In line with this, Wnt signaling activity in the small intestinal epithelium decreased in a gradient along the crypt-villus axis, with the highest Wnt signaling activity at the base of the crypt[49]. The Wnt ligand is mainly secreted by Panzzled cells and peripheral mesenchymal cells at the base of the crypt. LGR5-labeled small ISCs actively express Frizzled receptors to transmit Wnt signals[50]. A series of target genes downstream of Wnt signaling mediate its physiological function. A large part of the abovementioned small ISC stem cells are direct target genes of Wnt signaling, including LGR5, achaete-scute family bHLH transcription factor 2, and Musashi-1. Other target genes of Wnt signaling, including Myc, play an important role in the occurrence of colorectal cancer[51] (Figure 1).

Notch signaling pathway

Notch signaling is a functionally conserved signaling family that exists widely in multicellular animals (metazoans). Notch signaling is mainly transmitted through cell-cell contact and plays an important role in physiological processes such as cell proliferation, stem cell maintenance, cell fate determination, differentiation and apoptosis[52].

Unlike most cellular pathway transduction processes, Notch signaling does not rely on a second messenger (secondary messengers). Posttranslational Notch protein is localized to the cell membrane as an active receptor after O-Fut-mediated glycosylation and PC5-mediated protease cleavage[53]. When ligands located near the cell membrane, such as Dll1, Dll4 and jagged1, bind to the Notch receptor, the Notch receptor is sequentially cleaved by ADAM and gamma-secretase-mediated protease[54]. The Notch receptor NICD (Noch intracellular domain) is released. Gamma-secretase-mediated protease cleavage may occur at the cell membrane or at the surface of endosome membranes containing NICDs, but NICDs produced by the latter usually enter the proteasome degradation pathway[55]. The released NICD is transferred into the nucleus, where it interacts with the DNA binding protein CSL (an acronym for C BF-1/RBPJ-κ in Homo sapiens / Mus musculus respectively, S uppressor of Hairless in Drosophila melanogaster, L ag-1 in Caenorhabditis elegans) and recruits a transcriptional coactivator to activate the expression of downstream target genes[56].

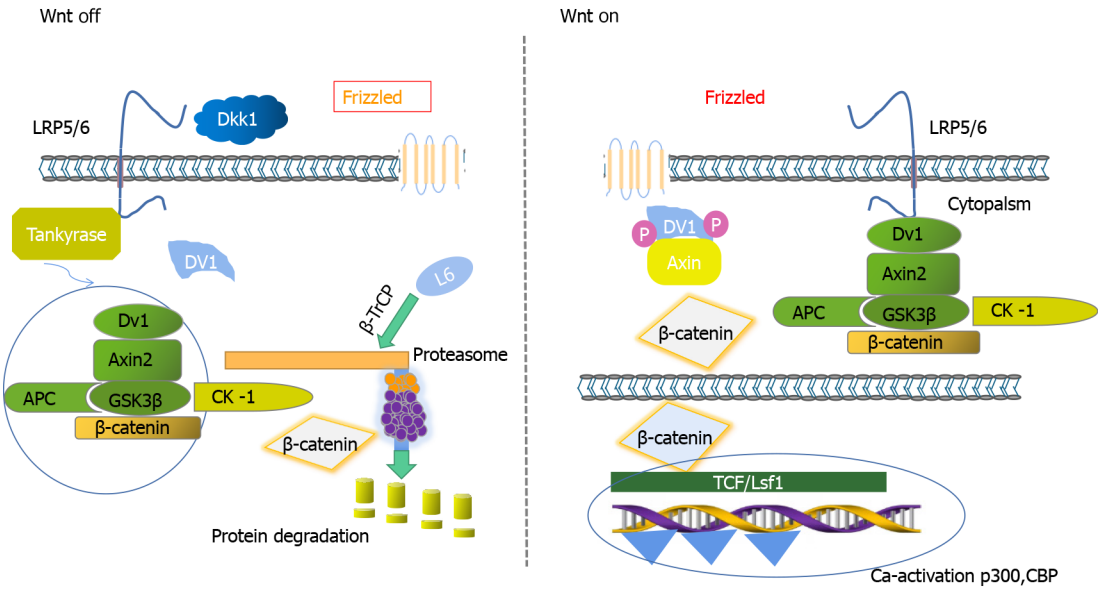

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Figure 1 Wnt signaling pathway. LRP5/6: LDL-receptor-related protein 5/6; Dvl: Dishevelled; APC: Adenomatosis polyposis coli; GSK-3β: Glycogen synthase kinase-3β; CK1: Casein kinase 1.

The Notch receptor is a single transmembrane protein that mainly includes Notch1, Notch2, Notch3 and Notch4 members in mammals. The Notch receptor extracellular end contains 29 to 36 EGF-like repeats, which may mediate Notch receptor and ligand interactions[57]. In mammals, Notch signaling ligands also contain multiple members, including Jagged1, Jagged2, Dll1, Dll3 and Dll4. The interaction of multiple ligands with multiple receptors increases the complexity of the Notch signaling pathway[58] (Figure 2).

BMP signaling pathway

BMP is a transforming growth factor (TGF-β). TGF-β is an important member of the TGF-β superfamily [59]. By regulating the activity of downstream genes, they play an important role in mesoderm formation, nervous system differentiation, bone development and cancer occurrence. BMP signal transmission occurs mainly through the specific binding of BMP protein to the BMP receptor (BMPR) on the cell membrane. Meanwhile, regulated Smads (R-SMAD) are regulated by activated type I receptors (BMPR1), which detach Smad molecules from cell membrane receptors[60]. After binding Smad4 [Common-mediator Smad (Co-SMAD)] in the cytoplasm, it enters the nucleus and coregulates the transcription of target genes with the participation of other DNA-binding proteins[61].

In contrast to Wnt signaling activity, BMP signaling activity increased gradually along the crypt-villus axis. In the small intestine, BMP ligands, including BMP2 and BMP4, are mainly secreted by mesenchymal cells around the crypt and inside the villi, while the BMP receptor Bmpr1a is expressed throughout the small intestine epithelium. Because peripheral mesenchymal cells also secrete BMP ligand inhibitors, including Noggin and Gremlin1, the BMP signal intensity in the crypt is low[62].

Hedgehog signaling pathway

The Hedgehog (Hh) signaling pathway is essential for embryonic development and cell growth and differentiation after embryogenesis[63]. Among mammals are three Hh family members: Sonic Hh, Indian Hh (Ihh), and Desert Hh. Ihh is the main Hh protein expressed in the intestine. It acts on mesenchymal cells through paracrine signaling by differentiated epithelial cells and negatively regulates the proliferation of crypt columnar cells by increasing BMP signals[64]. In addition, Ihh inhibits the lamina propria immune response. Without causing any damage to the upper cortex, Ihh knockout activates an immune response similar to the wage-healing response, epithelial remodeling, and recruitment of fibroblasts and macrophages[65]. Therefore, the decreased expression of Ihh caused by the injury or dysfunction of the upper cortex, thus triggering the damage repair of the interstitial cells, may be one of the main mechanisms of the wound healing response[66].

Hippo-YAP signaling pathway

The Hippo signaling pathway is a newly discovered cell signal transduction pathway whose main part is a kinase chain. Among them, kinase MST1/2 (mammalian Sterile 20-like kinases 1/2) can phosphorylate and activate LATS1/2 (large tumor suppressor 1/2)[67]. LATS1/2 phosphorylates and inhibits the key kinase Yes-associated protein (YAP)/Tafazzin (TAZ). YAP/TAZ are two homologous

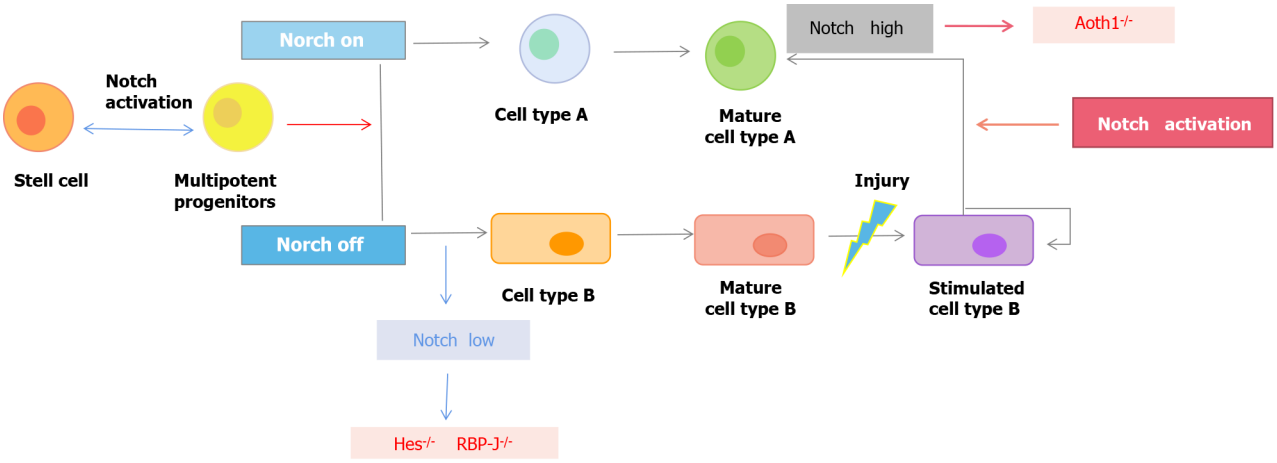

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Figure 2 Notch signaling pathway.

transcription cofactors that mediate most of the physiological and pathological functions of Hippo signaling pathways[68]. YAP is expressed in both the small intestine and large intestine, with low expression in the small intestine but high expression in the colon, especially in the terminal colon. At the cellular level, YAP was localized in the cytoplasm in intestinal villi and upper crypt cells and in the nucleus in LGR5+ ISCs at the bottom of crypts and was expressed at low levels in Pan's cells, indicating that YAP activity was negatively correlated with the degree of differentiation of intestinal epithelial cells [69].

The Hippo signaling pathway plays an important role in regulating the differentiation of ISCs. The proliferative ability of mouse intestinal epithelial-specific YAP transgenic stem cells increased, while the differentiation ability decreased[70]. Consistently, MST1/2 knockout in the mouse gut promoted stem cell proliferation, accompanied by abnormal crypt cell differentiation and reduced goblet cells[71]. Some studies have found that LATS1/2 double knockout in the intestine promotes the proliferation of crypt cells, an increase in ISCs, and the differentiation of goblet cells[72]. Further study showed that YAP/TAZ could cooperate with Klf4 to promote the differentiation of crypt cells into goblet cells. Regarding the effect of Hippo signaling pathway inhibition on goblet cell differentiation, the results of the above two experiments were different. Some scholars believe that this is because the intestine-specific gene transfer method they used can mildly express YAP exogenically or inhibit MST1/2 and LATS1/2, so that YAP can not only promote ISC proliferation but also cause differences in the activity of goblet cell differentiation. These results indicate that the regulatory effect of YAP on ISCs is closely related to its activity level[73] (Figure 3).

UC INTESTINAL INFLAMMATORY RESPONSE PROCESS

Intestinal mucosal mucus secretion decreased

The intestinal mucosa is covered with a thick layer of mucous substances containing a variety of antimicrobial molecules, which can play a protective role in the intestinal mucosa. It can lubricate the intestine and resist the invasion of microorganisms, pathogens and other harmful substances, acting as a chemical barrier and mechanical barrier protection[74]. One possible reason for the aggravation of UC is that the number of goblet cells in the intestinal mucosa is reduced, and the function of intestinal mucus secretion is impaired. Colon mucosal epithelial cells are mainly composed of goblet cells (GCs), secretory cells that secrete a large number of mucoproteins (MUCs) and intestinal trefoil factors (ITFs) and human intestinal resistin-like molecule β (resistin-like molecule β, RELM-β)[75].

The main component of the mucin layer is mucin, which is a high molecular weight glycoylated protein secreted by GCs. It is an important bioactive peptide that can coat bacteria and prevent direct contact between bacteria and epithelial cells[76]. Therefore, mice with insufficient mucus secretion easily develop UC, and studies have shown that MUC2-deficient mice or MUC2 gene mistranslation mice can spontaneously develop UC[77].

Studies have shown that the synthesis of MUC2 in the colon during MUC activity is 40% less than that in the normal colon, indicating that the decrease in mucin in the colon mucosa is one of the reasons for the weakening of intestinal mucosal barrier function and the pathogenesis of UC[78]. The Notch signaling pathway is one of the important ways to maintain the proliferation and differentiation of colon epithelial cells. Overactivation of Notch leads to increased expression of the transcription factor HE-1 in human colon cell lines, inhibits Hath-1 expression, and subsequently inhibits the differentiation

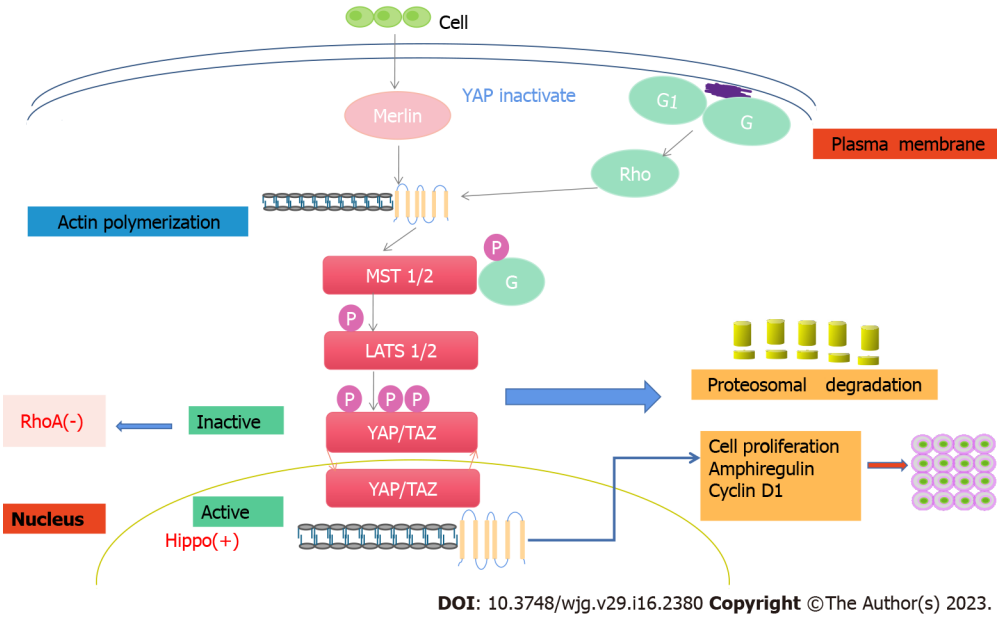

Figure 3 Hippo signaling pathway. MST: Mammalian sterile; LATS: Large tumor suppressor; YAP: Yes-associated protein; TAZ: Tafazzin.

of intestinal epithelial cells into goathous cells, resulting in a decrease in secretory cells and formation of the intestinal mucosal layer[79].

Intestinal mucosal oxidative stress response

Chronic intestinal inflammation can cause a large number of white blood cells to infiltrate the intestinal mucosa, including neutrophils and macrophages, which can produce inflammatory factors and excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing an intestinal mucosal oxidative stress response and intestinal mucosal damage together with an inflammatory response[80]. ROS content was positively correlated with the occurrence and development of UC. ROS consist of a variety of components, including peroxide, hydroxyl and a large amount of hydrogen peroxide. RNS include nitric oxide, nitrogen dioxide and peroxynitrite[81].

When UC occurs, colon mucosa produces a large number of inflammatory molecules and activates a large number of inflammatory response pathways, which jointly promote the production of a large number of peroxides and accumulate in the intestine, self-sustaining and amplifying intestinal oxidative stress, forming a vicious cycle[82]. A large number of ROS can destroy the structure of intestinal endothelial cytoskeleton proteins and cause intestinal mucosal barrier dysfunction. Finally, the structure and function of the intestinal mucosal barrier are damaged, which affects the protective effect of the intestinal tract[83]. ROS can also increase the permeability of the cell membrane. On the one hand, ROS can cause the inflow of extracellular Ca^{2+} into the cell to promote the apoptosis of intestinal cells; on the other hand, ROS can cause a peroxidation reaction with the cell membrane to damage the normal structure and function of intestinal mucosal cells and further lead to the impairment of intestinal mucosal function[84].

Intestinal mucosal barrier damage

The intestinal mucosal barrier has selective permeability. When the intestinal mucosal barrier is destroyed, mucosal inflammation can cause necrosis and shedding of epithelial cells, which increases intestinal mucosal permeability[85]. Structural damage to epithelial cells leads to changes in the tight connective structure and loss of protective effects so that various pathogenic substances in the intestinal cavity are absorbed into the body[86]. The intestinal immune system is repeatedly stimulated and misidentified with these harmful substances, which continuously activates intestinal macrophages and tissue lymphocytes and further stimulates or aggravates the release of inflammatory factors in the intestine, thus continuously initiating an excessive intestinal immune inflammatory response and ultimately damaging the intestinal mucosal barrier, resulting in the loss of protective function of the intestinal mucosal barrier and damage to intestinal tissues[87]. A large number of studies have shown that a large number of epithelial cells in the inflammatory site of the intestinal mucosa in UC patients suffer from apoptosis, and the resulting tight connection injury is considered to be an important cause of UC[88].

It was found that the goblet cells and mucus secreted by intestinal mucosa in patients with UC were reduced. Tight junctions are occlusive links formed by the binding of the outer layer of the adjacent intestinal epithelial cell membrane by specific transmembrane proteins[89]. Tight junctions are mainly

composed of tight junction proteins, including Occludin, the claudin family, Zonula occludens (ZO), the ZO family and junctional adhesion molecule (JAM), which are important structures of epithelial barrier function and play a decisive role in intestinal mucosal permeability by JAM[90]. As a transmembrane tight junction protein, Occludin can form the paracellular tight junction structure and is an important structural and functional protein involved in signal regulation of tight junction formation. Studies have shown that the silencing of occludin genes can increase the cell bypass permeability of intestinal epithelial cells, resulting in an increase in macromolecules and harmful substances in the intestine[91]. Several studies have shown that occludin gene expression in the colon mucosa of UC patients decreases, resulting in a decrease in occludin protein synthesis[92]. Claudins are one of the transmembrane proteins of intestinal epithelial cells. The extracellular part of Claudins acts as a ligand and interacts with transmembrane lectin receptors of adjacent epithelial cells to bind, thus filling the cellular gap and maintaining the tight connection function of the intestinal mucosa[93]. ZOs act as an "assembly platform" for tight junctions that link transmembrane proteins and the cytoskeleton to recognize and transmit signals[94]. The decreased expression of ZOs in intestinal mucosal epithelial cells indicated increased intestinal permeability and damage to the intestinal mucosal barrier[95]. Tight junctions are regulated mainly by protein phosphorylation. When the intestinal mucosa is stimulated by inflammation or oxidative stress, Occludin and ZO-1 phosphorylation are deactivated, and reallocation of the Occludin-Zo-1 complex affects the normal structure of tight junctions of intestinal epithelial cells, resulting in increased intestinal permeability and damage[96].

LGR5-labeled small ISCs

LGR5-labeled small ISCs are the most recognized small ISCs. The LGR5-labeled cells are located at the bottom of the crypt base columnar cell (CBC), which is also called the crypt base CBC because of its small size and elongated shape[97]. As early as 1974, the CBC stem cell model was proposed. According to the theory, CBC cells are small ISCs that live in a microenvironment formed by Paneth cells[98]. Once their offspring leave this microenvironment, they begin to differentiate into a variety of differentiated cells[99]. It was not until 2007, when the CBC cell-specific marker LGR5 was identified, that the theory was experimentally confirmed[100]. In the LGR5-enhanced green fluorescent protein (EGFP)-IRES-Cre ERT2 gene knockout mouse model, CBC cells were labeled with EGFP fluorescent protein. Lineage tracing experiments subsequently demonstrated that the progeny of CBC cells could differentiate into any kind of cell in the small intestinal epithelium, and such lineage markers could persist in the small intestinal epithelium, demonstrating the small ISCs property of CBC cells. EGFP fluorescent protein-labeled CBC cells were isolated using flow cytometry and were encapsulated in Matrigel for *in vitro* stem cell culture in the presence of three growth factors (EGF, Noggin, and R-spondin). Individual LGR5+ cells can grow into organoids, which closely resemble the structure and cellular composition of the intestinal epithelium in vivo. LGR5+ small ISCs can both self-renew and generate all progeny differentiated cells. This evidence suggests that LGR5-labeled cells represent small ISCs[101].

LGR5-labeled small ISCs are actively dividing stem cells that divide every 24 h on average. LGR5+ cells produce transient multiplication cells while generating new small ISCs[102]. TA cells migrate upward and differentiate gradually during subsequent rapid division. The present study suggests that self-renewal of LGR5+ small ISCs follows a "neutral competition" model. LGR5+ small ISCs can maintain their dry properties only when they are located in a microenvironment composed of Pan cells [103]. Because LGR5+ small ISCs divide symmetrically, the progeny cells forced out of the microenvironment due to space crowding will differentiate into TA cells, while the progeny cells left in the microenvironment will retain their stem cell properties[104].

The "+ 4 stem cell" model is another theory about the localization of small ISCs. The + 4 cells refer to the cells placed fourth from Paneth cells at the bottom of the crypt and are considered small ISCs because of their label retention ability[105]. Marker retention means that after marking the DNA of +4 cells, these markers remain in + 4 cells for a long time afterward and do not disappear with cell division [106]. This marker retention property is often thought to be unique to stem cells. At present, markers of + 4 stem cells have been identified, including Bmi and Lrig1, Hopx and Tert, *etc*. However, the specificity of these markers has been under great controversy. Studies have shown that cells at the bottom of the small intestine recess all express these genes; that is, the expression of these genes is not substantively specific[107].

The role of LGR5 and BMP pathways in UC mucosal injury

Significant expansion of LGR5+ small ISCs was detected under normal physiological conditions after BMP signaling was blocked by directly inducing the small intestinal epithelial receptor Bmpr1a, which specifically knocked out BMP signaling[108]. Specific knockout of the Bmpr1a receptor in LGR5+ small ISCs also led to rapid expansion of stem cell groups. In vitro culture and *in vivo* lineage tracing experiments showed that the self-renewal and proliferation abilities of LGR5+ small ISCs were significantly enhanced after BMP signaling inactivation[109]. In the case of long-term BMP signal inactivation, continuous and unrestricted expansion of LGR5+ small ISCs will lead to malignant proliferation of the small intestinal epithelium and the appearance of small intestinal polyps[110]. These phenotypes are very similar to the symptoms of human juvenile intestinal polyps. Finally, the radiation damage model also verified the upregulation of stem cell function after BMP signal inactivation[111].

That is, after BMP signal inactivation, a certain dose of radiation damage can only lead to the apoptosis of some LGR5+ small ISCs, while the remaining LGR5+ small ISCs actively participate in the process of damage repair, thus greatly accelerating the process of radiation damage repair[112].

The relationship between BMP signaling inactivation and self-renewal disturbances in the small intestine epithelium has long been noted. This is because inactivated mutations in the BMP signaling pathway, including the BMPR1A and SMAD4 genes, are found in most human genetic juvenile polyps [113]. In the small intestine, the BMP ligands BMP2 and BMP4 are mainly secreted by mesenchymal cells in the intestinal villi and mesenchymal cells around the small intestine recess. The BMP inhibitors Noggin and Gremlin1 are mainly secreted by mesenchymal cells around the crypts of the small intestine [114]. This secretion pattern results in higher BMP signaling activity in the villi and lower BMP signaling in the crypts of the small intestine. Similarly, however, cells in the mesenchyme responding to BMP stimulation should also be in a state of BMP signaling activation due to the abundance of BMP ligands in the mesenchyme[115]. In juvenile intestinal polyps, BMP signaling inactivation means that all cells no longer respond to BMP signaling, and malignant proliferative intestinal polyps appear[116]. Therefore, it is not clear whether BMP signaling in epithelial cells or in mesenchymal cells plays an important role in inhibiting the appearance and growth of small intestinal polyps. Earlier studies using a Noggin transgenic overexpression mouse model and a systemic Bmpr1a knockout mouse model failed to solve this problem[117].

The Wnt/β-catenin signaling pathway promotes ISC proliferation and maintains intestinal epithelial homeostasis

In the small intestine, the Wnt/β-catenin signaling pathway is thought to be critical for maintaining ISCs self-renewal and proliferation. Wnt was highly expressed in the stem cell area and around proliferating cells in the small intestine, and it decreased gradientally upward with the intensity of differentiation. Genes expressed in intestinal epithelial stem/progenitor cells, such as those that label ISCs LGR5 and Olfm4, are regulated by Wnt signals[118]. There are 19 different Wnt genes expressed in the small intestine. The main cell source of classical Wnt, such as Wnt3, Wnt6 and Wnt9b, is epithelial cells, not classical Wnt[119]. For example, Wnts2b, Wnt4, Wnt5a, Wnt5b, and Wnt antagonists [secreted frizzled related protein (SFRP)-1, SFRP-2, Dickkopf (DKK)2, and DKK-3] are derived from mesenchymal cells. Wnt secreted by epithelial or stromal cells first binds to the coreceptors LRP5/6 and Frizzled on the cell membrane in crypt cells, causing increased expression of β-catenin. Activated beta-catenin further binds to the nuclear transcription factor TCF4 to drive gene expression that supports stem cell maintenance, proliferation, and differentiation[120]. Blocking the Wnt/β-catenin signaling pathway leads to the stagnation of intestinal epithelial cell proliferation. Previous studies have demonstrated that knockout of TCF, DKK1 (Wnt antagonist), Ctnnb1 (β-catenin gene), or c-Myc (Wnt target gene) can significantly affect the proliferation of intestinal epithelial cells in mice. TCF4 knockout in embryonic intestinal epithelial cells resulted in no proliferation in the intervillus region of the small intestine in neonatal mice, while induced knockout of TCF4 and Ctnnb1 blocked crypt proliferation in adult mice[121]. In contrast, the addition of the Wnt agonist R-spondin (roof plate-specific spondin) or the elimination of APC resulted in small intestine or colorectal hyperplasia. Meanwhile, the deletion of Wnt key mediators ring finger protein 43 and zinc and ring finger 3 will also cause intestinal proliferation. Therefore, Wnt signaling plays an important role in the dry maintenance, proliferation and differentiation of small ISCs [122].

Although a large number of studies have confirmed that Wnt secreted by interstitial cells is essential in small intestine development, formation, and damage repair, the evidence that secreted Wnt regulates small intestine homeostasis in mice remains unclear[123]. Wnt3 derived from Pan's cells is necessary for the *in vitro* culture of LGR5-labeled ISCs organoids. Other studies have shown that Wnt generated from epithelial or mesenchymal cells supports intestinal epithelial growth in organoids in vitro[124]. Some scholars have demonstrated that purified stromal cells can support the formation of epithelial organoids that knock out Wnt3. However, mouse models with Paneth cells removed or Wnt3 knocked out in intestinal epithelial cells showed no obvious phenotype, and the types of interstitial cells secreting Wnt in the *in vivo* small ISCs microenvironment, as well as the mechanism of inducing secretion, remain unclear, so more *in vivo* evidence is needed to provide support[125] (Figure 4).

The Notch signaling pathway guides the differentiation of ISCs

Notch receptors and ligands are expressed only at the crypt site, and their signaling activity plays an important role in the self-renewal and differentiation of the small intestine epithelium[126]. First, Notch signaling regulates the differentiation process of small ISCs. In the process of upward migration, TA cells gradually differentiate into two types of cells, namely, secretory cells and absorptive cells. This differentiation process is mainly regulated by the Notch signaling pathway. Notch activation inhibited cell differentiation toward the secretory type and promoted cell differentiation toward the attractor type. Specific inhibition of Notch signaling in the small intestinal epithelium can rapidly transform all small intestinal crypt cells into secretase cell types by Notch receptor, ligand knockout, or gamma-secretase inhibitor treatment[127]. Conversely, activation of Notch signaling in the small intestinal epithelium significantly inhibited secretory cell differentiation. Second, Notch signaling regulates the

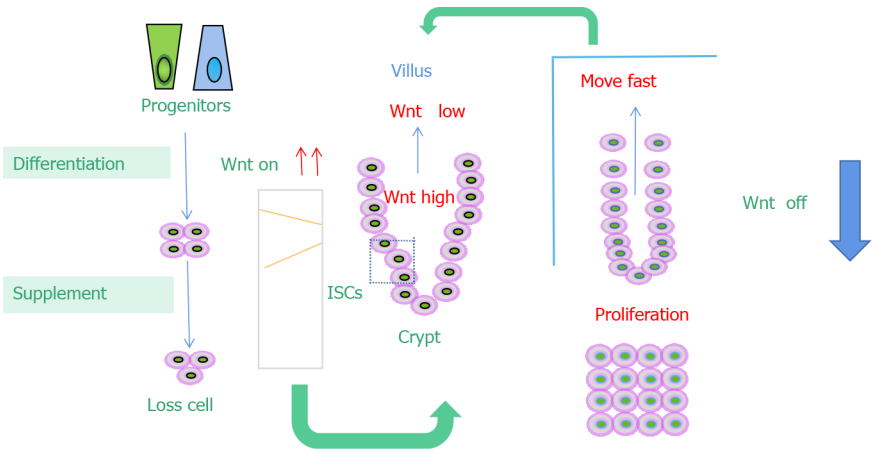

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Figure 4 The Wnt/β-catenin signaling pathway promotes intestinal stem cells proliferation and maintains intestinal epithelial homeostasis. ISCs: Intestinal stem cells.

self-renewal of small ISCs. In the small ISCs microenvironment, the ligands for Notch signaling are mainly provided by Paneth cells, and Notch receptors are actively expressed in small ISCs[128]. Using the mouse model, cells with high Notch signaling activity were specifically labeled. Using lineage tracing experiments, it was found that small ISCs belong to a type of cell with high Notch signaling, and these cells can form all cell types in the small intestine epithelium[129].

Interaction between the Wnt pathway and Notch pathway

The Wnt and Notch signaling pathways are two highly conserved signal transduction pathways that exist widely in multicellular animals. They regulate many life processes through different mechanisms and play an important role in cell proliferation, differentiation, and intestinal homeostasis[130]. However, the specific mechanism by which these two signaling pathways interact to regulate ISC activity and differentiation direction remains unclear. A large number of studies have reported crosstalk between Wnt and Notch signaling pathways[131]. The mechanisms are discussed as follows: (1) Wnt protein regulates downstream through binding to some Notch receptors, including Dfrizzled2, patched, shaggy, *etc.*, hairy and patched genes are expressed, and Dfrizzled2 and patched genes can mediate the Wnt pathway itself[132]; (2) Dvl can antagonize the Notch pathway through its direct interaction with Notch intracellular domain (NIC)[133]; (3) NIC can increase the activation potential of lymphoid enhancer factor under the action of some promoters[134]; (4) GSK-3β phosphorylates NIC, prevents its degradation by the proteasome, and prolongs its half-life[135]; and (5) C promoter binding factor-1 can promote the expression of some genes encoding Fz[136]. In addition to the direct crossover between pathways, there are also many indirect (for example, some pathways in both pathways are involved in the regulation of cyclin D1 and p21 expression) and mechanistic associations[137] (Figure 5).

CONCLUSION

In conclusion, intestinal mucosal injury is an important pathological change in UC. ISCs proliferation and differentiation are the main cytological basis for intestinal mucosal renewal. ISCs participate in normal physiological processes and some pathological processes of the intestine. They are located at the base of the crypts of the intestinal mucosa, which is the cell bank of ISCs. All cells of the intestinal epithelium were derived from crypt stem cells. Meanwhile, LGR5-positive ISCs are significantly regulated by the Notch signaling pathway and Wnt/β-catenin signaling pathway, which jointly maintain the function of LGR5-positive ISCs. More importantly, the surviving stem cells after intestinal mucosal injury accelerate division, restore the number of stem cells, multiply and differentiate into mature intestinal epithelial cells, and repair the damaged intestinal mucosa. Therefore, in-depth study of multiple pathways and transplantation of LGR5-positive ISCs may become a new target for the treatment of UC.

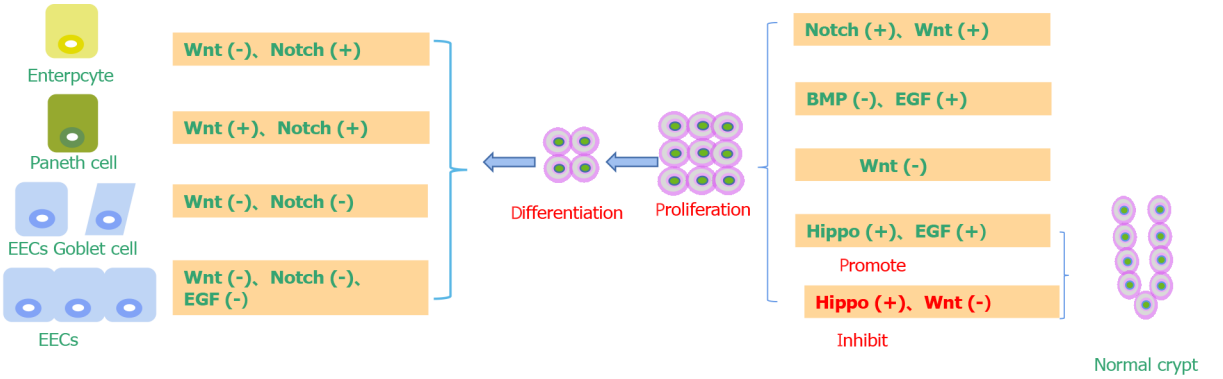

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Figure 5 Interaction between the Wnt pathway and Notch pathway. EGF: Epidermal growth factor; BMP: Bone morphogenetic protein.

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FOOTNOTES

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Country/Territory of origin: China

ORCID number: Lie Zheng 0000-0002-8243-3736; Sheng-Lei Duan 0000-0002-4151-1718.

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REVIEW

SARS-CoV-2 induced liver injury: Incidence, risk factors, impact on COVID-19 severity and prognosis in different population groups

George D Liatsos

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George D Liatsos, Department of Internal Medicine, Hippokration General Hospital, Athens 11527, Attiki, Greece

Corresponding author: George D Liatsos, MD, PhD, Chief Doctor, Department of Internal Medicine, Hippokration General Hospital, Vassilisis Sofias 114, TK 11527, Athens 11527, Attiki, Greece. geoliatsos@yahoo.gr

Abstract

Liver is unlikely the key organ driving mortality in coronavirus disease 2019 (COVID-19) however, liver function tests (LFTs) abnormalities are widely observed mostly in moderate and severe cases. According to this review, the overall prevalence of abnormal LFTs in COVID-19 patients ranges from 2.5% to 96.8% worldwide. The geographical variability in the prevalence of underlying diseases is the determinant for the observed discrepancies between East and West. Multifactorial mechanisms are implicated in COVID-19-induced liver injury. Among them, hypercytokinemia with "bystander hepatitis", cytokine storm syndrome with subsequent oxidative stress and endotheliopathy, hypercoagulable state and immuno-thromboinflammation are the most determinant mechanisms leading to tissue injury. Liver hypoxia may also contribute under specific conditions, while direct hepatocyte injury is an emerging mechanism. Except for initially observed severe acute respiratory distress syndrome corona virus-2 (SARS-CoV-2) tropism for cholangiocytes, more recent cumulative data show SARS-CoV-2 virions within hepatocytes and sinusoidal endothelial cells using electron microscopy (EM). The best evidence for hepatocellular invasion by the virus is the identification of replicating SARS-CoV-2 RNA, S protein RNA and viral nucleocapsid protein within hepatocytes using in-situ hybridization and immunostaining with observed intrahepatic presence of SARS-CoV-2 by EM and by in-situ hybridization. New data mostly derived from imaging findings indicate possible long-term sequelae for the liver months after recovery, suggesting a post-COVID-19 persistent live injury.

Key Words: COVID-19; SARS-CoV-2; Liver injury; Cytokine storm; Endotheliopathy; Immuno-thromboinflammation; Direct hepatocyte injury

Core Tip: Following respiratory system, liver is the second most involved organ in coronavirus disease 2019 (COVID-19). Besides the well-observed cholangiocyte tropism, typical severe acute respiratory distress syndrome corona virus-2 (SARS-CoV-2) Lesions indicated by ultrastructural and histological evidence, identification of replicating SARS-CoV-2, S and nucleocapsid proteins RNAs within hepatocytes, as well as intrahepatic virus observation by electron microscopy and in-situ hybridization, converge to the conclusion that SARS-CoV-2 may also be hepatotropic. Most prevalent mechanisms of COVID-19-related liver injury are hypercytokinemia with "bystander hepatitis", cytokine storm syndrome with subsequent oxidative stress, endotheliopathy and immuno-thromboinflammation. Depending on the grade of their abnormalities, increased serum aspartate aminotransferase, (mostly peak) alanine aminotransferase, alkaline phosphatase, total bilirubin, inflammatory markers (C-reactive protein, ferritin, interleukin-6, -10) and decreased albumin levels are independent discriminators of COVID-19 severity and mortality. Age, male gender, chronic liver disease, liver cirrhosis, obesity, diabetes, and non-alcoholic fatty liver disease are independent prognostic factors of unfavorable COVID-19 outcomes.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA determined by quantitative rt-polymerase chain reaction is widely spread outside the respiratory tract, including the liver[1]. Regardless of pre-existing chronic liver disease (CLD), coronavirus disease 2019 (COVID-19)-induced liver injury (LI) is mainly reflected by hypertransaminasemia, elevations of gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) (less frequently), and hypoalbuminemia[2-8], with the later being a negative acute phase reactant rather than manifestation of liver failure and is one of the most prevalent abnormalities. COVID-19-induced LI is secondary than primary[9,10], mostly mild, transitory and self-limiting[11], it does not impact the majority of patients[12], and is common in absence of CLD[13]. In asymptomatic/subclinical cases randomly diagnosed by computed tomography (CT) scans a mild increase in transaminases (8.8%) is observed[14]. LI definition varies among values just above the upper limit of normal (ULN)[15,16] up to 2–5 × ULN[17,18]. Substantial transaminases increases are linked to unfavorable outcomes, such as death, invasive mechanical ventilation (IMV), and intensive care unit (ICU) admission[7,18-22]. The prognostic relevance of higher liver function tests (LFTs) may result from a more vigorous host immunological and inflammatory response to infection[12], particularly in younger individuals[12,23]. The pattern of LI is typically hepatocellular rather than cholestatic[24]. Severe LI (SLI) defined as alanine aminotransferase (ALT) elevations > 10–15 × ULN with or without jaundice, occurs in 2% of COVID-19[25], while acute liver failure (ALF) without underlying CLD is extremely rare and is typically associated with severe pneumonia and multiple organ dysfunction syndrome (MODS)[26]. SARS-CoV-2-induced ALF has been described in case reports[21].

PATHOPHYSIOLOGY OF SARS-COV-2 INFECTION

The SARS-CoV-2 spike (S) protein is recognized by the angiotensin converting enzyme-2 (ACE2), whilst the androgen-induced transmembrane serine protease-2 (TMPRSS2) and paired basic amino acid cleaving enzyme (FURIN) are necessary for cell tropism and entry[27,28]. ACE2 cleaves the vasoconstrictor peptide angiotensin II to vasodilator angiotensin I[29]. S protein interacts with ACE2[30] and with an identified co-receptor neuropilin-1[31]. FURIN evades immune surveillance thus promoting transmission[28]. SARS-CoV-2 binding to ACE2 causes inflammation, oxidative stress, and pro-apoptotic reactions, ultimately leading to LI[30]. According to single-cell RNA sequencing studies of healthy livers, cholangiocytes exhibit the highest expression of ACE-2, with modest expression found in hepatocytes, sinusoidal endothelial cells, and resident Kupffer cells[32]. Luminal immunohistochemical staining for ACE2 is observed in the bile ducts[33]. A few hepatocytes co-express both TMPRSS2 and FURIN[34,35]. Liver ductal organoids that express ACE2 and TMPRSS2 have been shown to recapitulate SARS-CoV-2 infection[36], whereas liver organoids generated from pluripotent stem cells also express ACE2 and allow SARS-CoV-2 pseudoparticle entry[36,37]. A small population of TROP2+ liver epithelial progenitors express both ACE2 and TMPRSS2. In healthy livers *vs* cirrhotics, 1.8/10000 cells *vs* 10.6/10000 expressed ACE2 and 97.2/10000 *vs* 216/10000 expressed TMPRSS2 representing a significant ($P < 0.001$) increase in the number of TMPRSS2+ cells in cirrhotics[38]. In untreated hepatitis B virus

(HBV) infected livers, only 1.4/10000 and 48.3/10000 cells expressed ACE2 and TMPRSS2 respectively, significantly fewer than both healthy and cirrhotics[38]. ACE2 expression is 30 times higher in hepatitis C virus (HCV)-related cirrhosis than in healthy liver[39]. As ACE2 has been identified as an interferon-inducible gene[40,41], LI and inflammation may therefore enhance SARS-CoV-2 hepatotropism by modifying viral receptor expression, which is consistent with the damage to the respiratory epithelia [41]. In non-infected individuals with obesity and Non-alcoholic Steatohepatitis (NASH), ACE2 and TMPRSS2 Liver mRNA co-expression is likewise upregulated[42]. Non-alcoholic fatty liver disease (NAFLD) and cirrhotic livers have much higher TMPRSS2+ progenitor cells indicating a susceptibility to SARS-CoV-2, findings consistent with the sc-RNA-seq results[38]. Given the recognized link between obesity and NAFLD[43], the finding of a larger abundance of TMPRSS2+ progenitor cells in NAFLD livers may offer a potential explanation for why obese people experience more severe COVID-19[38].

RATIONALE AND MECHANISMS OF LIVER INJURY

The variability in prevalence and severity of LI among COVID-19 patients suggests that the mechanisms of LI are multifactorial (Figure 1).

Direct liver injury

Many studies propose that SARS-CoV-2 hepatotropism and its direct liver function impairment is implicated in COVID-19-induced LI, while only a few speculate that definite evidence is lacking[44,45]. Liver progenitor cells, particularly those destined to become cholangiocytes, contain ACE2[46], in addition to virus isolation in bile[47] imply a direct invasion by SARS-CoV-2. Its infection triggers cell apoptosis factors resulting in cholangiocyte death[36] by lysis and/or by inducing necrosis and apoptosis[48-50]. SARS-CoV-2 virions have been seen within hepatocytes and sinusoidal endothelial cells using electron microscopy (EM)[51]. The best evidence is the identification of replicating SARS-CoV-2 RNA, S, and nucleocapsid proteins RNA within hepatocytes using in-situ hybridization and immunostaining[51,52]. In hepatocytes and sinusoidal endothelial cells, SARS-CoV-2 virions have been seen using EM[51]. The strongest supporting data were found employing in-situ hybridization and immunostaining to identify replicating SARS-CoV-2 RNA, S, and nucleocapsid proteins RNA within hepatocytes[51,52].Viral genomic RNA was also identified in postmortem COVID-19 Liver examinations[53,54], with observed intrahepatic presence of SARS-CoV-2 by EM and by in-situ hybridization [55-57], and viral replication within hepatocytes[58,59], thus reinforcing the role of direct SARS-CoV-2 hepatocyte injury. SARS-CoV-2 particles without membrane-bound vesicles were found in the hepatocyte cytoplasm of COVID-19 patients with aberrant LFTs[51], which is additional proof.

Liver hypoxia

Hypoxia can cause hepatocytes inflammatory cells infiltration, lipid accumulation, an increase in reactive oxygen species (ROS), and death[60,61]. ROS peroxidation products act as a second messenger amplifying the release of multiple cytokines[62]. In COVID-19, hypoxia and cytokine storm syndrome (CSS) are considered as risk factors for LFT abnormalities[63]. Hypoxic hepatitis features (*e.g.* centrilobular necrosis) are widely shown in postmortem liver biopsies[64]. In severe COVID-19, IMV, positive end-expiratory pressure, and/or vasopressor support negatively impact hepatic perfusion by lowering cardiac output, raising hepatic vascular resistance, and increasing portal vein pressure, which obstructs venous drainage, leading to acute LI (ALI) and/or cholestasis[58,65,66]. Gut ischemic injury on the other hand, results in intestinal endotoxinaemia and activation of the sympathetic nervous and adrenocortical systems furthermore contributing to LI[58,66]. Additionally, Kupffer cells can stimulate cytokines due to ischemia[67], while mitochondrial damage by SARS-CoV-2 results in aspartate aminotransferase (AST) release[68-70]. Direct interaction of mitochondrial proteins with the virus nonstructural protein 5 provides a probable reason for the AST-dominant liver profile[71]. In addition, unlike the hepatic preponderance of ALT, zone 3 of the hepatic acini containing higher AST concentrations is more susceptible to hypoxic injury[72]. In COVID-19 aminotransferases elevations, typically mild, are incompatible with very high AST/ALT elevations of primary hypoxic hepatitis[73]. Secondary hypoxic LI owing to the presence of acute respiratory distress syndrome (ARDS) as well as to an overactive inflammatory response to SARS-CoV-2 and MODS[73] might be implicated. In addition, cell iron overload might play a role, and hepcidinmimetic action of S protein may induce ferroportin blockage[25].

Cytokine storm syndrome

In order to maintain homeostasis, the body activates the immunological defense system and the oxidative stress response with the release of many cytokines when activated by endogenous or external stimuli like viruses[74]. Severe COVID-19 exhibits a distinct immunological dysregulation with two essential characteristics: Lymphocyte dysregulation with lymphopenia and overproduction of pro-inflammatory cytokines by monocytes[75,76]. The relationship between lymphopenia and CSS in COVID-19 pathogenesis was described in previous coronaviruses outbreaks[77,78]. Severe hypercy-

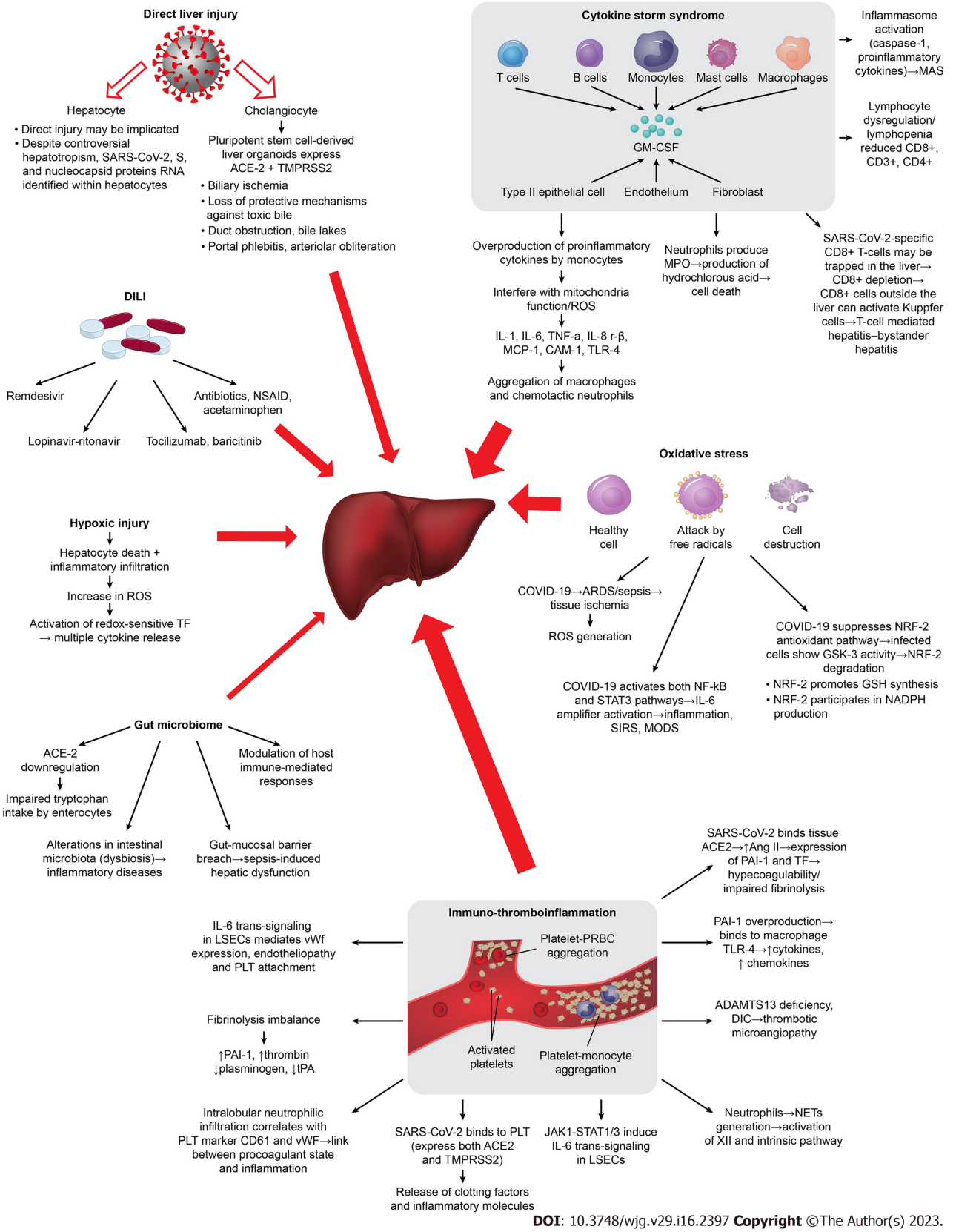

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Figure 1 Mechanisms implicated in COVID-19-induced liver injury. The different width of the red arrows represents the different contribution/significance of each separate mechanism in coronavirus disease 2019-associated liver injury. ACE2: Angiotensin converting enzyme-2; ADAMTS 13: A disintegrin and metalloproteinase with a thrombospondin type 1 motif; Ang II: Angiotensin II; ARDS: Acute respiratory distress syndrome; CAM-1: Cell-adhension molecule-1; DIC: Disseminated intravascular coagulation; GM-CSF: Granulocyte-macrophage colony-stimulating factor; GSK: Conserved serine/threonine kinase; IL: Interleukin; JAK1: Janus kinase 1; LSECs: Liver sinusoidal endothelial cells; MAS: Macrophage activation syndrome; MPO: Myeloperoxidase; MCP-1: Monocyte chemotactic protein-1; NADPH: Nicotinamide adenine dinucleotide phosphate; NETS: Neutrophil extracellular traps; NRF2: Nuclear factor erythroid 2-related factor 2; NSAIDS: Non-steroidal anti-inflammatory drugs; PAI-1: Plasminogen activator inhibitor-1; PLT: Platelet; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of

transcription 3; TF: Tissue factor; TLR4: Toll-like receptor 4; TMPRSS2: Transmembrane serine protease-2; TNF-α: Tumor necrosis factor alpha; TPA: Tissue plasminogen activator; vWF: Von Willebrand factor; COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

tokinemia results in a cascade of actions leading to tissue (especially liver) damage and MODS[79]. Lymphopenia, decreased CD4+, early and persistent elevation of cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-2, -6, -7, -10, -18, granulocyte-colony stimulating factor, interferon gamma (IFN-γ), interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory-protein-1a, chemokines], lactate dehydrogenase (LDH), C-reactive protein (CRP), ferritin, D-dimer and of coagulopathy markers (thrombopoietin), are independent risk factors for SLI (Table 1), and are linked to unfavorable outcomes[23,37,50,59,80-87]. CSS in severe COVID-19 is also associated with reduced CD8+, CD3+ and CD4+ T-cells[88,89]. Depletion of circulating CD8+ T-cells, the main determinant of LI in viral infections (influenza, measles, and SARS), reflects their trapping in the liver[90,91]. The syndrome known as "bystander hepatitis," which is frequently seen in systemic viral infections[92] and in COVID-19[93], is caused by circulating cytokines activating hepatic immune cells without compromising liver function. By activating Kupffer cells in the absence of viral antigens in the liver, viral-specific CD8+ T-cells that are confined to locations outside the liver may cause T-cell-mediated hepatitis[94]. Also, T-cells depletion cannot control the viral infection, leading to macrophage activation and more secondary inflammatory reactions[95,96]. In severe situations, the SARS-CoV-2 virus may cause a hyperinflammatory disease known as macrophage activation syndrome (MAS) or secondary hemophagocytic lymphohistiocytosis[75,76]. This syndrome is characterized by CSS, cytopenias, disseminated intravascular coagulation (DIC) and MODS. The pathogenesis of cytokine-driven hyperinflammatory disorders is heavily dependent on IL-6 signaling[76], that strongly correlates with elevated transaminases[88]. Inflammasome, a complex intracellular protein that SARS-CoV-2 may produce, helps promote caspase-1's autocatalytic activation (apoptosis/pyrolysis) and the exudation of pro-inflammatory cytokines[97], triggering the expression of other genes involved in the immune process[98], therefore resulting in MODS[87]. However, patients with mild COVID-19 may experience LFT abnormalities regardless of their inflammatory condition, probably because the unique inflammation brought on by SARS-CoV-2 is more likely to do so than inflammation brought on by other pathogens[99].

Endotheliopathy– hypercoagulable state– immuno-thromboinflammation

COVID-19 is considered to affect the endothelium, one of the largest organs in the human body[82]. SARS-CoV-2 may worsen microcirculation and encourage thrombus formation, tissue oedema, and organ ischemia by encouraging endothelial cell damage in the arteries, veins, arterioles, capillaries, and venules of all major organs[87,100-101]. Hepatic artery branches in the portal tract with endothelial enlargement and luminal constriction, as well as portal vein endophlebitis, and endotheliitis (leukocyte attachment to the vascular wall) with thrombotic material[102-105], are pathology findings indicative of endotheliopathy in COVID-19-related LI. The observed network of sinusoids decorated by CD34 suggests abnormal hepatic blood circulation[102]. In deceased patients with elevated ALT, significantly higher fibrinogen, factors VIII and II activity, and platelet marker CD61 liver staining was morphologically shown, in accordance with their serum levels (fibrinogen, D-dimer, von Willebrand factor (vWF) activity and antigen, and CRP[45,46,106,107]), thus resembling a microangiopathy thrombotic state[86,106,107]. Additionally, vWF-positive areas correlate with CD61-positive areas[60,101] and with intralobular neutrophil infiltration suggesting a link between the procoagulant state and liver inflammation[45,107]. Endotheliopathy, vWf expression on cell surfaces, and platelet adhesion are all mediated by IL-6 trans-signaling in liver sinusoidal endothelial cells (LSECs), which also plays a role in LSECs inflammation and activation of coagulation therefore being involved in COVID-19-related LI[45,107,108]. As LSECs are endothelial cells and do not express IL-6Ra, trans-signaling is thought to be the main method of IL-6 signaling to LSECs[109,110]. The Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway may also be used to promote IL-6 trans-signaling[45,107], which is essential for inducing a procoagulant and proinflammatory LSECs phenotype[111,112]. Activated neutrophils may generate neutrophil extracellular traps[113]. Decreased ADAMTS13 Levels, another typical finding in severe COVID-19 can induce increased platelet-endothelial interaction[111,114-118], while DIC may also result from CSS in critical/fatal COVID-19[119,120]. A significant imbalance between inhibitors and activators of fibrinolysis is also demonstrated. Reduced action of endogenous anticoagulants [antithrombin, tissue factor (TF) pathway inhibitor, and proteins C and S[121]] is a hallmark of hemostasis dysregulation. As the pulmonary inflammation worsens, hypofibrinolysis is caused by the consumption of plasminogen, high levels of plasminogen activator inhibitor-1 (PAI-1) and a decrease in tissue plasminogen activator, which prolongs the prothrombotic state[122,123].The pathological state involving platelet hyper-reactivity, hypercoagulability and hypofibrinolysis during COVID-19 is named “immuno-thromboinflammation”[123]. As platelets express both ACE2 and TMPRSS2 on their surface[124], it is intriguing that SARS-CoV-2 can attach to them directly and activate them, causing the release of clotting factors and inflammatory chemicals. Endothelial injury triggers the release of TF in circulation which may be also derived from macrophage/monocyte cells as a

Table 1 Liver function tests and factors associated significantly with clinical outcomes in COVID-19

Author/yr	Type of study (*n* of patients)	Factor	Outcome - statistical significance (severity/mechanical ventilation/ICU/mortality)
Krishnan *et al*[321], 2022, United States	Retrospective (*n* = 3830)	TBIL[1]	
		2–5 × ULN	Mortality risk significantly increased 6-fold ($P < 0.001$)
		> 5 × ULN	Mortality risk increased 7.86-fold ($P = 0.005$)
		AST[1]	
		2–5 × ULN	All-cause mortality HR, 1.49; $P < 0.001$
		> 5 × ULN	All-cause mortality HR, 2.19; $P = 0.005$
		ALP[1]	
		1–2 × ULN	All-cause mortality risk increased 1.42-fold ($P = 0.009$)
		> 2–5 × ULN	All-cause mortality risk increased 1.81-fold ($P = 0.032$)
		Inflammatory markers	
		CRP	aHR, 1.04 associated with mortality ($P = 0.001$)
		Ferritin	aHR, 1.0 associated with mortality ($P = 0.001$)
		IL-6	aHR, 1.0 associated with mortality ($P = 0.001$)
		neutrophil count	aHR, 1.0 associated with mortality ($P = 0.008$)
		D-Dimer	aHR, 1.03 associated with mortality ($P = 0.004$)
		LDH	aHR, 1.0 associated with mortality ($P < 0.001$)
		AST, ALT, TBIL	Significantly increased for those who received MV ($P < 0.0001$)
Kodavoor *et al*[180], 2022, India	Retrospective (*n* = 708)	AST[1]	aOR 1.007, per 1 IU/L increase for SD
		AST[1]	aHR 1.002 per 1 IU/L increase for mortality
		Sensitivity/specificity	90.6%/67% to predict mortality
		PPV/NPV	17.5%/95.73% to predict mortality
		Albumin[1]	aOR 0.217 per 1 g/dL increase for SD
			aHR 0.396 per 1 g/dL increase for mortality
Lombardi *et al*[230], 2022, Italy	Retrospective (*n* = 382)	Transaminases[1]	
		> 2 × ULN	OR 2.6, 95%CI: 1.3–6.7 for SD
		FIB-4 score < 1.45[1]	(OR 0.4; $P = 0.04$) protective factor for mortality
Hartl *et al*[326], 2022, Austria	Retrospective (*n* = 900)	AST[1]	aHR: 1.47; $P = 0.043$ for mortality
		TBIL[1]	aHR: 2.20; $P = 0.009$ for mortality
Siddiqui *et al*[229], 2022, United States	Retrospective (*n* = 1935)	Abnormal LFTs	
		Liver injury defined as: (AST/ALT > 3 × ULN or ALP/TBIL > 2 × ULN)	RR, 4.26; $P < 0.0001$ risk for mortality
		Mild elevated enzymes	RR, 5.52; $P < 0.0001$ for ICU admission
		(Levels lower than LI)	RR, 11.01; $P < 0.0001$ for MV
			RR, 2.16; $P < 0.0001$ for mortality
			RR, 2.48; $P < 0.0001$ ICU admission
		Cirrhotics	RR, 3.76; $P < 0.0001$ for MV

			RR, 2.19; P = 0.0022 for mortality
Cai *et al*[20], 2020, China	Retrospective (n = 417)	Hepatocellular LI	OR, 2.73; P = 0.02 for severe disease
		Mixed LI	OR, 4.44; P < 0.001 for severe disease
		LI[1]	aOR, 9.04; P < 0.001 for severe disease
Huang *et al*[191], 2020, China	Retrospective (n = 675)	AST[1] 3-fold ULN	aOR, 19.27; P < 0.0001 for mortality
			aOR, 116.72; P < 0.0001 for MV
Lei *et al*[192], 2020, China	Retrospective (n = 5771)	AST[1] 40-120 U/L	aOR, 4.81; P < 0.001 for all-cause mortality
		AST[1] > 120 U/L	aOR, 14.87; P < 0.001 for all-cause mortality
Ding *et al*[22], 2020, China	Retrospective (n = 2073)	Abnormal AST[1]	aHR, 1.39; P = 0.027 for mortality
		Abnormal DBIL[1]	aHR, 1.66; P = 0.001 for mortality
		LI during hospitalization[1]	aHR, 4.63; P < 0.001 for in-hospital mortality
		LI at admission[1]	aHR 1.87; P = 0.003 for in-hospital mortality
		Mixed LI[1]	aHR, 4.77; P < 0.001 for in-hospital mortality
		Cholestatic LI[1]	aHR, 3.99; P = 0.008 for in-hospital mortality
Phipps *et al*[23], 2020, United States	Retrospective (n = 3381)	Ferritin[1]	OR, 2.40; P < 0.001 for SLI
		IL-6[1]	OR, 1.45; P = 0.009 for SLI
		Peak ALT[1]	OR, 1.14; P = 0.044 for mortality
		Older age[1]	OR, 1.07; P < 0.001 for mortality
		DM[1]	OR, 1.30; P = 0.045 for mortality
Medetalibeyoglu *et al*[221], 2020, Turkey	Retrospective (n = 554)	AST/ALT > 1	AUC = 0.713, P = 0.001 marker of mortality risk
			AUC = 0.636, P = 0.001 for ICU admission
Chen *et al*[199], 2020, China	Retrospective (n = 502)	Grade of Liver damage[1]	aHR, 1.377; P = 0.049 risk factor for mortality
Mishra *et al*[200], 2021, United States	Retrospective (n = 348)	AST[1] (1 unit increase) IU/L Peak AST[1] (1 unit increase)	OR, 1.011; P = 0.006 for mortality
		Peak ALT[1] (1 unit increase)	OR, 1.007; P < 0.001 for mortality
		TBIL[1] (1 unit increase) mg/dL	OR, 1.005; P = 0.003 for mortality
		Alb[1] (1 unit increase) g/dL	OR, 1.997; P = 0.04
		Male[1]	OR, 0.5; P = 0.01
		BMI > 40 kg/m^2	OR, 1.94; P = 0.001
		LI[1]	OR, 2.17; P = 0.003
			OR, 1.79; P = 0.008
Chew *et al*[190], 2021, United States	Retrospective (n = 834)	Ischemic disease state[1]	OR, 2.4; P = 0.001 for mortality
		Hypecoagulable[1]	OR, 1.7; P = 0.02 for mortality
		Hyperinflammatory[1]	OR, 1.9; P = 0.02 for mortality
Ponziani *et al*[327], 2021, Italy	Retrospective (n = 515)	ALP[1] peak value	aOR, 1.007; P = 0.005 for mortality
		CRP[1]	aOR, 1.007; P = 0.008 for mortality
Piano *et al*[246], 2020, Italy	Retrospective (n = 565)	Abnormal LFTs[1]	OR, 3.53; P < 0.001 for ICU admission/death
Yip *et al*[287], 2021, China	Retrospective (n = 1040)	ALT/AST[1] ≥ 2 × ULN	aOR, 7.92; P < 0.001 for ICU/MV/death
Marjot *et al*[237], 2021, multinational	Retrospective (n = 785)	Age[1]	OR, 1.02; P = 0.011 for mortality
		Cirrhotics CTP-A[1]	OR, 1.90; P = 0.040 for mortality
		Cirrhotics CTP-B[1]	OR, 4.14; P < 0.001 for mortality

		Cirrhotics CTP-C[1]	OR, 9.32; $P < 0.001$ for mortality
		ArLD[1]	OR, 1.79; $P = 0.040$ for mortality
Lee *et al*[328], 2020, South Korea	Retrospective ($n = 1005$)	Age[1]	aHR = 4.96; $P < 0.001$ for mortality
		Liver cirrhosis[1]	aHR = 2.86; $P = 0.042$ fro mortality
		DM[1]	aHR = 2.29; $P < 0.001$ for mortality
		COPD[1]	aHR = 4.52; $P = 0.001$ for mortality
Singh *et al*[236], 2020, United States	Retrospective ($n = 2780$)	CLD[1]	RR, 2.8; $P < 0.001$ risk of mortality
		propensity matching	RR, 3.0; $P = 0.001$ risk of mortality
		Cirrhotics[1]	RR, 4.6; $P < 0.001$ risk of mortality
Hashemi *et al*[232], 2020, United States	Retrospective ($n = 363$)	CLD[1]	aOR 1.77; $P = 0.04$ for ICU admission
			aOR, 2.08; $P = 0.0092$ for IMV
		Cirrhotics[1]	aOR, 12.5; $P = 0.009$ mortality risk
Sarin *et al*[235], 2020, Asian	Retrospective ($n = 228$ CLD)	Cirrhotics[1]	
		AST/ALT > 1.4	HR = 1.4; $P = 0.02$ for mortality
		Obesity	OR = 8.1; $P = 0.002$ for LI
		Decompensated	OR = 2.5; $P = 0.05$ for mortality
		CTP score > 8	HR = 19.2; $P < 0.001$ for mortality
		DM in CLD non-cirrhotics	OR = 2.1; $P = 0.01$ for LI
Wang *et al*[51], 2020, China	Retrospective ($n = 657$)	Male gender[1]	OR, 2.038; $P < 0.001$ for LI
		hsCRP ≥ 10 mg/L	OR, 1.733; $p = 0.014$ for LI
		NLR ≥ 5	OR, 2.154; $P < 0.001$ for LI
Zhang *et al*[183], 2020, China	Retrospective ($n = 218$)	Male[1]	OR, 6.203; $P < 0.001$ risk for LI
		Neutrophil percentage[1]	OR, 1.004; $P = 0.003$ risk for LI
		CRP[1]	$P < 0.001$ in LI patients
		D-dimer[1]	OR, 1.486; $P < 0.001$ risk for LI
Shauly-Aharonov *et al*[329], 2021, Israel	Retrospective ($n = 37121$)	Age	OR = 1.1 for every year increase; $P < 0.001$) risk for severity
		Male gender	OR = 1.34; $P = 0.012$ risk for severity
		BMI	OR = 1.02 for 1 kg/m^2 increase; $P = 0.025$ risk for severity
Kovalic *et al*[208], 2020, United States	Meta-analysis ($n = 24299$)	CLD[1]	Pooled OR, 1.48; $P = 0.001$ for severity
			Pooled OR, 1.78; $P = 0.02$ for mortality
Kulkarni *et al*[6], 2020, India	Meta-analysis Multinational ($n = 20874$)	Increased LFTs	OR, 3.46; $P < 0.001$ for mortality
			OR, 2.87; $P < 0.001$ for severe disease
Sharma *et al*[207], 2021, United States	Meta-analysis ($n = 12882$)	AST[1]	Pooled OR, 2.98; $P < 0.00001$ for poor outcomes
		ALT[1]	Pooled OR, 1.73; $P < 0.0001$ for poor outcomes
Del Zompo *et al*[323], 2020, Italy	Meta-analysis ($n = 20724$)	ALT[1]	OR 1.54, 95%CI: 1.17-2.03 for severity
		ALT[1]	OR 1.48, 95%CI: 1.12-1.96 for mortality
		AST[1]	OR 3.17, 95%CI: 2.10-4.77 for severity
		AST[1]	OR 4.39, 95%CI: 2.68-7.18 for mortality
		TBIL[1]	OR 2.32, 95%CI: 1.18-4.58 for severity

	TBIL[1]	OR 7.75, 95%CI: 2.28-26.40 for mortality

[1]Age, gender, ethnicity, race, BMI, and all the pre-existing comorbidities were adjusted as confounders.

Results come from multivariate analysis and logistic regression (studies with more than 200 individuals are included). aHR: Adjusted hazard ratio; ALB: Albumin; ALI: Acute liver injury; ALT: Alanine transaminase; ALP: Alkaline phosphatase; aOR: Adjusted odds ratio; ArLD: Alcohol related liver disease; AST: Aspartate transaminase; BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; CRP: C-reactive protein; CTP: Child-Turcotte-Pugh; CLD: Chronic liver disease; DM: Diabetes mellitus; FIB-4: Risk of fibrosis score; GGT: Gamma-glutamyl transferase; HR: Hazard ratio; ICU: Intensive care unit; IMV: Invasive mechanical ventilation; LDH: Lactate dehydrogenase; LI: Liver injury; LFTs: Liver function tests; NLR: Neutrophil-to-lymphocyte ratio; NPV: Negative predictive value OR: Odds ratio; RR: Relative risk; PPV: Positive predictive value; SLI: Severe liver injury; MOF: Multiple organ failure; TBIL: Total bilirubin; ULN: Upper limit of normal.

consequence of MAS[125]. When PAI-1 is overproduced, it binds to TLR4 on macrophages and triggers the release of cytokines and chemokines[126], which in turn promotes inflammation, steatosis, and microvascular thrombosis[127]. SARS-CoV-2 binds to the ACE2 receptor on tissues inceasing Ang II levels, favoring PAI-1 and TF expression thus promoting hypercoagulability and impairing fibrinolysis [115]. Extensive pericyte activation during LI contributes to the recruitment of inflammatory cells, and their conversion into cells that resemble myofibroblasts results in the creation of extracellular matrix proteins and the ensuing fibrosis of the vessel wall[128].

Oxidative stress

The expression of antioxidant proteins is regulated by nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that is triggered by oxidative stress[129,130,131]. One of the most significant mechanisms of antioxidant and anti-inflammatory element signaling is the complex Kelch-like ECH-associated protein 1-NRF2-antioxidant response element[132]. The NRF2 antioxidant pathway is suppressed in COVID-19, while infected cells show a GSK-3 (conserved serine/threonine kinase) activity that degrades NRF2[133]. NRF2 interacts with NF-kB, a proinflammatory signal transduction pathway[134] driving the initial proinflammatory response[135], to reciprocally regulate redox metabolism[136-139]. When NRF2 activity reaches its maximum level, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation is inhibited. In response to viral infection or other stimuli, inhibitor kappa B (IB) is phosphorylated, which releases and translocates NF-B to the nucleus, causing inflammatory cascades and the generation of inflammatory mediators[140]. *Via* the TNFR1-NF-B signaling axis, TNF- may activate NF-B[141], and NF-kB in turn enhances the release of inflammatory cytokines[135]. This creates a vicious loop that feeds CSS and exacerbates LI[142-144]. Oxidative stress is mostly caused by ROS. The NF-B pathway is activated by COVID-19, ARDS, and sepsis as they cause tissue ischemia and ROS production[145]. In the early stages of COVID-19, ACE2 is the most critical component, whereas the IL-6-STAT3 axis is crucial in the late stages and in CSS[146]. Indeed, both NF-κB and STAT3 pathways are activated in COVID-19 promoting inflammation by activating the IL-6 amplifier[147]. NRF2 promotes Glutathione synthesis[148], and participates in the tricarboxylic acid cycle by regulating the production of NADPH, a key co-factor of antioxidant reactions[149,150]. NRF2 inhibits liver fibrosis and promotes liver regeneration[151-153] therefore protecting liver cells in viral hepatitis[154], drug-induced LI (DILI)[155-157], cholestasis[144,158,159], and NAFLD[160,161], by reducing gluconeogenesis and fat deposition, restoring insulin resistance, and boosting the anti-inflammatory and antioxidant effects[162].

Drug-induced liver injury

The large use of antiviral drugs may contribute to COVID-19-related LI especially in individuals with increased baseline ALT[163]. The pooled incidence of DILI in COVID-19 is reported 25.4%[6]. In DILI, AST usually peaks before ALT, a biochemical pattern also observed in severe COVID-19. In some cases, observed microvascular steatosis and mild hepatic inflammation are consistent with DILI[20,49]. In remdesivir-treated patients, 23%-35% show increased LFTs[164,165] indicating hepatotoxicity, while 2%–3% required treatment discontinuation[164]. Lopinavir/ritonavir incidence of DILI is 37.2%[9] with a significant increased risk (OR = 4.44) for severity[20]. Medicines with possible antiviral effects should only be administered on patients who have risk factors for severe illness[82] and early in the course of the disease[166]. In tocilizumab-treated patients, 15%-51% presented a transitory but not significant hypertransaminasemia between 9-13 d, some of which showed surprisingly higher mortality[167,168]. The liver's IL-6-mediated endotheliopathy should be improved by treatment with the JAK inhibitor baricitinib. A significant disadvantage of all those treatments those treatment clinical trials is the frequent exclusion of patients with AST/ALT > 5 × ULN[169]. Moreover, immunosuppressive drugs, such as tocilizumab, tofacitinib, and dexamethasone, can potentially induce LI *via* HBV reactivation in patients with occult infections[170,171], therefore antiviral prophylaxis should be administered. Dexamethasone may ameliorate endothelial injury[172] by dampening of endothelial IL-6 production [173]. The most typical contributors to DILI in the general population, antibiotics and nonsteroidal anti-inflammatory medications, may also cause LI, while acetaminophen can cause alterations in aminotransferases even at therapeutic doses[174].

Gut microbiome

In COVID-19, dysbiosis of the gut microbiota may have a significant impact on the clinical outcome of patients with comorbid conditions such diabetes, hypertension, and obesity and may lead to liver damage[175,176]. For example, older people often have less variety in their gut microbiota, and COVID-19 is more severe in this age group, supporting the possibility that microbes play a role in outcomes [177]. Hepatic dysfunction brought on by sepsis may result from disruption of the gut microbiota and a breach of the gut-mucosal barrier[178]. Moreover, the diversity of the gut microbiota influences how the host immune system responds[178]. It is hypothesized that changes in the gut-liver axis may contribute to the severity of COVID-19 seen in cirrhotics. Cirrhosis is characterized by changes to intestinal permeability, gut microbiota composition, and function[179].

INCIDENCE

The earliest available epidemiological data of COVID-19 patients came from China. Abnormal LFTs were first reported in a cohort from Wuhan, China[9], making liver the most frequently damaged outside of the respiratory system. It's interesting to note that Wuhan, the COVID-19 epicenter, had a substantially greater incidence of elevated aminotransferases than the surrounding areas (21% *vs* 10%) [18,63] possibly because of higher SARS-CoV-2 doses exposure in Wuhan[80]. Western populations show abnormal LFTs more clearly than Eastern populations (Figure 2). The timing of LFT determination during disease course, different definitions, but mostly, the geographical variability in the prevalence of underlying diseases are the determinants for the observed discrepancies[18]. With respect to worldwide published data the overall prevalence of abnormal LFTs ranges from 2.5% to 96.8% (Table 2), while SLI accounts for 4.94%-21.8% of COVID-19 patients[7,20,22,23,38,80,180]. Patients with SLI are younger and more likely to be male[23]. Younger patients may exhibit a more robust immune response to infection, causing LI and determining its degree[23,38]. Aminotransferases are higher in severe COVID-19 cases, in accordance with the 2002-2004 SARS outbreak[181]. Concerning cholestatic enzymes, elevated GGT and ALP range between 15%-47.3% and 4%-58.5% respectively (Table 2). GGT, a surrogate marker for increased oxidative stress and chronic inflammation[182], usually increases in severe cases[58,59] implying cholangiocyte injury[183,184]. The GGT elevation without accompanied by ALP elevation[59] may also develop in DILI more frequently than obstruction. ALP elevation is rare, usually < 2 × ULN [185], and is mostly observed in MODS or death from COVID-19[186]. The joint trajectory of GGT, ALP, and bilirubin points towards a cholestatic LI seen in impaired survival[187]. The prevalence of total bilirubin (TBIL) elevations ranges between 3.1% and 52.1%. Concerning longitudinal changes, LFTs become more frequently, and more severely deranged during hospitalization[20,188,189]. The median time to peak AST levels is 3 d after admission, normalizing within 4.4 d[190]. ALT elevations peak between 4-17 hospital day[188]. In deceased patients, ALT levels are normal in the first week but subsequently rise rapidly along with AST at the third week. In survivors, slightly elevated ALT levels occur at 2-3 wk after symptoms onset when AST levels might remain normal[191]. A biphasic pattern with early aminotransferase onset, culminating around days 10-15 of hospitalization, and then gradual normalization accompanied by rising ALP is also suggested[187]. ALI (ALT > 3 × ULN) occurs between 17-18.5 d after symptoms onset[28,192]. AST is diffusely represented in many tissues while ALT is considered liver-specific[193]. Greater AST levels may be related to mitochondrial damage or damage to other organs[194]. In the liver, ALT is only found in the cellular cytoplasm[72,195] whereas AST is both cytosolic (20%) and mitochondrial (80%) localized, and is in higher concentrations in zone 3 of the hepatic acinus therefore ischemic or toxic damage to this zone may result in greater AST elevations.

UNDERLYING DISEASES, SEVERITY, PROGNOSIS

Underlying diseases

The median age of COVID-19-induced LI patients ranges between 51.5-56 years with male predominance[65,191]. The incidence of hypertension, diabetes, and coronary heart disease ranges between 23.08%-31,8%, 11.54%-15.3%, and 7.8%-11.54%, respectively[192]. Age, male sex, hypertension, and diabetes are negatively correlated with SLI in COVID-19[23]. The association of LI with hypertension and poorer prognosis is more significant in the absence of pre-existing CLD[38]. CLD prevalence varies widely with Chinese studies being reported between 1.4%-15.3%[9,184,196], lower than in Western countries (5%-37.6%)[197,198]).

Severity

Risks of severity for specific LFTs indices are shown in Figure 3. Aminotransferases levels > 5 × ULN correlate to mortality[23,192], while incidence of LI is higher in ICU than non-ICU patients (61.5% *vs* 25.0%)[199]. Elevated LFTs on admission show a 3-fold greater risk of severe disease and 3.5-fold risk for mortality[6,200]. After adjustment, patients with LI are at a 9-fold greater risk of severe COVID-19

Table 2 Incidence of abnormal liver function tests (liver injury)

Author/citation LFTs performed	Type of study (*n* = participants)	Incidence (%)	Country/year of publication
Cai *et al*[20]	Study (*n* = 417)		China/2020
Abnormal LFTs			
SLI (AST/ALT > 3 × ULN		76.3	
or ALP/γGT > 2 × ULN)		21.8	
ALT (> 3 × ULN)		37	
GGT (> 3 × ULN)		41	
AST (> 3 × ULN)		20	
TBIL (> 3 × ULN)		10	
MOF		23.3	
Phipps *et al*[23]	Study (*n* = 2273)		United States/2020
Mild (peak ALT < 2 × ULN)		45	
Moderate (peak ALT 2-5 × ULN)		21	
SLI (peak ALT > 5 × ULN)		6.4	
Huang *et al*[191]	Study (*n* = 675)		China/2020
Abnormal LFTs		37.5	
SLI		7.7	
Guan *et al*[284]	Study (*n* = 1099)		China/2020
AST/ALT			
mild disease		18.2–19.8	
severe disease		28.1–39.4	
Hundt *et al*[185]	Study (*n* = 1827)		United States/2020
LFTs (on admission)			
AST		66.9	
ALT		41.6	
TBIL		4.3	
ALP		13.5	
Wang *et al*[65]	Study (*n* = 657)		China/2020
Liver injury		46.1	
ALT		42.2	
GGT		24.4	
TBIL		4.9	
Chu *et al*[320]	Study (*n* = 838)		China/2020
Liver Injury		51.2	
Yip *et al*[287]	Study (*n* = 1040)		China/2021
Aminotransferases		22.5	
ALP		58.5	
TBIL		52.1	
Ding *et al*[22]	Study (*n* = 2073)		China/2021
Survivors		90.3	
Any abnormal LFT		61.8	
Mild abnormal LFT		47.5	

SLI		14.3	
LI type			
Hepatocellular		25.8	
Cholestatic		6.7	
Mixed		25.7	
Specific liver indices			
ALT		43.3	
AST		38.9	
GGT		31.8	
Shao *et al*[38]	Study (n = 1520)		China/2021
SLI		17.9	
Mishra *et al*[200]	Study (n = 348)		United States/2021
New-onset LI		52.8	
Sikkema *et al*[204]	Study (n = 382)		Netherlands/2021
LI		41.6	
Moderate LI (ALT > 100 or ALP > 200)		6.5	
Cholestatic LI		9.2	
Chew *et al*[190]	Study (n = 834)		United States/2021
AST		62.5	
ALT		33.7	
ALP		11.9	
TBIL		3.1	
Richardson *et al*[25]	Study (n = 5700)		United States/2020
AST		58.4	
ALT		39	
Bernal-Monterde *et al*[187]	Study (n = 540)		Spain/2020
Abnormal LFTs		64.3	
ALT		28.6	
AST		40.9	
GGT		47.3	
Krishnan *et al*[321]	Study (n = 3830)		United States/2022
ALT		70.4	
AST		44.4	
ALP		16.1	
TBIL		5.9	
Kodavoor *et al*[180]	Study (n = 708)		India/2022
AST		69.91	
< 1–2 times ULN		42.51	
2–3 times ULN		14.26	
3–5 times ULN		8.19	
> 5		4.94	
ALT		80.22	
< 1–2 times ULN		42.93	

2–3 times ULN		17.93	
3–5 times ULN		12.14	
> 5		7.2	
Russo *et al*[234]	Study (*n* = 1641)		Italy/2022
AST		27.7	
ALT		23	
TBIL		12.6	
Marjot *et al*[44]	Review		United Kingdom/2021
AST		29–39	
ALT		38–63	
Cai *et al*[82]	Review		China/2021
ALT		11–56.3	
AST		15–86.8	
TBIL		2.7–30.6	
CLD		2–11	
Ekpanyapong *et al*[322]	Review		Multinational/2022
Aminotransferases		10–58	
ALP		1–10	
TBIL		3–23	
GGT		13–54	
Esteban *et al*[209]	Review		United States/2022
Aminoransferases (admission)		20–67	
Aminoransferases (hospitalization)		61–83	
ALP		23–30	
TBIL		4–16	
Garrido *et al*[59]	Review		Portugal/2020
ALT		2.5–50	
AST		2.5–61.1	
TBIL		0–35.3	
Kullar *et al*[2]	Meta-analysis (*n* = 3046)		United States/2020
ALT		21	
AST		24	
TBIL		9	
Wijarnpreecha *et al*[198]	Meta-analysis (*n* = 64 studies) (*n* = 11245 pts)		United States/2021
AST		23.2	
ALT		21.2	
TBil		9.7	
GGT		15	
ALP		4	
AST			
Severe cases		45.5	
Non-severe		15	

Wu *et al*[253]	Meta-analysis (*n* = 45 studies)		Multinational/2018
Admission			
Any abnormal LFT		27.2	
ALT		20.4	
AST		21.8	
ALP		4.7	
GGT		35.8	
TBIL		8.8	
Hospitalization			
Any abnormal LFT		36	
ALT		38.4	
AST		28.1	
TBIL		23.2	
Del Zompo *et al*[323]	Meta-analysis (*n* = 36 studies) (*n* = 20724 patients)		Italy/2020
At admission (pooled prevalence)			
Abnormal LFT		46.9	
ALT		22.8	
AST		26.5	
GGT		22.5	
ALP		5.7	
TBIL		8	
Zhu *et al*[262]	Meta-analysis (*n* = 38 studies) (*n* = 3063 pts)		China/2020
Abnormal LFTs		29	
Mao *et al*[18]	Meta-analysis (*n* = 1267)		China/2020
Abnormal LFTs		19	
Alqahtani *et al*[324]	Meta-analysis (*n* = 30 studies)		Multinational/2020
Abnormal LFTs		61.1	
Sultan *et al*[325]	Meta-Analysis (*n* = 47 studies) (*n* = 10,890 pts)		United States/2020
Pooled prevalence			
ALT		15	
AST		15	
TBIL		16.7	
Kumar *et al*[210]	Meta-analysis (*n* = 128 studies)		India/2020
Pooled prevalence			
TBIL		13.71	
ALT		31.1	
AST		33.95	
ALP		6.99	
GGT		30.62	
ALB		61.57	
Severe *vs* non-severe pts			

TBIL	18.80 *vs* 9.24
ALT	39.58 *vs* 24.15
AST	49.68 *vs* 19.40
ALP	11.33 *vs* 4.0
GGT	46.90 *vs* 18.66
ALB	75.91 *vs* 31.04

The table encompasses several studies and meta-analyses which included more than 200 individuals.
ALB: Albumin; ALI: Acute liver injury; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; GGT: Gamma-glutamyl transferase; LI: Liver injury; LFTs: Liver function tests; SLI: Severe liver injury; MOF: Multiple organ failure; TBIL: Total bilirubin; ULN: Upper limit of normal; γGT: Gamma-glutamyltransferase; LFT: Liver function tests; pts: Patients.

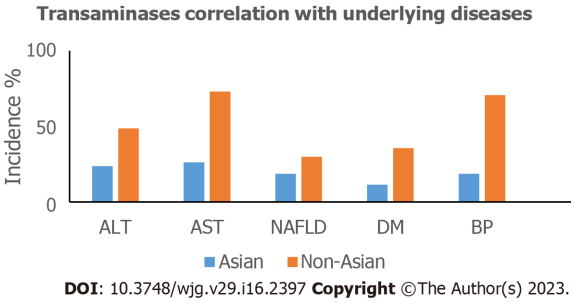

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Figure 2 Transaminases correlation with underline disease in Asian and non-Asian COVID-19 populations. BP: Blood pressure; DM: Diabetes mellitus; NAFLD: Non-alcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; COVID-19: Coronavirus disease 2019.

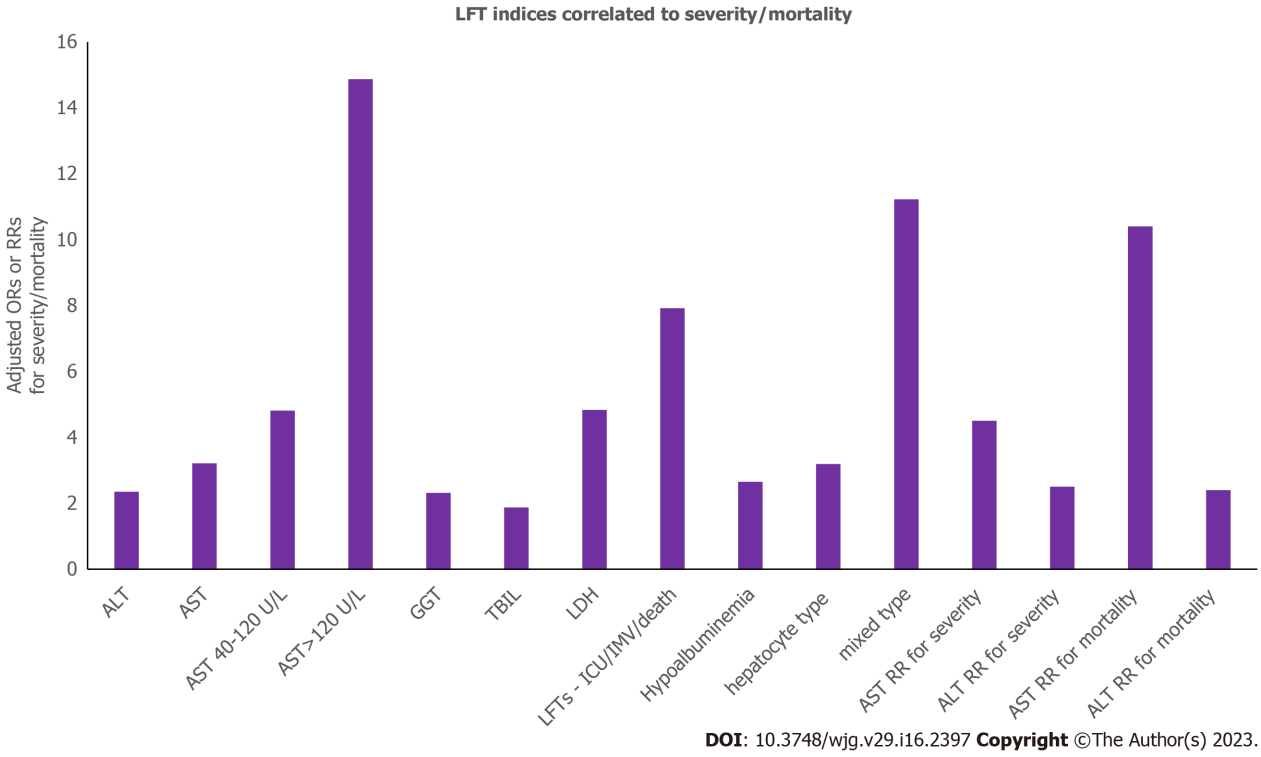

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Figure 3 Adjusted liver function test indices and type of liver injury correlated to COVID-19 severity and mortality. ICU: Intensive care unit; IMV: Invasive mechanical ventilation; LFTs: Liver function tests; OR: Odds ratio; RR: Relative risk; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase LDH: Lactate dehydrogenase; TBIL: Total bilirubin; COVID-19: Coronavirus disease 2019.

[20], and at a 7.5-fold risk for mortality[201]. A few studies however, failed to show such an association with severity/mortality[202-205], disease progression[17], and ICU admission[202,206]. These differences could be attributed to the nature of the studies that were included and the various outcomes definitions[207,208]. It seems that LI portends the need for ICU care and IMV[209]. CLD is also associated with severity [odds ratio (OR)/relative risk (RR) = 1.48, 1.70][208,210-213], and mortality (OR/RR = 1.08, 2.65)[208,212,214] while in a few studies such an association was not observed[6,207,215-219] (Table 1). Age, male gender, higher body mass index (BMI), corticosteroids, antifungals, lymphocyte decrease, neutrophil increase, and CLD are factors positively associated with ALT/AST increase[6,18,20,192,194,220]. ALP levels are tightly associated with male gender, antifungals, neutrophil count increase, and CLD. Antifungals, antivirals, systemic corticosteroids, and platelet count reduction are positively correlated with increased TBIL levels.

Within five weeks, patients with SLI are significantly more likely to have been intubated, to require renal replacement therapy, or to decease compared to moderate/mild LI[23]. LFTs elevations during hospitalization correlate well with the severity of inflammatory indexes (CRP, procalcitonin, ferritin, LDH, GGT, lactate, and D-dimer)[16,200,221]. In essence, LFTs can be used as a surrogate for the monitoring of inflammation. ALT levels correlate well with the CSS inflammatory markers[317,222-225] (Table 3). This immunological response is consistent with that reported for other viral respiratory tract infections[226]. IL-6, -8, TNF-α are positively correlated with the increased AST, TBIL, and ALP, therefore, cytokines contribute to COVID-19-induced LI[65]. Significantly higher white blood cell (WBC) and neutrophils, and lowered lymphocytes are observed in LI[65,227,228].

Prognostic factors

In mixed-effect Cox model adjusted for age, gender, and comorbidities patients with AST > 3 × ULN compared to normal AST exhibited increased risks of death and IMV (19.27-fold and 116.72-fold, respectively)[191]. Risk of severity and all-cause mortality of LFTs abnormalities are shown in Figure 3. Patients with LI have a 4-fold higher rate of mortality, 7-fold higher rate of ICU admission, and 11-fold higher rate of intubation[20,229], while hepatocellular and cholestatic type LI increases the risk by 3-fold [20]. LI is suggested as an independent prognostic factor of COVID-19[200,211]. AST/ALT ratio > 1 predicts mortality, severe pneumonia and ICU admission[221]. While hepatic steatosis is considered to have no impact on disease course, fibrosis (FIB-4) score < 1.45 is a significant protective factor[230]. Vasopressor use (ischemia), and hyperinflammatory/hypercoagulable state are also independent predictors of death[190]. ALP peak value is a risk factor for in-hospital mortality[231]. All-cause adjusted mortality risk is 6-fold significantly increased in patients with an elevated TBIL > 2-5 × ULN and 1.42-fold in patients with ALP > 1-2 × ULN[232]. Serum albumin is negatively associated with severity. Hyperglycaemia at admission is associated with severity/mortality[233]. In fully models adjusted for confounders, increasing age, non-white and non-black race, hypertension, overweight/obesity, kidney disease, cardiovascular disease, diabetes, cancer, and dementia, are independently associated with an increased risk of in-hospital mortality[232-234]. Higher state of inflammation is also significantly associated with mortality[232], while peak ferritin and IL-6 Levels are associated with SLI (Table 1)[23].

COVID-19 IN DIFFERENT CLD POPULATIONS

Cirrhosis

In COVID-19 the presence of cirrhosis, mostly of decompensated, is an independent predictor of liver-related (OR = 3.24) and overall complications, as well as mortality (aOR; 11.3-12.5)[232,235]. In patients with CLD, the 30-d cumulative overall mortality is higher in cirrhotics (RR = 4.6)[236], with respiratory complications being the main cause of death[208,237-239].In terms of CLD etiology, more frequent is viral (60.5%), followed by NAFLD (32.6%), alcohol-related liver disease (ArLD) (4.7%), and autoimmune hepatitis (2.3%). ArLD, NAFLD and hepatocellular carcinoma (HCC) but neither viral nor autoimmune hepatitis are associated with increased mortality[237,240-242]. Cirrhosis-associated immune dysfunction (CAID), is a condition that affects patients with CLD, particularly cirrhosis, who display a variety of immune dysfunctional mechanisms that enhance their vulnerability to infection and abnormal inflammatory responses[44]. The CAID phenotypes represent a continuum of dynamic events that shift from being primarily pro-inflammatory to being primarily immunodeficient. Reduced bacterial opsonization, phagocytosis, protein C activity, antigen T-lymphocyte dependent responses, vaccination responses, hypoalbuminemia, hypocomplementaemia, and intestinal dysbiosis are some of the characteristics of this condition[44,243,244]. CAID is also associated with increased serum levels of IL-1β, -6, -17, -18, TNF-α, and IFN-γ[245], and predisposes to a variety of viral or fungal-related diseases[246]. Despite the increased risk of infection[247], cirrhotics exhibit a lower risk of acquiring SARS-CoV-2[209,248,249], whereas in large population studies patients with CLD are not over-represented[250]. In patients with CLD, a condition known as acute-on-chronic liver failure (ACLF) is characterized by abrupt hepatic decompensation and extrahepatic organ failures and is linked with a significant short-term mortality [251]. While being typically linked to bacterial infections, COVID-19, among other viruses may cause

Table 3 Serum parameters alone or in combination associated with specific outcomes

Parameters	Associated conditions
ALT	CSS inflammatory markers
Elevated serum IL-2R, IL-6, TNF-α	LI
IL-6, ferritin, CRP, ESR, Procalcitonin, hypoalbuminemia, low PLTs, low CD4+ T-cells and B-lymphocytes	Non-favorable course of LI
Simultaneous increase in IL-6 + ferritin + ALT + hypoalbuminaemia	Significant LI
On admission increased inflammatory markers + AST + GGT + LDH + lymphopenia+eosinopenia	More Severe clinical course
Lymphopenia, Thrombocytopenia	Disease severity
Thrombocytopenia	Consumptive coagulopathy
Low Hb	Controversial data

ALI: Acute liver injury; ALT: Alanine transaminase; AST: Aspartate transaminase; CRP: C-reactive protein; GGT: Gamma-glutamyl transferase; LDH: Lactate dehydrogenase; LI: Liver injury; CSS: Cytokine storm syndrome; IL: Interleukin; ESR: Equivalent series resistance; TNF-α: Tumor necrosis factor-α; PLT: Platelet; Hb: Hemoglobin.

ACLF[251-253].The incidence of ACLF in COVID-19 patients with CLD ranges from 10% to 50%[235,237], with a reported 65% mortality rate[237]. ACLF may also occur in compensated cirrhotics with severe COVID-19[254]. Also, as the severity of cirrhosis is assessed by the Child-Turcotte-Pugh (CTP) score, there is a stepwise rise in morbidity and mortality[237]. After adjusting for baseline characteristics, COVID-19-related mortality is significant across the CTP stages (aORs): A = 1.90, B = 4.14 and C = 9.32[237], similar to non-COVID-19 hospitalized cirrhotics[235,238]. CTP score > 9 at presentation independently predicts mortality, in accordance with MELD and chronic liver failure consortium scores [235,238,239]. In cirrhotics LI is evident at presentation (OR = 6.2)[200]. The non-survivors cirrhotics have higher AST levels, AST/ALT ratio > 1.4 (aHR = 1.4), and a low R value that predicts mortality [235]. Development of liver decompensation during COVID-19 exhibits increased mortality compared to maintenance of hepatic compensation (63.2% *vs* 26.2%)[239].

Despite suffering higher mortality, those cirrhotics who survive the initial insult show re-admission/death rates at 90-d comparable to cirrhotics without COVID-19[254]. SARS-CoV-2 does not appear to accelerate the progression of liver disease beyond cirrhosis' normal course after acute infection. The interaction of severe COVID-19, pulmonary illness, and ACLF is probably mediated by CAID. Cirrhosis is linked to an increase in cytokine production and baseline endotoxinaemia, which may cause an increased inflammatory response to infection[44]. Hypercytokinemia, another characteristic of COVID-19, causes hepatocyte apoptosis and necrosis, which, in the presence of depleted liver reserves, may result in hepatic decompensation[244,255]. Therefore, cirrhosis and COVID-19 may have detrimental effects on each other. Cirrhosis decompensation in COVID-19 is characterized by deteriorating ascites, worsening of jaundice and hepatic encephalopathy more frequent than variceal bleeding or spontaneous bacterial peritonitis[235,240,241]. Despite compensated cirrhotics having greater problems, mortality among them and non-cirrhotics is comparable, supporting the idea that there is adequate hepatic reserve for recovery. The more significant predictor of death in COVID-19[237] is hepatic decompensation as opposed to cirrhosis *per se*. The data indicate that non-traditionally hepatotropic infections, such as SARS-CoV-2, may directly precipitate ACLF in cirrhotic patients[256] as also observed in influenza[252]. The median platelet (PLT) count and IFN-γ are significantly decreased in CLD than non-CLD patients[226,257], and cirrhotics are at higher risk for thrombotic events[105].

Non-alcoholic fatty liver disease

NAFLD has been advocated as a risk factor for severe COVID-19, it is present in the majority of COVID-19 patients with CLD worldwide, and shows longer viral shedding time[258]. Patients with NAFLD assessed by CT scan and with intermediate/high risk of FIB-4 have higher risk for severe COVID-19 (OR = 2.95) *vs* patients without NAFLD fibrosis score (NFS), suggesting a pathogenetic role for advanced liver fibrosis in severe COVID-19 with worse outcomes[232,250-260]. Increasing FIB-4 or NFS values when included as continuous measures in multivariable regression, they are significantly associated with COVID-19 severity (aORs = 1.90, and 2.57, respectively). NAFLD is epidemiologically associated with an increased risk of severe COVID-19[261,262], independently of BMI[263]. Hepatic steatosis is an interesting pathological characteristic that frequently occurs in COVID-19. It is possible that activation of coagulation, which is capable of causing hepatic steatosis in NAFLD, represents a unique mechanism connecting thrombosis and steatosis, two diseases that are both common in COVID-19[264]. Given that high PAI-1 has been linked to NAFLD and NASH[265,266], the involvement of PAI-1 in COVID-19 may be noteworthy. Lastly, obesity, a major risk factor for thrombosis owing to adipocy-

tokine-mediated processes, increases inflammatory molecules, Ang II/Ang 1,7 imbalance, ROS-mediated endothelial dysfunction, and alterations in lipid/glucose metabolism[267-269]. Greater risk of severe COVID-19 is found in non-diabetics patients < 60 years with NAFLD (aOR = 4.07)[259,270]. Additionally, underlying diabetes with NAFLD shows a 2-fold higher risk of severe COVID-19, much higher when LI is present (OR = 6.4), or in obese NAFLD patients (aOR = 6.32)[271]. NAFLD is associated with an increased risk of severity when it coexists with obesity[272], in non-diabetics[270], in younger patients[259], and in individuals with high hepatic fibrosis scores[260]. Although it is unknown how obesity and NAFLD might worsen COVID-19, comparable mechanisms are frequently present in both disorders comprising alterations in the immune response, macrophage activation, and (low-grade) inflammation[260,273,274]. Obesity-related chronic inflammation impairs macrophage activation *via* antigen presentation and pro-inflammatory cytokine synthesis[275]. Moreover, obesity reduces B- and T-cell responses, which leads to greater susceptibility and delayed clearance of viral infections[275,276]. The advancement of COVID-19 is favored in NAFLD patients when hepatic macrophage polarization changes from M1 (which promotes inflammation) to M2 (which suppresses inflammation)[58,274,277]. The polarization states of macrophages may be unbalanced, influencing the host's inflammatory or tolerance response to SARS-CoV-2 signals provided *via* the gut-liver axis. ACE2 expression is elevated in CLD/NASH patients, and cytokine secretion is enhanced in association with COVID-19[42]. Conversely, in a few studies NASH was not associated with severe disease[278]. It is also demonstrated that COVID-19 patients exhibit increased serum levels of MCP-1, which exacerbates steatohepatitis [279]. Age, gender, obesity, and other comorbidities are thought to be less important risk factors for COVID-19 than NAFLD. NAFLD progression may also be hastened by COVID-19.

Alcohol-related liver disease

The secure-cirrhosis and COVID-Hep registries identified ArLD as a risk factor for COVID-19 mortality (aOR = 1.79) related to advanced disease and CAID[237]. Increased mortality is also seen in alcohol-related cirrhosis[238,239]. Immune dysregulation, particularly changes in the gut-liver axis, is accentuated in ArLD, with increased endotoxinaemia and Kupffer cell activation leading to proinflammatory cytokine transcription and superoxide production[44,178]. Moreover, alcohol impairs adaptive immunity, including both cell-mediated and humoral responses[179]. The inflammatory state caused by danger-associated molecular patterns is linked to ArLD leading to the production of pro-inflammatory cytokines[238,256]. It is postulated that CSS could aggravate the increased inflammatory process in ArLD, resulting in worse outcomes[119]. In the COVID-19 period, hospitalized patients with ArLD appear at more advanced disease stages, with acute decompensation, higher MELD scores, and greater rates of ICU and mortality[185].

Autoimmune hepatitits

Immunosuppressed patients exhibit higher SARS-CoV-2 viral titres, prolonged viral shedding but do not exhibit increased risk of complications[241-242,280,281]. Analysis of AIH *vs* non-CLD patients demonstrates increased risk of hospitalization, but equivalent risk of all other outcomes including death [281]. As a result, in stable patients, immunosuppression should not be lowered as a COVID-19 risk reduction strategy[11,282]. Immunosuppression may also reduce the risk of new-onset LI[242,281,282].

Viral hepatitis

Persons with chronic HBV or HCV infection who do not have cirrhosis are not more likely to get COVID-19 or have a worse outcome[9,59]. Conversely, patients with SARS-CoV-2 and HBV co-infection are prone to a worse prognosis[283] and tend to have 2.2-fold more severe COVID-19[284]. The in-hospital mortality in COVID-19 patients with HBV is 6.0%[22] with ACLF precipitation < 1%, while LI prevalence in non-decompensated is comparable with non-CLD patients[22]. Significantly lower monocyte and WBC counts, higher levels of CD8+ T-cells and thrombocytopenia in HBV with COVID-19 are observed compared to COVID-19 alone[285]. The likelihood of HBV reactivation during SARS-CoV-2 infection is mentioned, however the risk appears to be low[286]. Those with HCV who have COVID-19 are more susceptible to hospitalization, but not at a higher risk of death[287]. Because recently treated HCV patients were less prone to contracting with SARS-CoV-2, HCV antibodies may be indicative of "protection" against COVID-19[288].

Liver transplantation

Liver transplant (LT) recipients are more frequently diagnosed with COVID-19 than general population. This might be attributable in part to more careful surveillance and a lower viral testing criterion in LT recipients[289]. The hospitalization rate for COVID-19-positive LT recipients is 50%–70%[290]. Immunocompromised individuals above the age of 60 are more prone to develop SARS-CoV-2 infection with protracted viral clearance[291,292]. LT recipients however, are not at increased risk of severity/death as compared to non-LT recipients[44,290]. LT can involve the donor-to-recipient transmission of SARS-CoV-2[293]. COVID-19 is linked to worse postoperative transplant outcomes, particularly in elderly and obese patients[294]. Most LT patients with COVID-19[295,209] should continue to receive systemic immunosuppression with stable dosages, except for immunomodulators[209], as mycophenolate

treatment is considered an independent risk factor for severe COVID-19[289]. The case-fatality rates (17%-18%) are consistent with the expected mortality rates[296]. Those with underlying cancer may require particular consideration[297]. The mortality risk does not change between early (1year) and remote transplantation[296,297].The rate of graft dysfunction during COVID-19 infection is estimated 2.3%-5%[294,298]. The inflammatory reaction in COVID-19 solid organ transplant (SOT) patients is not more severe while IL-6 Levels and incidence of ARDS are lower in SOT suggesting that immunosuppressive medication might limit the COVID-19 hyperinflammation[92].

Hepatocellular carcinoma

Data on HCC patients with COVID-19 infection are limited. COVID-19 in cancer patients is associated with poor outcomes especially if antitumor treatment was received within 14 d[299]. The all-cause mortality in the HCC subgroup is reported 52.4%, almost 7-fold higher than in patients without HCC [238]. COVID-19 patients with HCC may exhibit exacerbated progression with aggravation of existing liver disease[300].

SARS-COV-2 VACCINATION IN CLD

Because neither the adenoviral vector nor the mRNA vaccines contain live or attenuated virus, it is unlikely that vaccination poses a special safety risk for CLD patients. Vaccine trials of both Pfizer-BioNTech[301] and Moderna[302] demonstrated consistent efficacy among subgroups with coexisting conditions, but small numbers of CLD patients were included. It is unknown if SARS-CoV-2 vaccinations are as effective in cirrhotic/transplanted/immunocompromised CLD patients as they are in the general population, especially against quickly evolving viral variations. Preliminary findings suggest that transplant patients are safe[303]. Whilst historically there have been anxieties that vaccination in SOT recipients might develop alloimmunity and graft rejection, no clinical evidence support this concern[44]. LT recipients should be prioritized for immunization since the advantages exceed the risks [44].

LONG COVID-19 LIVER INJURY

Patients who recovered from COVID-19 and followed for months post-infection show increased risk of LFTs abnormalities, suggesting some possible long-term sequelae for the liver[304].The likelihood of persisting liver inflammation and fat deposition (magnetic resonance imaging) following COVID-19 [305] is discussed, as is the prospect of growing liver stiffness over time[306]. In a cohort, ninety randomly selected participants were enrolled three to nine months post-COVID-19 infection and were compared to healthy individuals (negative anti-SARS-CoV-2 immunoglobulin M/immunoglobulin G). The patterns of LI were defined using multiparametric ultrasound (mpUS)[307]. MpUS examination of post-COVID-19 hepatic parenchyma demonstrated higher liver stiffness and steatosis (attenuation imaging-ATI) scores suggestive of LI compared to healthy controls. The most notable change is increased liver stiffness, as measured by greater shear-wave elastography values[307]. Metabolomic analysis of individuals three months after COVID-19 infection reveals higher taurine concentrations, which may be suggestive of LI[308]. Additionally, persistent viral protein and RNA infection of enterocytes[309] in intestinal biopsies for several months after infection renders the small intestine a reservoir of long-term viral replication[34]. Despite mechanisms of long-term LI remain speculative, the sustained endotheliopathy following COVID-19[310] suggests endothelial-mediated inflammation as a possible mechanism. A new entity, post-COVID-19-cholangiopathy, resembling secondary sclerosing cholangitis has also been described in critical COVID-19 cases and typically presents several weeks (mean 118 d) after COVID-19 diagnosis, implying direct LI from COVID-19[311-313].

IMAGING FINDINGS

Increased liver stiffness correlates well with increased ALT/GGT values indicating underlying hepatocellular/cholangiocellular damage on a biochemical level[308]. Patients with increased liver echogenicity and increased ATI values have increased risk for severity (8-fold, 5-fold, respectively). Increased BMI and CRP levels are also associated with hepatic steatosis (ORs = 1.459, 1.387, respectively), while patients with higher steatosis scores present more severe disease[308]. Sonographic findings of ALI, including signs of acute hepatitis and vascular complications, appear in 37.3% of COVID-19 and in 48.7% of ICU COVID-19 patients[314]. CT scan findings are liver hypodensity (26%) and pericholecystic fat stranding (21%).

Table 4 Most frequent liver-related histopathological findings in COVID-19 patients

Findings	Frequency (%)
Portal and sinusoidal microthrombosis	29.4–100
Hepatic/macrovesicular steatosis	50–75
Mild portal inflammation	13.2–66
Centrilobular necrosis	50
Mild acute hepatitis	50
Congestion/dilation of hepatic sinuses	34.7
Portal fibrosis	20.5
Kupffer cell hyperplasia	13.5
Lobular inflammation	11.6
Inflamed cells within the sinusoids (neutrophils, plasmatocytes and Kupffer)	N/A
Panacinar hepatitis, zone 3 necrosis	N/A

N/A: Not applicable; COVID-19: Coronavirus disease 2019.

HISTOPATHOLOGICAL FINDINGS

Most frequently observed liver-related histopathological findings (Table 4) are associated with underlying comorbidities (*e.g.*, NAFLD), rather than of SARS-CoV-2 itself. In initial pathology reports some authors cautioned about the observed "spiked virions", "corona-like" inclusions that could be artifacts of tissue autolysis or of an alternative origin (*e.g.*, intrahepatic cholesterol crystals, lamellations, "crown-like" structures seen in NAFLD, multi-vesicular bodies, exosomes)[12,315,316]. Further investigations found evidence of apoptosis, an abundance of mitosis, mixed inflammatory infiltration in the portal region, and extensive bile duct damage. The hypothesis of direct cell damage was strengthened by identifiable viral particles, viral RNA in the liver, and hepatocytes, together with the intrahepatic detection of SARS-CoV-2 by EM and in-situ hybridization[55-57]. Hepatocytes' cytoplasm contained characteristic coronavirus particles with their spikes, according to ultrastructural analysis[317]. While EM reveals mitochondrial enlargement and apoptosis, in situ hybridization may identify SARS-CoV-2 in 68% of samples[36]. Mitochondrial swelling, endoplasmic reticulum dilatation, and cell membrane dysfunction, document SARS-CoV-2 ability to replicate within hepatocytes[102,317]. Proteomic analysis of autopsy tissue showed mitochondrial dysfunction with dysregulated fatty acid oxidation and oxidative phosphorylation[68]. Biopsies from LT recipients who tested positive for COVID-19 revealed endotheliitis, bile duct damage, and mixed inflammatory portal infiltrates, which are findings of T-cell-mediated rejection[318]. Steatosis is predominant in obesity and overweight patients[102], and its high prevalence is attributed to population's baseline characteristics (severe COVID-19 and steatosis share common risk factors). DILI and CSS may also contribute to the development of hepatic steatosis. Direct vascular damage, portal endotheliitis, portal vein herniation, terminal vessel dilations, and thrombosis with luminal ectasia are examples of acute vascular alterations. Chronic alterations in the portal and sinusoidal vessels, characterized by fibrous thickening of the vascular wall (thrombotic bodies), are sinusoidal inclusions positive for the platelet marker CD61[56,102,108,319]. Lobular ductal pathology is common showing the presence of moderate nuclear pleomorphism in cholangiocytes and canalicular cholestasis[108].

CONCLUSION

This review sheds light on issues raised by early COVID-19 studies concerning discrepancies in prevalence of LI and CLD, and the role of direct SARS-CoV-2 hepatocyte/cholangiocyte injury. The weighty implication of COVID-19-induced LI mechanisms comprising CSS, endotheliopathy/immuno-thromboinflammation, liver hypoxia, and oxidative stress with respect to histopathological and immunohistochemical findings is meticulously discussed. Finally, an emerging entity, long-COVID-19 persistent LI is also studied.

FOOTNOTES

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Country/Territory of origin: Greece

ORCID number: George D Liatsos 0000-0002-8203-2748.

S-Editor: LI L
L-Editor: A
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REVIEW

Ferroptosis regulates key signaling pathways in gastrointestinal tumors: Underlying mechanisms and therapeutic strategies

Ravichandiran Rabitha, Sethuraman Shivani, Yahya Showket, Ganapasam Sudhandiran

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Ravichandiran Rabitha, Sethuraman Shivani, Yahya Showket, Ganapasam Sudhandiran, Department of Biochemistry, University of Madras, Cell Biology Research Laboratory, Chennai 600 025, Tamil Nadu, India

Corresponding author: Ganapasam Sudhandiran, MPhil, PhD, Full Professor, Department of Biochemistry, University of Madras, Cell Biology Research Laboratory, Gandhi Mandapam Road, Chennai 600 025, Tamil Nadu, India. sudhandiran@unom.ac.in

Abstract

Ferroptosis is an emerging novel form of non-apoptotic, regulated cell death that is heavily dependent on iron and characterized by rupture in plasma membrane. Ferroptosis is distinct from other regulated cell death modalities at the biochemical, morphological, and molecular levels. The ferroptotic signature includes high membrane density, cytoplasmic swelling, condensed mitochondrial membrane, and outer mitochondrial rupture with associated features of accumulation of reactive oxygen species and lipid peroxidation. The selenoenzyme glutathione peroxidase 4, a key regulator of ferroptosis, greatly reduces the lipid overload and protects the cell membrane against oxidative damage. Ferroptosis exerts a momentous role in regulating cancer signaling pathways and serves as a therapeutic target in cancers. Dysregulated ferroptosis orchestrates gastrointestinal (GI) cancer signaling pathways leading to GI tumors such as colonic cancer, pancreatic cancer, and hepatocellular carcinoma. Crosstalk exists between ferroptosis and other cell death modalities. While apoptosis and autophagy play a detrimental role in tumor progression, depending upon the factors associated with tumor microenvironment, ferroptosis plays a decisive role in either promoting tumor growth or suppressing it. Several transcription factors, such as *TP53*, activating transcription factors 3 and 4, are involved in influencing ferroptosis. Importantly, several molecular mediators of ferroptosis, such as p53, nuclear factor erythroid 2–related factor 2/heme oxygenase-1, hypoxia inducible factor 1, and sirtuins, coordinate with ferroptosis in GI cancers. In this review, we elaborated on key molecular mechanisms of ferroptosis and the signaling pathways that connect ferroptosis to GI tumors.

Key Words: Ferroptosis; Gastrointestinal cancers; Nuclear factor erythroid 2–related factor 2; Apoptosis; Autophagy

Core Tip: Gastrointestinal tumors contribute to the majority of cancer-related deaths. Ferroptosis is a novel form of non-apoptotic cell death that plays a vital role in reducing the invasiveness and metastasis of gastrointestinal tumors. Herein, we discussed the regulatory mechanisms involved in ferroptosis through the hallmark pathways of glutathione peroxidase 4, iron metabolism, lipid peroxidation, and redox signaling that provide a novel therapeutic approach for gastrointestinal cancers.

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INTRODUCTION

Cancer is a notorious disease that causes numerous disorders and deaths worldwide, particularly gastrointestinal (GI) cancers comprising esophageal cancer, gastric cancer (GC), liver cancer, pancreatic cancer, and colorectal cancer[1]. A recent report stated that there are approximately 4 million new cases each year, which is far greater than breast and lung cancers combined; thus, there is a need to improve the therapeutics of GI cancers[2]. Although there has been an improvement in the prognosis of GI cancers in the last decade due to development of intensive therapies, such as incorporating cytotoxic drugs and targeted therapies, GI cancers are one of the leading causes of cancer deaths in developed and developing countries[3-5]. With varying risk factors, incidence, prevalence and prognosis, early diagnosis with increased screening may facilitate therapies to fight against GI cancers.

Biomarkers, including epigenetic markers, also play an effective role in decreasing the progression of GI cancers by early diagnosis and decreasing risk assessment by predicting the tumor response from specific therapies in patients[6]. Apart from internal stimulation, external exposures, such as dietary intake, tobacco use, alcohol consumption, obesity, and pathogens, are considered to increase the risk of GI tumors[7]. Various improvements in the therapeutic aspects to decrease the burden of GI cancer, including the chemoprevention of these cancers by using antioxidants, have drawn much attention[8]. Murphy *et al*[9] estimated that by 2025, the pervasiveness of obesity will be increased in men and women by 18% and 21%, respectively, which could escalate the encumbrance of GI cancers worldwide [9].

The induction of cell death by ferroptosis has increased in the recent past. Cell death is an endogenous mechanism that regulates homeostasis, and it is perceived as a requisite bodily process. As an imperative system, it exterminates useless or unwanted cells and bolsters the defense system of the body. Many forms of cell death have been observed in the recent past, particularly accidental cell death and regulated cell death (RCD)[10,11]. Accidental cell death occurs suddenly in response to stress, heat shock, or mechanical nature, while RCD is orchestrated by exhibiting a precise signaling cascade provided by a defined group of effector molecules. Many forms of RCD have been observed, such as apoptotic, autophagic, pyroptotic, necrotic, entotic, and ferroptotic cell death[12].

Investigating RCD introduces a new path for developing cancer therapeutics. Among the various forms of RCD, ferroptosis, an iron dependent, non-apoptotic RCD, is fascinating because of its ability to manage cancer cells that develop resistance to apoptosis and drugs. Although a link between iron and lipid peroxidation has been found in cancer research, this form of RCD has a peculiar combination of morphological, biochemical, and genetic characteristics distinct from necrosis, apoptosis, and autophagy [13]. Dixon *et al*[14] proposed the term ferroptosis in 2012, and its emerging role in several other disease settings was observed[14]. Ferroptotic cell death is characterized by the occurrence of oxidative stress and membrane lipid peroxidation, thereby causing mitochondrial atrophy, increased density of the mitochondrial membrane, and membrane damage[15]. Therefore, this review summarizes the role of ferroptosis in regulating key cancer signaling and cell death pathways in GI tumors for better prognosis.

GASTROINTESTINAL CANCERS: INCIDENCE AND MORTALITY

GI cancers contribute to significant morbidity and mortality rates worldwide[16] (Table 1), accounting for 26% of all cancer-related incidence and 35% of all cancer-related fatalities. It was noted that there is a two-fold increase in men compared to women. It has been reported that by 2040, there will be an increase in new cases and deaths from GI cancers by 58% and 73% to 7.5 million and 5.6 million, respectively[17]. Incidence and mortality rates of GI cancers worldwide is presented in Table 1[18-22].

Table 1 Incidence and mortality rates of gastrointestinal cancers worldwide

No	Gastrointestinal cancers	Incidence	Mortality	Ref.
1	Esophageal cancer	604100	544100	[18]
2	Gastric cancer	1100000	770000	[19]
3	Liver cancer	905700	830200	[20]
4	Pancreatic cancer	495773	466003	[21]
5	Colorectal cancer	1930000	900000	[22]

While cell death modalities play a decisive role in eradication of tumor cells and maintenance of homeostasis, the role of RCD pathways, particularly ferroptosis, in tumor progression and metastasis of cancers has been the subject of interest for the last decade.

DISTINCTIVE FEATURES OF FERROPTOSIS

The various features of ferroptosis include the morphological, biochemical, and epigenetic alterations that describe this novel form of cell death.

Morphological distortion of ferroptosis

Ferroptosis is a non-apoptotic form of cell death because it lacks the classical features of apoptosis, such as mitochondrial cytochrome-C release, caspase activation, and chromatin fragmentation[23]. In contrast, ferroptosis induces mitochondrial membrane disintegration, which results in cell enlargement, plasma membrane rupture, volume reduction, increased density, and disappearance of cristae observed under the electron microscope. These changes are potentially caused by permeability loss due to increased lipid peroxidation[24].

Biochemical changes involved in ferroptosis

The main biochemical features of ferroptosis are iron accumulation, reactive oxygen species (ROS) production, and induction of lipid peroxidation. Iron is the most abundant metal and is essential to all life on earth. As an iron-dependent form of cell death, increased circulating iron leads to the Fenton reaction through activated iron-containing enzymes that act as biochemical markers of ferroptosis[25]. Intracellular iron and iron-containing enzymes are indispensable for the execution of ferroptosis. Molecular regulators associated with iron homeostasis, such as transferrin, lactotransferrin, transferrin receptor, nuclear receptor coactivator 4-dependent degradation of ferritin, iron regulatory protein, and iron responsive element regulatory network, facilitate ferroptotic cell death[26,27].

ROS are the major culprits in many disease settings. ROS can damage essential biomolecules such as DNA, protein, carbohydrates, and lipids, thereby causing denaturation, peptide s-s bond breaking, crosslinking, enzyme inhibition, and permeability changes in tissues and cells. Therefore, increased ROS promote several perturbances leading to altered cell death pathways as well as induction of ferroptosis [28].

Lipid peroxidation is a seminal process in which the generated free radicals (H_2O_2, super peroxides, peroxy radicals) target long-chain fatty acids[29]. Extensive lipid peroxidation affects the permeability, fluidity, and curvature of the membrane, thereby stimulating cell death by forming micelles and pores in the biological membrane. A close connection between lipid peroxidation and ferroptosis exists in cancers. Importantly, to overcome drug resistance in chemotherapy, ferroptosis plays a crucial role in tumor microenvironment, thereby controlling cell proliferation through redox signaling pathways[30].

Epigenetic alterations

Epigenetics is a genetic mechanism that can influence gene expression by DNA methylation, histone modifications, and the effects of noncoding RNA without altering the DNA sequence[31,32]. DNA methylation is an epigenetic modification process that uses DNA methyltransferases to covalently transfer the methyl group to the C-5 position in the cytosine ring of DNA[33]. It has been reported that hypermethylation of the *CDH1* gene promoter could increase ferroptosis susceptibility in head and neck cancer cells[34]. Recently, epigenetic regulation through H2B monoubiquitination and p53 has been determined[35].

Histone acetylation is controlled by histone acetyltransferases, bromodomains (BRDs), and histone deacetylases (HDACs) to regulate ferroptosis. In the histone acetylation process, histone acetyltransferases play the writer role, BRDs the reader role, and HDACs the eraser role[36,37]. The tumor suppressive role of *Tp53* is well known. For p53-induced ferroptosis, acetylation plays a crucial role by regulating solute carrier family 7 member 11 *(SLC7A11)* expression[38]. Acetylated and mutant $p53^{3KR}$

suppresses *SLC7A11* expression and inhibits cysteine uptake, which alleviates ferroptosis and lipid peroxidation by decreasing glutathione (GSH) synthesis[38,39]. BRD4 induces the expression of anti-ferroptotic genes, and it has been observed that the BRD4 inhibitor JQ1 induces ferroptosis by downregulating the expression of ferroptosis-related genes such as glutathione peroxidase 4 (*GPX4*), *SLC7A11*, and solute carrier family 3 member 2 in breast and lung cancer cells[40]. HDACs were initially identified as an eraser for removing acetyl groups from histones, but it was later discovered to be involved in many important biological functions. Recently, HDAC inhibitors have been endorsed as potential therapeutics for various cancers[41].

As a multifarious group of noncoding transcripts, noncoding RNAs, microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs were first considered nonfunctional junk, but they participate in a broad domain of gene-regulatory pathways[42]. Beyond this, it has been suggested that circular RNAs play a role in ferroptosis by regulating multiple signaling pathways either directly or indirectly by acting on the key regulating factors and upstream targets of ferroptosis[43]. miRNAs tend to act as stimulants in ferroptosis by targeting ferroptosis-associated factors by downregulating the expression of activating transcription factor 4 (ATF4), which is a stress signal that tends to inhibit ferroptosis by miR-214-3p in hepatocellular carcinoma (HCC) by inducing *SLC7A11* and several other miRNAs, including miR-101-3p, at high expression levels. They profoundly enhance the activity of nuclear factor kappa B, which regulates ferroptosis *via* GPX4 and prostaglandin endoperoxide synthase 2 in lung cancer, and miR-324-3p at high expression levels induces ferroptosis in lung adenocarcinoma by targeting GPX4[44]. Furthermore, miRNAs have also been found to have inhibitory effects on ferroptosis; therefore, they play diverse roles in ferroptosis. The alterations they cause to ferroptosis-associated factors make them a potential target in cancer therapeutics.

FERROPTOSIS INDUCERS

Over the past few years, several small molecules and plant-based compounds that target transporters or enzymes involved in ferroptosis have been described.

In principle, GSH synthesis and GPX4, a selenoenzyme, are the major regulators of ferroptosis. GSH is an important substrate for GPX4. Therefore, any depletion in GSH leads to substantial lipid peroxidation, leading to ferroptosis. By inhibiting GPX4, accumulation of lipid peroxidation takes place [45]. To produce GSH, system Xc^- regulated cysteine activity is needed. Certain compounds, such as sorafenib, glutamate, and erastin, induce ferroptosis through inhibition of system Xc^-. Ras-selective lethal 3 (RSL3), a known GPX4 inhibitor and other compounds containing electrophilic chloroacetamides, covalently binds and restricts selenocysteine activity inside the active site of GPX4 to initiate ferroptosis[46]. Other nitrile oxide electrophiles include ML210, JKE-1674, and JKE-1716, which attach to selenocysteine and cause ferroptosis[46,47]. By directly oxidizing lipids and indirectly impairing GPX4 action, FINO2 causes ferroptosis, and FIN56 drives the destruction of GPX4 to induce ferroptosis[48]. To induce ferroptosis, FINO2 harnesses either a direct or indirect iron oxide to induce suppression of GPX4 activity. Organic peroxides are compounds with multiple O_2 bonds that are cleaved, resulting in the production of RO anions. These organic peroxides are often used as models to induce ROS. A commonly held view is that the lipid peroxide analogue tert-butyl hydroperoxide stimulates lipid peroxidation-dependent ferroptosis[49].

Excessive iron accumulation is a precursor of ferroptosis in a plethora of cell types. *In vitro*, hemoglobin causes ferroptosis, and *in vivo*, it causes intracerebral hemorrhage in other disease states[50-52]. Furthermore, the rise in cellular iron levels and consequent ferroptosis caused by pharmacological stimulation of selective autophagy through degradation of ferritin is known as ferritinophagy[53,54]. Apart from these, there are many other ferroptotic inducers; for example, in human pancreatic cancer cells, zalcitabine causes autophagy-dependent ferroptosis, pointing to a link between DNA sensor pathways, autophagy activation, and mitochondrial malfunction[55].

FERROPTOSIS INHIBITORS

The inhibition of ferroptosis by small molecular compounds takes place through the following mediators.

Iron chelators: The important role of iron is to promote lipid peroxidation by either activating iron-containing lipid oxygenases (LOX) or nonenzymatic iron-mediated Fenton action[56-58]. Iron chelators like deferoxamine inhibit ferroptotic cell death. Iron chelators reduce H_2O_2-induced necrosis but not ferrostatin-1, suggesting that iron may be potentially involved in multiple cell death modalities[59,60].

Enzyme inhibitors: The enzyme inhibitor acyl-CoA synthetase long chain family member 4 (ACSL4) mediates addition of CoA to long-chain fatty acids, particularly arachidonic acid, and appears to be a crucial precursor to further arachidonate lipoxygenase-dependent lipid peroxidation[57]. ACSL4 and

arachidonate lipoxygenase inhibitors are known to inhibit the ferroptotic cell death process by preventing LOX accumulation[57,61]. In addition, NAD phosphate oxidase inhibitors comprising of diphenylene iodonium and GKT137831 prevent erastin-induced ferroptosis[62].

Protein degradation inhibitors: GPX4 may be degraded by a variety of ferroptosis activators, which results in lipid peroxidation. However, FIN56 and erastin-induced GPX4 breakdown is inhibited by the molecular chaperone heat shock protein 90, 5-(tetradecyloxy)-2-furoic acid, and dopamine[63-65].

Other inhibitors: The mitogen-activated protein kinase kinase inhibitor, U0162 is commonly used to reduce ferroptosis owing to its broad antioxidant activity[66]. The strong inhibitory effects of exogenous monounsaturated fatty acids (MUFAs) or deuterated polyunsaturated fatty acids (PUFAs) on ferroptosis may be attributed to their displacement of PUFAs from phospholipids, which lowers the accumulation of lipid peroxidation[67].

REGULATION OF FERROPTOSIS

A deeper knowledge of how ferroptosis is regulated by metabolic pathways, including iron, GSH, and lipid metabolism, has been the focus of research in proposing therapeutic drugs. In this context, several metabolic and cancer signaling pathways that connect ferroptosis have been described.

GPX4

Ferroptosis is primarily caused by the inactivation of the cellular antioxidant system, particularly the system that is dependent on the antioxidant defense Xc^- GSH-GPX4 that leads to toxic lipid ROS accumulation[68]. GSH peroxidases are pivotal enzymes that enable the reduction of peroxides *via* GSH. The system Xc^- family mediates transport of cysteine and glutamate, where glutamate is exported outside the cell and cysteine is imported inside the cell, which initiates the production of GSH, hence inhibiting ferroptosis in cancer cells[69] (Figure 1). The GPX protein comprises different types, among which GPX4, a selenoprotein, is an elemental antioxidant mediator known for its capability of reducing large peroxides such as PUFAs[68]. It is primarily involved in the maintenance of lipid metabolism and defense against the accumulation of toxic lipid ROS. Since GPX4 activity is directly impacted by conditions that reduce intracellular cysteine and subsequently GSH levels and because GSH is the dominant antioxidant in mammalian cells, these conditions trigger ferroptosis[70]. Small molecules such as erastin can block GSH or GPX4 expression to activate ferroptosis. In many cells, the rate at which GSH is synthesized is limited by the internal reduction of cystine to cysteine[71].

Lipid metabolism

The process in which free radicals and non-free radicals cleave the C-C double bonds in lipids such as PUFAs, phospholipids, glycolipids, and cholesterol is known as lipid peroxidation.

According to lipidomic research, arachidonic acid and adrenic acid are key driving factors in inducing ferroptosis. Elongation of very long-chain fatty acid protein 5 and fatty acid desaturase 1 are associated with the synthesis of fatty acids, where both enzymes are upregulated in mesenchymal GC cells, resulting in ferroptosis sensitivity, according to studies on the disease[72]. The process by which these PUFAs become coenzyme-A derivatives that are integrated into phospholipids triggers ferroptosis. Both autophagy and deubiquitylation can result in the reduction of intracellular GPX4[73]. Specialized sequences in GPX4 are distinctly recognized by heat shock protein 90 and facilitate the breakdown of GPX4 by chaperone-mediated autophagy[63]. On the other hand, the inhibition of chaperone-mediated autophagy by the mTOR pathway is relieved by its suppression, which may have caused GPX4 to degrade and cause ferroptosis[74].

Numerous investigations have revealed ACSL4 as a crucial sensitizer in ferroptosis machinery. ACSL4 mediates the esterification of PUFAs into phospholipids by adding CoA to the polyunsaturated bond of arachidonic acid[75] (Figure 1). Lysophosphatidylcholine acyltransferase 3, which is activated by ACSL4, contributes to ferroptotic lipid signaling by adding acyl groups to lysophospholipids, particularly to the phospholipids phosphatidylcholine and phosphatidylethanolamine[75]. Apart from PUFAs, MUFAs can promote ferroptosis resistance by initiating mutations in the PI3K-AKT-mTOR pathways, sterol regulatory element-binding protein 1 can mediate the production of MUFAs, which aids cancer cells in resisting ferroptosis[76]. MUFAs can modulate the hydroxycarboxylic acid receptor 1-sterol regulatory element-binding protein 1-stearyl-coenzyme A desaturase 1 pathway and lactate in the tumor microenvironment, which might increase the production of MUFAs and lower the expression of ACSL4 to avoid ferroptosis[77]. As functional lipids in ferroptosis, polyunsaturated ether phospholipids have a significant regulatory effect in changing cancer cells from a ferroptosis-sensitive state to a ferroptosis-resistant state[78]. Thus, lipid metabolism has a notable effect on the capability of cancer cells to induce ferroptosis.

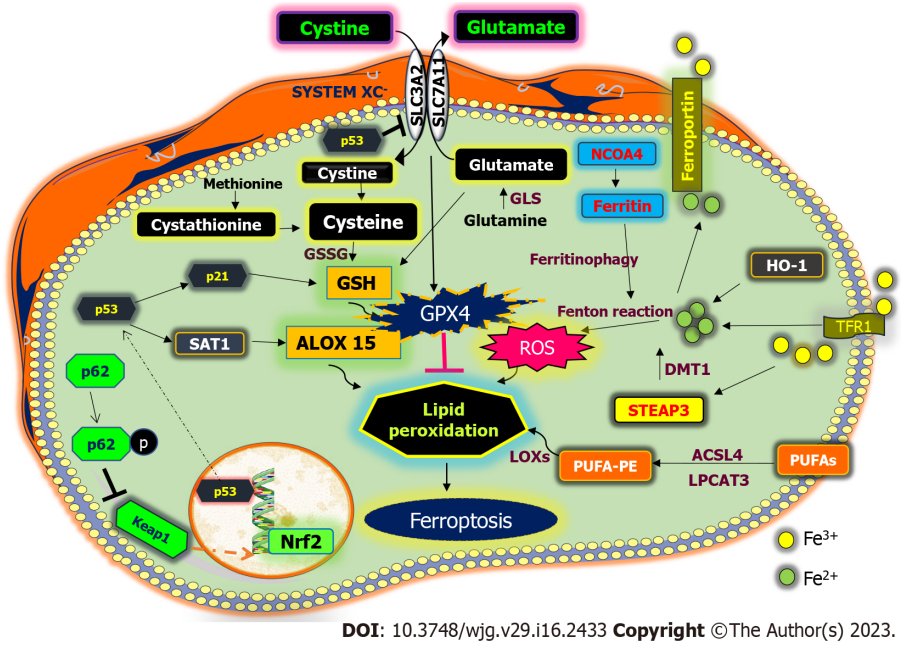

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Figure 1 Mechanistic insights of ferroptosis regulated pathways are represented. Glutathione peroxidase 4 (GPX4) is central in ferroptosis and works through the initiation of system Xc^-, which mediates the export of glutamate and import of cystine. Cystine is required for the synthesis of glutathione (GSH). p53 induces the expression of p21 following oxidative stress and further induces GSH leading to GPX4 expression. Alternatively, p53 increases the expression of spermidine/spermine N1-acetyltransferase 1 (SAT1), which serves as the transcriptional target of p53. SAT1 joins ferroptotic pathway through induction of arachidonate lipoxygenase (ALOX) 15 leading to lipid peroxidation. In addition, p62 phosphorylation targets Kelch-like ECH-associated protein 1 (Keap1) leading to accumulation of nuclear factor erythroid 2–related factor 2 (Nrf2) in the nucleus to subscribe antioxidant enzymes during oxidative stress. Acyl-CoA synthetase long chain family member 4 (ACSL4) is an isozyme catalyzes polyunsaturated fatty acids (PUFAs) contributes to ferroptosis through induction of lipoxygenases. Ferritinophagy induction *via* ferritin contributes to the Fenton reaction leading to accumulation of reactive oxygen species (ROS) and lipid peroxidation. DMT1: Divalent metal transporter 1; GSSG: Glutathione disulfide; HO-1: Heme oxygenase-1; LOX: Lipid oxygenase; LPCAT3: Lysophosphatidylcholine acyltransferase 3; PUFA-PE: Polyunsaturated fatty acid-phosphatidylethanolamine; SLC3A2: Solute carrier family 3 member 2; SLC7A11: Solute carrier family 7 member 11; STEAP3: Six-transmembrane epithelial antigen of the prostate 3; TFR1: Transferrin receptor 1.

Iron metabolism

To carry out ferroptosis and allow lipid peroxides to build up, iron is particularly necessary. The capacity of iron to catalyze numerous metabolic events and flip between the various ionic states depends largely on how it can absorb and give electrons[79]. In the Fenton reaction, Fe^{2+} is converted to Fe^{3+} in the presence of H_2O_2, and $HO^{\cdot}$ is produced as a result of electron transfer to H_2O_2[80]. In contrast, the Haber-Weiss process could convert Fe^{3+} back to Fe^{2+} by reacting with O_2, causing O_2 to lose one electron and become O^{2-}[81].

The transfer of iron takes place in the following ways. Transferrin synthesized by the liver as a chelator of Fe^{3+} ions. In contrast to apo-transferrin, which is sans iron transferrin that is not recognized by transferrin receptor 1 (TfR1) and not internalized, transferrin recognizes and binds to TfR1 when it reaches the cell membrane, which is then internalized by clathrin-mediated endocytosis[82]. Accumulating evidence suggests the role of iron metabolism in ferroptosis[83,84]. Fe^{3+} is liberated from the transferrin TfR1 complex due to the acidic pH of endocytic vesicles and is converted to Fe^{2+} by the six-transmembrane epithelial antigen of the prostate (STEAP) family. STEAP1 and STEAP2 are implicated in multiple human malignancies, including Ewing sarcoma bladder, ovary, colon, breast, prostate, pancreatic, and cervical cancer[85-87]. Malignant gliomas have high levels of STEAP3, which triggers the cancer epithelial-mesenchymal transition[88]. Under hypoxic circumstances, STEAP4 is activated, resulting in an imbalance of mitochondrial iron and increased ROS generation[89].

The intracellular labile iron pool is subsequently created by divalent metal transporter 1, which mediates the release of Fe^{2+} into the cytoplasm[90]. Apo-transferrin is released back to the plasma membrane post recycling, while TfR1 and apo-transferrin remain linked[91]. Fe^{2+} is then directly transported into cells by divalent metal transporter 1 during the conversion of Fe^{3+} to Fe^{2+} (Figure 1). Another mechanism includes the absorption of porphyrin-bound Fe^{2+} that contains hemoglobin, particularly in macrophages, and involvement of cell membrane receptors of iron-storing protein ferritin, such as scavenger receptor class A member 5, as observed in kidney and embryonic development, which absorbs iron to regulate the ferroptosis mechanism[92,93].

Role of ferritin in Fe transport

Ferritin, an iron-sequestering protein with 4500 iron atoms, plays a crucial role in iron transport, cellular multiplication, angiogenesis, and immune suppression[94]. By using NCOA4, ferritin can also be split

up to liberate free iron, through "ferritinophagy"[95]. Ferroportin (FPN), recognized as the sole iron release pump that works with ceruloplasmin to export iron, is primarily in charge of moving Fe^{2+} out of cells[96]. Ceruloplasmin controls HepG2 and Hep3B cell iron regulation to prevent ferroptosis, and its loss causes a buildup of intracellular Fe^{2+} and lipid ROS and enhances ferroptotic death caused by erastin and RSL3[97]. Prominin-2 promotes the growth of multivesicular structures that comprise ferritin and exosomes that transfer iron from cells, enabling ferroptosis resistance in breast cancer[98]. FPN is severely reduced in many cancer types, suggesting that cancer cells may contain large amounts of iron[99]. Decreased FPN levels promote proliferation and epithelial-mesenchymal transition in triple-negative breast cancer cells[100]. Hepcidin, produced by tumors of the liver, promotes the breakdown of FPN and aids in the spread and development of cancer[101].

TRANSCRIPTION FACTORS

Activating transcription factor 3 and activating transcription factor 4

The unfolded protein response is activated in mammalian cells due to endoplasmic stress and has a two-dimensional functional role in cell survival and death[102]. Activating transcription factor 3 (ATF3) is a member of the ATF/cAMP-responsive element-binding protein family of transcription factors. It binds to the ATF/cAMP-responsive element-binding protein cis-regulatory element and coordinates gene expression. ATF3 has tumor suppressive roles and inhibits cancer malignancy in GI cancers[103]. The context-dependent role that ATF3 plays in cancer is likely due to complex protein-protein interaction networks in which ATF3 is involved.

Indeed, in addition to transcriptional regulation, ATF3 has been found to interact with many critical cellular proteins and regulate their functions. One of the well-characterized ATF3-binding proteins is wild-type p53, and ROS signals are necessary for this aberrant production of endoplasmic reticulum stress markers; however, the antioxidant N-acetyl L-cysteine prevents overexpression and consequent ferroptotic cellular death. Although the ATF3-TP53 complex helps to initiate DNA damage, *TP53* is not necessary for ATF3-regulated suppression of *SLC7A11* transcription[104]. Nuclear factor erythroid 2-related factor 2 (Nrf2) can, in contrast, regulate the expression of ATF3 by creating complex feedback loops for the activation of a number of transcription factors in coordinating the ferroptotic response [105].

ATF4 is a double-sided sword that plays a dual role in ferroptosis. In numerous cancer cells, including human glioblastoma as well as pancreatic cancer cell lines, the depletion of ATF4 increases erastin-induced or RSL3-induced ferroptosis, and the inhibition of ATF4 increases artesunate-induced ferroptosis in the DAUDI cell line[106,107]. The classes of genes targeted by ATF4, such as *SLC7A11*, heat shock protein 5 (*HSPA5*), and tribbles pseudokinase 3, may have an impact on this ATF4-dependent mediation of ferroptosis in various cancer types. ATF4 is inhibited by the erastin-induced overexpression of HSPA5, which results in the sequential breakdown of GPX4 and lipid peroxidation [108-112]. Thus, ATF3-dependent and/or ATF4-dependent pathway dysregulation might influence ferroptosis in a tumor-specific phenotype.

Nrf2

Binding to antioxidant response elements, Nrf2, a transcription factor from the Cap-N-Collar family, is essential in regulating antioxidant genes and maintaining redox homeostasis[113]. Nrf2 transcriptional activity is normally attached to Kelch-like ECH-associated protein 1 (Keap1), retaining it in the cytoplasm. Nrf2 separates from Keap1 during environmental conditions (such as oxidative stress) and subsequently translocates into the nucleus, where it stimulates the expression of ARE-dependent target genes[113]. Nrf2, however, depends on the expression of the target genes that are involved in the control of cell proliferation, migration, and death to have a double impact on carcinogenesis and tumor treatment[114].

Initially, researchers showed that ferroptosis resistance may be promoted by activating the Nrf2 pathway using a model of HCC. In human HCC cell lines, ferroptosis activators like erastin and sorafenib boost Nrf2 stability by inhibiting the development of the Nrf2-Keap1 complex. The autophagy receptor SQSTM1/p62 elevates Nrf2 expression by inactivating Keap1, which is another regulator of this process[115]. By boosting GSH production and function, Nrf2-dependent genes such as glutathione synthetase, GPX4, and *SLC7A11* contribute to ferroptosis inhibition[115]. Control of NADPH synthesis, a vital electron donor required for reduction of oxidized substrates[116], which is also a ferroptosis sensitivity biomarker[117], is another way that Nrf2 intermediate metabolism is connected to the regulation of ferroptosis. Overall, Nrf2 is an important transcription factor that regulates ferroptosis.

TP53

p53, a tumor suppressor encoded by the *TP53* gene, is involved in the mediation of DNA damage, oncogene activation, and hypoxia. p53 promotes ferroptosis by transcriptional or posttranscriptional pathways in addition to its impacts on apoptosis, autophagy, and cell cycle. p53 can both induce and inhibit ferroptosis in a context dependent manner. To cause ferroptotic cell death, p53 increases the

production of spermidine/spermine N1-acetyltransferase 1, which sequentially causes increased 15-LOX expression responsible for oxidation of PUFAs (Figure 1)[118]. Simultaneously, p53 decreases the expression of ELAV-like RNA-binding protein and its action with LINC00336, by limiting the capability of cells to fight ferroptosis by increasing the activity of cystathionine β synthase (CBS)[119]. Additionally, p53 interaction with ubiquitin-specific protease 7, a deubiquitinating enzyme, facilitates its nuclear translocation by altering histones, favorably controlling ferroptosis[120]. Human colon cancer cells, such as HCT116 and SW48, are inhibited against ferroptosis by p53 in a transcription-independent manner[121] The p53-p21 axis prevents ferroptosis, allowing cancer cells to withstand stressful metabolic situations. Thus, this dual role of p53 in ferroptosis can be explored for therapeutic treatment of cancers.

Heme oxygenase-1

Heme oxygenase-1 (HO-1) is a significant redox-mediating enzyme that is activated in reaction to oxidative stress, cellular stress, neurodegeneration, and other diseases. HO-1 has a dual personality. The use of HO-1 antagonist zinc protoporphyrin IX and HO-1 knockdown animals demonstrated that HO-1 increases erastin-induced ferroptosis[121]. Conversely, because HO-1 expression is knocked down, erastin and sorafenib more effectively limit cell proliferation in HCC caused by these drugs[121]. In addition, HO-1 mediates iron and ROS levels where Nrf2-derived HO-1 provides a cytoprotective effect by scavenging ROS when HO-1 is moderately active[121]. In contrast, given that cancer cells produce more HO-1 than normal cells, a high level of HO-1 activation may increase fragile Fe^{2+}, resulting in an excess of ROS and eventual oxidative cell death[121]. Hence, the employment of ferroptosis by HO-1 activation can define the fate of cancer cells.

Sirtuins

Sirtuins are NAD^+-dependent deacetylases that are involved in DNA repair, cellular metabolism, and cancer development. As a key oxidizing agent, sirtuins tend to play a crucial role in regulating redox signaling pathways[122]. Seven sirtuins have been identified in mammals so far, and each family has been found to regulate cellular homeostasis. SIRT1 activation increases the antioxidant activity mediated by oxidative stress-related transcription factors[122]. Studies have found that increased expression of SIRT1 induces Nrf2-mediated antioxidant activity, thereby increasing GPX4 and GSH levels in HCC. Conversely, their downregulation by protocadherin 20 mediates ferroptotic cell death by lowering the expression of GPX4 and GSH and increasing intracellular iron levels and ROS[123]. SIRT6 promotes ferroptosis in pancreatic cells through upregulation of the ROS levels[124], and its downregulation causes GC cells to resist sorafenib-induced ferroptosis[125].

Hypoxia inducible factor

Hypoxia is a prominent factor involved in the progression and metastasis of numerous cancers. Prolyl hydroxylase hydroxylates the hypoxia inducible factor (HIF)-a subunits HIF-1α and HIF-2α under normoxic circumstances; subsequently, they are subjected to ubiquitin-mediated proteolysis and destruction. HIF target genes are activated in hypoxia as a result of HIF-1α and HIF-2α failing to hydroxylate and translocate into the nucleus[126]. Our laboratory's previous studies found that under hypoxic conditions, hypoxia-inducible factors such as HIF-1α and HIF-2α are activated and stimulate the activation of matrix metalloproteinases 2 and 9, thereby increasing tumorigenesis[127]. Singhal *et al*[128] demonstrated the mechanism by which activation of HIF-2α raises cellular iron to accelerate ferroptosis or irreversible cysteine oxidation that causes cell death.

FERROPTOSIS AS A NOVEL TARGET FOR GI CANCER RESEARCH

The leading cause of cancer deaths and incidence from GI tumors has become the most essential health concern, so improving the therapeutic aspects provides an important way to decrease the burden of GI cancers. Several studies have used ferroptotic cell death as a key modulator to inhibit the growth of GI cancers such as colorectal cancer, liver cancer, pancreatic cancer, GC, and esophageal cancer.

Ferroptosis in colorectal cancer

Recent studies by Wang *et al*[129] suggested that ferroptosis can have a potential modulatory role in the targeted killing of chemoresistant colon cancer cells, and ferroptosis-related genes could also be harnessed as biomarkers in colorectal cancer therapeutics. Genes involved in the colorectal cancer tumor microenvironment are intricately linked to ferroptosis, and some of them are involved in the lipid peroxidase and GPX/GSH enzyme system. Iron metabolism genes are also powerful prognostic markers in colorectal cancer; for example, elevated expression of thio-redoxin tumor suppressing protein is closely concomitant with iron accumulation in mitochondria[130]. The above findings clearly state that targeting ferroptosis-inducing genes could provide a novel avenue for treating colorectal cancer. p53 tumor-suppressing protein and heme oxygenase have been implicated as key factors in regulating ferroptosis in colorectal cancer.

Ferroptosis in liver cancer

HCC is the most prevalent type of liver cancer, accounting for approximately 90% of cases[131]. Multiple studies have reported that ferroptosis plays a role in ameliorating the burden of HCC progression. Sorafenib, an anticancer agent that is used for the treatment of advanced HCC, has been found to induce ferroptosis by initiating the translation of rapamycin kinase signaling pathway, thereby initiating ferroptosis. Iron chelators such as deferoxamine inhibit sorafenib-induced ferroptosis by reducing the oxidative stress created by sorafenib in HCC cells[132].

The finding of a potent inhibitor of CBS, which is the main source of cystathionine gamma lyase by metabolizing homocysteine to cystathionine to increase intracellular L-cysteine, called CH004, selectively inhibits CBS but not human cystathionine gamma lyase in both *in vivo* and *in vitro* experiments, thereby increasing lipid ROS and decreasing the viability of HepG2 cells, indicating their role in ferroptosis[133].

Modulating the function of key regulators of ferroptosis also plays a significant role in the induction of ferroptotic cell death in HCC cells. Nrf2 is a key regulator of the antioxidant response. It plays a negative regulatory role in ferroptosis by actuating p62-Keap1-Nrf2 pathway, which upregulates the expression of quinone oxidoreductase 1, HO-1, and ferritin heavy chain 1. Therefore, inhibiting the p62-Keap1-Nrf2 pathways by erastin and sorafenib significantly enhances the ferroptosis-mediated cell death of liver cancer cells[134].

The mitochondrial membrane protein CDGSH iron sulfur domain 1 serves as a target to treat diabetes using glitazone, but it acts as a negative regulator of ferroptosis by modulating mitochondrial iron uptake; therefore, the pharmacological or genetic inhibition of CDGSH iron sulfur domain 1 enhanced mitochondrial lipid peroxidation, increasing erastin-induced ferroptosis in liver cancer cells[135].

The highly reactive metabolite NAPQ1 from acetaminophen, an analgesic and antipyretic agent, is involved in the ferroptotic cell death of HepG2 cells by decreasing their viability through GSH depletion and GPX inhibition[136]. The expression of retinoblastoma protein (Rb) in HCC cells determines the susceptibility of cancer cells to sorafenib treatment and regulates ferroptosis. Louandre *et al*[137] showed that the decrease in the levels of Rb protein exhibits an increase in cell death when cells were treated with sorafenib compared with controls; thus, determining the Rb status of HCC patients treated with sorafenib will provide novel insight into the HCC treatment.

LncRNAs represent a vital class of molecules that have regulatory effects in both physiological and abnormal conditions, but lncRNA GABPB1-AS1 has a role in regulating oxidative stress, confirming its involvement in the ferroptosis-mediated cell death of HCC cells. The expression of this lncRNA was upregulated by erastin to inhibit the translation of GA binding protein transcription factor subunit beta 1, resulting in the rapid accumulation of ROS in HepG2 cells by decreasing the expression of the *PRDX5* peroxidase gene[138].

Ferroptosis in pancreatic cancer

Several molecules were demonstrated to induce ferroptosis in pancreatic cancer cells in which the first-line drug gemcitabine is used to treat advanced pancreatic cancer, but HSPA5 causes resistance to gemcitabine treatment by inhibiting ferroptosis. Therefore, genetically or pharmacologically inhibiting HSPA5 enhanced the sensitivity of gemcitabine to pancreatic ductal adenocarcinoma cells by inducing ferroptosis[139]. Inhibiting cytosolic aspartate aminotransferase, which is predominant for redox balance, represses the growth of pancreatic cancer cells by enhancing labile iron release, thereby inducing sensitivity to ferroptosis[140].

Several studies have shown that certain natural plant extracts possess potential anticancer effects by inducing ferroptosis in pancreatic cancer cells. A saponin called ruscogenin represses cell viability and induces ferroptotic cell death in a pancreatic cancer cell line by increasing the concentration of intracellular ferrous iron and ROS production; it also exerts anti-tumor effects in *in vivo* experiments with less toxicity[141].

The combinatorial regimen using plant derivatives also expresses effective anticancer effects in pancreatic cancer cells by inducing ferroptosis; cotylenin A (a plant growth regulator) in combination with phenethyl isothiocyanate, an anti-carcinogenic compound, stimulated ferroptotic cell death in PANC-1 cells (Figure 2)[142]. Artesunate, an antimalarial agent, induced ferroptosis in Kras-activated pancreatic ductal adenocarcinoma cell lines driven by ROS generation and lysosomal iron-dependent cell death without affecting normal pancreatic cell lines[143]. Piperlongumine alone, as well as in combination with cotylenin A and sulfasalazine, generates ferroptotic death of pancreatic cancer cells [144].

Ferroptosis in GC

GC is a heterogeneous disease among GI cancers, with over 1 million new cases worldwide where surgery is the primary treatment to prevent progression[145]. The factors involved in PUFA biosynthesis play an essential role in inducing ferroptosis sensitivity in GC[72]. Apatinib, an anti-tumor agent, decreases the expression of GPX4 and results in apatinib-mediated ferroptotic cell death in GC cells [146]. GC cells resistant to sorafenib-induced ferroptosis treatment by silencing SIRT6, one of the sirtuin proteins that plays a vital role in the regulation of metabolism, DNA repair, and cancer development

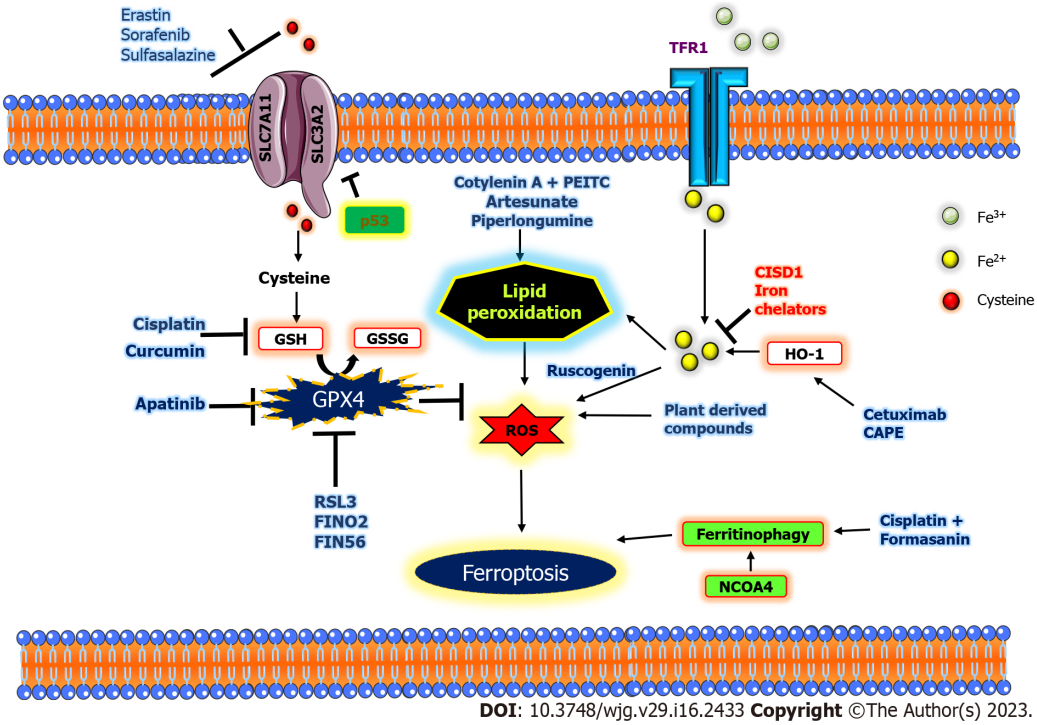

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Figure 2 Regulation of ferroptosis in gastrointestinal tumors is schematically illustrated. The factors cotylenin A, phenethyl isothiocyanate, artesunate, and piperlongumine induce lipid peroxidation and thereby ferroptosis. Small molecules such as erastin, sorafenib, and sulfasalazine inhibit system Xc^- thereby inducing ferroptosis through glutathione peroxidase 4 (GPX4). Cisplatin, curcumin, and apatinib inhibit GPX4 thereby attenuating reactive oxygen species. Several inducers of ferroptosis such as RAS-selective lethal 3, FINO2, and FIN56 inhibit GPX4. Several synthetic and natural compounds regulate ferroptosis through hemoxygenase leading to iron overload. Transferrin receptor 1 mediates endocytosis of iron particles and facilitates ferroptosis. CDGSH iron sulfur domain 1, an iron chelator, blocks iron production and inhibits ferroptosis. Several compounds mediate ferritin degradation *via* ferritinophagy and modulate ferroptosis. CAPE: Caffeic acid phenethyl ester; CISD1: CDGSH iron sulfur domain 1; GSH: Glutathione; GSSG: Glutathione disulfide; HO-1: Heme oxygenase-1; PEITC: Phenethyl isothiocyanate; RSL3: Ras-selective lethal 3; ROS: Reactive oxygen species; SLC3A2: Solute carrier family 3 member 2; SLC7A11: Solute carrier family 7 member 11; TFR1: Transferrin receptor 1.

and is primarily located in the cell nucleus. SIRT6 sensitizes GC cells to sorafenib-induced ferroptosis by the Keap1/Nrf2/GPX4 signaling pathway[125,147]. The ingredient from the Chinese medicine tanshinone IIA, isolated from the rhizome of *Saliva miltiorrhiza Bunge*, exhibits an anticancer effect on GC cells by inducing ferroptosis and downregulating p53-mediated *SLC7A11*[148].

Ferroptosis in esophageal cancer

Targeting ferroptosis will provide new avenues for esophageal cancer diagnostics and treatment strategies. Sulfasalazine, a ferroptotic inducer, inhibits the progression of esophageal cancer, and plant-derived compounds such as oridonin, a diterpenoid, have also been reported to stimulate ferroptotic cell death in esophageal cancer cells[149].

FERROPTOSIS AND LINK TO OTHER RCD PATHWAYS

Ferroptosis is intricately intertwined with other forms of cell death through various iron and lipid peroxidation proteins and several transcription factors. However, molecular mechanisms underlying the role of other forms of RCD remain poorly understood. For example, *TP53*, a critical mediator of tumor suppressive response involved in apoptosis, ferroptosis, and antioxidant response machinery like Nrf2, has a significant role in ferroptosis as well as autophagy. The molecular mechanisms associated with other forms of RCD pathways and ferroptosis are discussed herein.

Apoptosis and ferroptosis: Molecular switch

Numerous studies have reported the interconnection between apoptosis and ferroptosis. Apoptosis, typically through p53, induces cell cycle arrest, thereby preventing tumorigenesis; likewise, *TP53* is known to sensitize cells to ferroptosis, leading to a reduced tumor burden[150]. The widely known ferroptosis inducer erastin has the potential to induce the unfolded protein response and promote p53 expression through apoptotic markers PUMA, CHOP, and TRAIL, and TRAIL-dependent apoptosis implies an augmented link between apoptosis and ferroptosis[151]. A similar study on a metal-

encapsulated p53 plasmid construct by Zheng *et al*[152] was found to release ions of iron, instigating the Fenton reaction and leading to ROS oxyradical overload, thereby leading to ferroptosis-dependent apoptosis in the liver. This consequently reduced the tumor burden and prevented metastasis in mice. An imbalance in the ferroptotic process is implicated in severely hindering apoptosis induction; for example, cancer cells subjected to ferroptosis by cysteine starvation were found to have reduced GSH levels but failed to induce caspase activation, which is seminal in apoptosis[153].

Autophagy-dependent ferroptosis

Autophagy-dependent ferroptosis is putatively ferritinophagy; under an excessive Fe^{2+} milieu, ferritin degradation is mediated by Atg5, an autophagy regulator protein. Ferritin is a seminal protein complex with light and heavy chain polypeptides (ferritin light chain 1 and ferritin heavy chain 2) predominantly controlling iron metabolism. Atg5 and Atg7 knockdown is implicated in preventing erastin-induced ferroptosis, facilitating tumorigenesis[154]. Similarly, BECN1 or Atg6 is known to induce ferroptosis by regulating glutamine and cysteine and inhibiting system Xc^- through the BECN1-*SLC7A11* complex in cancer cells. Additionally, studies have reported BECN1 facilitates lipid peroxidation through malondialdehyde (MDA) stress modulation[155]. Observations from our laboratory demonstrated that Eupatilin exhibits anticancer effects in part through regulation of autophagy-mediated ferroptosis (data not shown).

In summary, while apoptosis, ferroptosis, and autophagy are all different cellular pathways, they can be linked in the sense that autophagy can play dual roles by promoting apoptosis and protecting against ferroptosis. In contrast, both apoptosis and ferroptosis are forms of RCD mediated by different enzymes and signals that manifest different morphological outcomes.

THERAPEUTIC ASPECTS OF FERROPTOSIS

The listed drugs have potential therapeutic applications and have been reported to regulate ferroptosis (Table 2)[156-164].

Although conventional drugs are implicated in inducing or modulating ferroptosis, resistance to these drugs supersedes the benefits. Therefore, the identification of compounds with neutral toxicity profiles and natural origins has garnered tremendous attention in ferroptosis and iron metabolism. The section below briefly discussed the role of natural compounds in ferroptosis.

Formasanin C (FC) is known to induce ferroptosis in p53-null and p53 wildtype cellular phenotypes of HCC, and FC treatment increases the mitochondrial morphology and membrane potential in HepG2 cells, as a hallmark of cells undergoing ferroptosis[165]. FC and cisplatin synergistic treatment is known to induce ferritinophagy and enhance therapeutic potential of cisplatin. Similarly, gallic acid (GA) is known to prompt lipid peroxidation and ferroptosis. Upon exposure to an Fe^{2+} chelator, GA activity is suppressed, which in part signifies its role in ferroptosis. GA exhibited anti-tumor effects in colorectal cancer by deterring GPX4 and elevating MDA expression[166]. Celastrol, a tri-terpenoid is reported to induce ROS production, thereby promoting ferroptosis in liver cancer cells. Structural protein activity revealed that celastrol directly binds to multiple orthologues of PDXs. PDX knockdown in turn elevates ROS production, ensuing ferroptosis[167]. Chen *et al*[168] reported that curcumin could induce ferroptosis in colorectal cancer by modulating expression of key ferroptotic markers Fer1, *SLC7A11*, GSH, MDA, and ROS through PI3K/mTOR pathway.

The mechanisms orchestrating ferroptosis has been the subject of interest in several cancers. Many investigators have contributed to identifying key molecules that regulate ferroptosis in GI tumors[169]. The above findings are helpful in understanding the mechanism of many synthetic and natural compounds in inducing iron-dependent cell death in GI cancers. There are ample avenues to further elucidate the mode of action and mechanistic aspects of how natural compounds could be synergistically used to induce ferroptosis.

CONCLUSION

The increasing incidence and mortality imposed by GI cancers, a dangerous malignancy, warrants novel therapeutic strategies. Ferroptosis, a form of non-apoptotic RCD, has been found to play a significant role in regulating the progression of GI cancers. This review delineated the major regulatory mechanisms involved in ferroptosis for better understanding to create a new opportunity for diagnosis and therapeutic intervention. The involvement of ferroptosis-associated factors and the effect of several drugs, including the first discovered ferroptotic inducers erastin, sorafenib, cisplatin, artesunate, piperlongumine, haloperidol, baicalein, bromelain, and saponins have been found to induce ferroptotic cancer cell death in GI cancers. Ferroptotic inducers synergized with various anticancer drugs in clinical trials have demonstrated effective therapeutic results in GI cancers. Thus, inducing ferroptosis may have significant potential for treating GI cancers and related malignancies. Overall, this review provides insights into the regulatory mechanisms involved in ferroptotic cell death for development of novel

Table 2 Conventional drugs and their mode of action in ferroptosis

GI cancer	Drugs	Pathway involved	Mode of action	Ref.
Colorectal cancer	Paclitaxel	p53, *SLC7A11*	Lipid peroxidation by suppressing GPX4 expression	[156]
	5-fluorouracil	Lipocalin2 (LCN2), xCT	Stimulates GPX4 expression by diminishing circulating iron levels	[157]
	Cisplatin	Ferrostatin-1	Conjugates with GPX and GSH modulation	[158]
	Cetuximab	GSH, GPX4, HO-1, *SLC7A11*, KRAS, RSL3	Through β-elemene synergism by inducing iron dependent ROS, GSH accumulation, and EMT regulation	[159]
	Dihydro-artemisinin (DAT)	Iron metabolism, GPX4	Sensitizes cells to ferroptosis by iron overload and lysosomal degradation	[160]
Hepatocellular carcinoma	Artesunate	Ferritin	Elevates lysosomal degradation partially through autophagy along with sorafenib; it induces cathepsin activation	[161]
	Sorafenib	Nrf2	Through metallothionein-1 activation (MT-1G–Nrf2) axis	[162,125]
	Deferoxamine	Iron metabolism	Through peroxidation and iron storage depletion	[163]
Gastric cancer	Cisplatin	Nrf2/Keap1-xCT	Through transcription factor ATF3 overexpression	[164]

ATF3: Activating transcription factor 3; EMT: Epithelial-mesenchymal transition; GI: Gastrointestinal; GPX4: Glutathione peroxidase 4; GSH: Glutathione; HO-1: Heme oxygenase-1; Keap1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid 2–related factor 2; ROS: Reactive oxygen species; RSL3: Ras-selective lethal 3; *SLC7A11:* Solute carrier family 7 member 11.

therapeutic strategies. The mechanism of ferroptosis with other RCD, such as autophagy and apoptosis, to induce cancer cell death may also provide new development in the therapeutic aspects of treating GI tumors. Therefore, further investigation of ferroptosis in GI cancers will improve the prognosis and therapeutic aspects of GI cancers.

FOOTNOTES

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Country/Territory of origin: India

ORCID number: Yahya Showket 0000-0001-6509-7032; Ganapasam Sudhandiran 0000-0003-0066-7801.

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REVIEW

Updates on global epidemiology, risk and prognostic factors of gastric cancer

Wen-Juan Yang, He-Ping Zhao, Yan Yu, Ji-Han Wang, Lei Guo, Jun-Ye Liu, Jie Pu, Jing Lv

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Wen-Juan Yang, He-Ping Zhao, Yan Yu, Jun-Ye Liu, Jing Lv, Department of Clinical Laboratory, Honghui Hospital, Xi'an Jiaotong University, Xi'an 710054, Shaanxi Province, China

Ji-Han Wang, Institute of Medical Research, Northwestern Polytechnical University, Xi'an 710072, Shaanxi Province, China

Lei Guo, Department of Spinal Surgery, Honghui Hospital, Xi'an Jiaotong University, Xi'an 710054, Shaanxi Province, China

Jie Pu, Department of Cardiology, Shaanxi Provincial People's Hospital, Xi'an 710068, Shaanxi Province, China

Corresponding author: Jing Lv, MD, Doctor, Department of Clinical Laboratory, Honghui Hospital, Xi'an Jiaotong University, No. 555 Youyi Dong Road, Xi'an 710054, Shaanxi Province, China. lvjing-1219@163.com

Abstract

Gastric cancer (GC) is defined as the primary epithelial malignancy derived from the stomach, and it is a complicated and heterogeneous disease with multiple risk factors. Despite its overall declining trend of incidence and mortality in various countries over the past few decades, GC remains the fifth most common malignancy and the fourth leading cause of cancer-related death globally. Although the global burden of GC has shown a significant downward trend, it remains severe in certain areas, such as Asia. GC ranks third in incidence and mortality among all cancer types in China, and it accounts for nearly 44.0% and 48.6% of new GC cases and GC-related deaths in the world, respectively. The regional differences in GC incidence and mortality are obvious, and annual new cases and deaths are increasing rapidly in some developing regions. Therefore, early preventive and screening strategies for GC are urgently needed. The clinical efficacies of conventional treatments for GC are limited, and the developing understanding of GC pathogenesis has increased the demand for new therapeutic regimens, including immune checkpoint inhibitors, cell immunotherapy and cancer vaccines. The present review describes the epidemiology of GC worldwide, especially in China, summarizes its risk and prognostic factors, and focuses on novel immunotherapies to develop therapeutic strategies for the management of GC patients.

Key Words: Gastric cancer; Epidemiology; Risk factors; Prognosis; Treatment;

Immunotherapy

Core Tip: As a malignant disease with decreasing trends in incidence and mortality, gastric cancer (GC) remains a public health issue worldwide. Various risk factors have been suggested, and the prognosis of GC is related to various factors, such as tumor location, lymph node metastasis, gene polymorphisms and therapeutic strategies. Therefore, novel treatments have been proposed, and immunotherapy has attracted more attention. The present review discusses the epidemiology, risk and prognostic factors of GC with a focus on immunotherapy to better inform the management of GC patients.

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INTRODUCTION

Despite its global declines in incidence and mortality over the past several decades, gastric cancer (GC) remains responsible for 1.089 million new cancer cases and 0.769 million deaths in 2020 worldwide, which makes it the fifth-most common malignancy and the fourth leading cause of cancer-related deaths, according to Global Cancer Statistics (GLOBOCAN) 2020[1]. The global age-standardized incidence and mortality rates for GC were 11.1/100000 and 7.7/100000 in 2020, with wide geographical variations[2]. These data highlight that GC remains a major global health challenge. As a primary epithelial malignancy derived from the stomach, the initiation of GC is a multistage process and is generally associated with various risk factors[3], and some elements are related to its prognosis and survival[4,5]. Thanks to advances in preventative, screening and therapeutic strategies, the incidence and mortality of GC has been decreasing gradually worldwide. However, certain challenges still exist in the management of GC, such as the clinical applications of surgical treatment and chemotherapy. Recent immunotherapy for GC has drawn much attention, and it improved the current therapeutic situation. The present review describes the epidemiology of GC in different regions in the world, especially in China, summarizes its risk and prognostic factors, and focuses on new immunotherapies to develop therapeutic strategies for the management of GC patients.

GLOBAL EPIDEMIOLOGY OF GC

Incidence and mortality rates of GC around the world

The GLOBOCAN 2020 database (https://gco.iarc.fr/)[1] estimated that there were 1089103 newly diagnosed GC cases in 185 countries, with GC ranking fifth in incidence among all cancer types globally (Figure 1A). According to the anatomical locations, GC is classified as cardia GC and noncardia GC with different epidemiological profiles[1], and noncardia GC is the most common subtype[6]. There is a significant difference in GC incidence in sex distribution, and the age-standardized incidence rate (ASIR) is 15.8/100000 in males and 7.0/100000 in females, which indicates that GC incidence is approximately 2-fold higher in males than females[1]. GC incidence ranked fourth in males and seventh in females among all cancer types[1] (Figure 1A). Geographic variations in the ASIR in GC are up to 1- to 4-fold worldwide[7]. The ASIR is highest in Asia (14.3/100000), followed by Latin America and the Caribbean, Europe and Oceania, and it is lowest in Africa and North America[7] (Figure 2A). Most GC cases are diagnosed in countries with a high and very high human development index (HDI), such as eastern and southeastern Asian countries, central and eastern European and South American countries, and the ASIRs in these countries were higher than countries with a medium and low HDI[2] (Figure 2B). The five countries with the highest ASIRs in Asia were Mongolia (32.5/100000), Japan (31.6/100000), Republic of Korea (27.9/100000), Tajikistan (23.4/100000) and China (20.6/100000) (Figure 3A), which indicated that greater than 69% of the total GC cases in 2020 occurred in eastern and south-central Asia. In addition, Figure 3B and C show the detailed information about the male and female ASIRs in Asia.

GC is the fourth most common cause of mortality among all cancer types, followed by lung, colorectal and liver cancers[1] (Figure 1B). A total of 768793 deaths were estimated to be related to GC, with an overall mortality of 7.7/100000 globally, and sex differences exist with males being twice as likely as females to exhibit the disease (Figure 1B)[1]. The age-standardized mortality rate (ASMR) of GC was

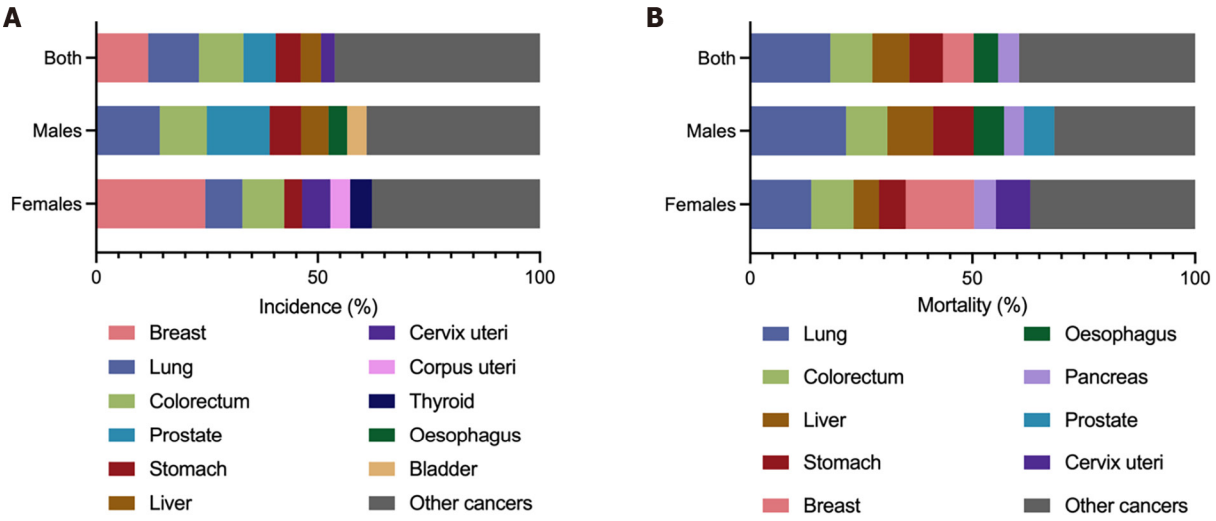

Figure 1 The composition of incidences and mortalities of all cancer types in 2020 globally. A: The composition of incidences of all cancer types in 2020 globally; B: The composition of mortalities of all cancer types in 2020 globally. Bar plots show the composition of incidences or mortalities of all cancer types in both sexes, males and females, respectively. Citation: Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; 71: 209-249. Copyright ©International Agency for Research on Cancer 2020. Published by International Agency for Research on Cancer[1].

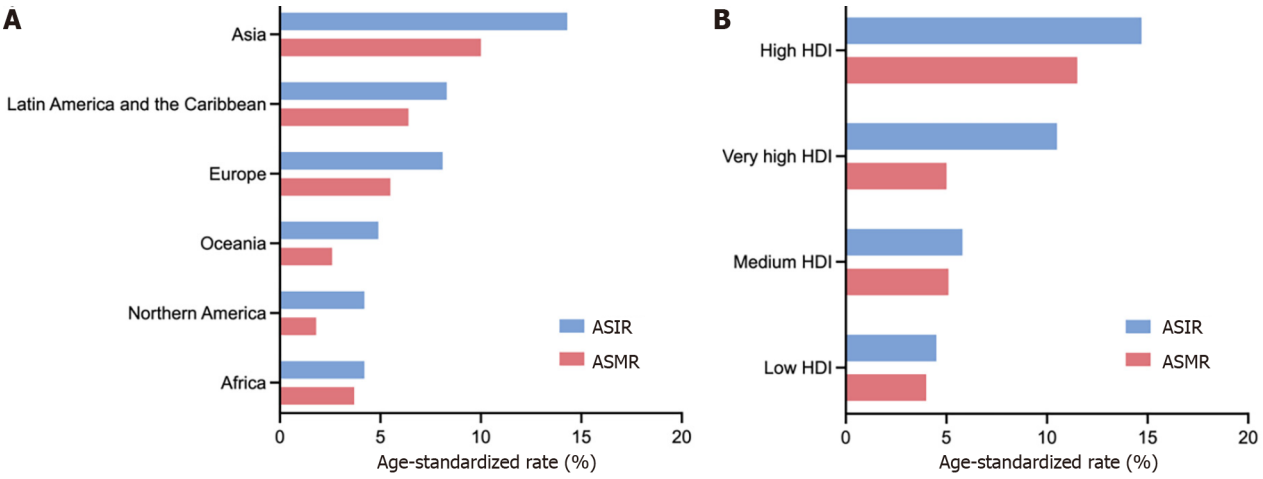

Figure 2 Age-standardized incidence and mortality rates of gastric cancer in 2020 worldwide. A: Age-standardized incidence and mortality rates of gastric cancer (GC) in 2020 in the five continents; B: Age-standardized incidence and mortality rates of GC in countries classified by human development index in 2020 worldwide. ASIR: Age-standardized incidence rate (1/100000); ASMR: Age-standardized mortality rate (1/100000); HDI: Human development index. Citation: Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; 71: 209-249. Copyright ©International Agency for Research on Cancer 2020. Published by International Agency for Research on Cancer[1].

highest in Asia (10.0/100000) (Figure 2A). Countries with a very high HDI have higher mortality rates, and countries with a medium and low HDI have lower mortality rates, which is consistent with GC incidence (Figure 2B). The five Asian countries with the highest ASMRs were Mongolia (24.6/100000), Tajikistan (19.7/100000), China (15.9/100000), Bhutan (15.9/100000) and Kyrgyzstan (15.7/100000) (Figure 3D), and the male and female ASMRs in Asia are shown in Figure 3E and F, respectively. Mongolia has the highest incidence and mortality rates, primarily due to the lack of endoscopy and professional endoscopists[8].

The overall GC incidence and mortality rates have steadily declined in most countries during the past several decades, with evident decreases in males and females[1,9-13], as preventative, screening and therapeutic programs have been implemented worldwide[14-16]. For example, the ASIR of GC in Korea decreased significantly from 2011 (ASIR 43.0) to 2019 (ASIR 29.6)[17]. Similar to most other cancers, GC is generally rare in adults aged < 50 years, and its incidence increases with aging[9,10]. However, GC incidence has presented an increasing trend in younger generations (below age 50 years) in high- and low-incidence areas, such as the United States and the United Kingdom, compared to older individuals who exhibited a decreasing trend in GC incidence[9,10,18]. One United States study reported a more

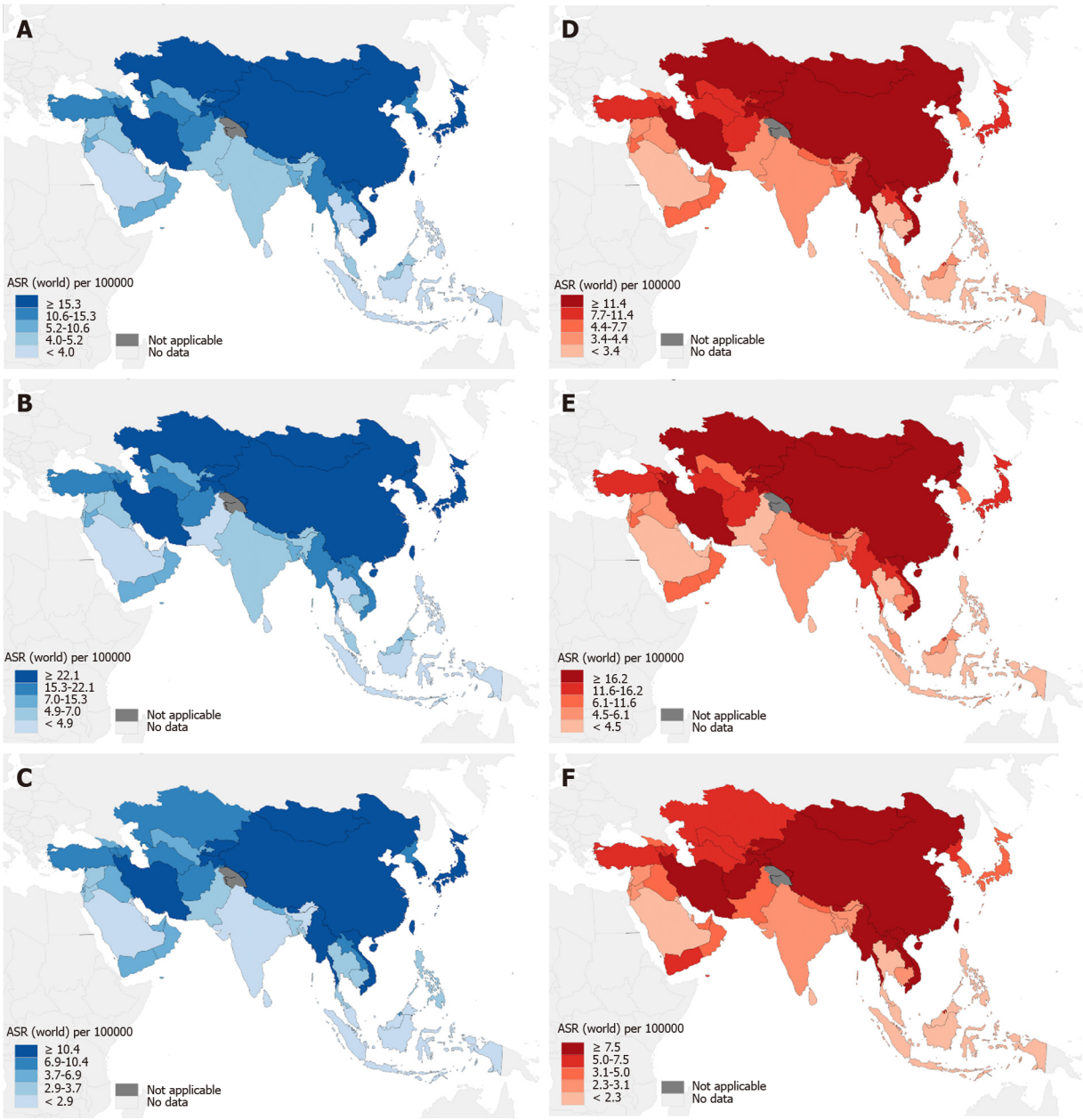

Figure 3 Estimated age-standardized incidence and mortality rates of gastric cancer in 2020 in Asian countries. A: Estimated age-standardized incidence rates of gastric cancer (GC) in 2020 in Asian countries; B: Estimated age-standardized incidence rates of GC in males in 2020 in Asian countries; C: Estimated age-standardized incidence rates of GC in females in 2020 in Asian countries; D: Estimated age-standardized mortality rates of GC in 2020 in Asian countries; E: Estimated age-standardized mortality rates of GC in males in 2020 in Asian countries; F: Estimated age-standardized mortality rates of GC in females in 2020 in Asian countries. ASIR: Age-standardized incidence rate (1/100000); ASMR: Age-standardized mortality rate (1/100000). Citation: Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; 71: 209-249. Copyright ©International Agency for Research on Cancer 2020. Published by International Agency for Research on Cancer[1].

pronounced increase in incidence in younger females than males and predicted that the overall incidence may no longer be decreasing, and the GC incidence in females may exceed males if this pattern continues[18]. The 5-year overall survival (OS) rate shows that GC survival has improved due to advances in diagnostic and therapeutic strategies, especially with early detection from national screening programs using endoscopic and/or radiographic methods[9,19]. For example, one study found that the 5-year survival rate of GC in Korea increased from 55.7% in 1999-2005 to 77% in 2013-2019[17], which is consistent with the previous cancer statistics in Korea in 2015[20]. However, GC maintains a high case fatality rate, and it is a main contributor to the global burden[21].

Epidemiology of GC in China

GC is also one of the major malignances in China. The ASIR and ASMR of GC were higher in 2012 (ASIR 22.06/100000, ASMR 15.16/100000) than 2016 (ASIR 17.59/100000, ASMR 12.30/100000), with a

decreasing trend from 2012 to 2016 and decreasing trends in males and females[22-26] (Figure 4). The National Cancer Center of China reported 396500 new GC cases and 288500 GC-leading deaths in China in 2016[26], and the ASIR and ASMR of GC ranked fifth (17.59/100000) and third (12.30/100000) among all cancer types, respectively[26,27]. The number of new GC cases in males was nearly 276300 (ASIR 25.14/100000), and GC-related deaths (200200, ASMR 17.77/100000) accounted for approximately 13% of all male cancer-related deaths. The number of new GC cases in females was approximately 120200 (ASIR 10.31/100000), and GC-related deaths (88400, ASMR 7.13/100000) accounted for approximately 10% of all female cancer-related deaths[26]. GLOBOCAN 2020 indicated 479000 new GC cases and 374000 GC-related deaths in China in 2020, which ranked third in incidence and mortality among all cancer types[28] and accounted for 44.0% and 48.6% of new GC cases and GC-related deaths worldwide, respectively[1]. Current data in China (https://gco.iarc.fr/) showed that the ASIR and ASMR of GC were greater than 2-fold in males (ASIR 29.5/100000, ASMR 22.8/100000) than females (ASIR 12.3/100000, ASMR 9.5/100000) in 2022, and more GC cases and deaths occurred in patients over 60 years. Notably, geographic variations also exist in different areas of China. Specifically, the ASIRs and ASMRs of GC in urban areas were higher than rural areas[26]. The ASIR and ASMR were highest in northwestern China (ASIR 25.8/100000, ASMR 18.1/100000), followed by eastern (ASIR 21.9/100000, ASMR 14.8/100000) and central (ASIR 18.7/100000, ASMR 13.8/100000) areas of China, and southern China had the lowest ASIR of 9.2/100000 and ASMR of 6.4/100000[26].

Because the early symptoms of GC are insidious, most GC patients are metastatic in the advanced stage at the time of diagnosis[6]. The proportion of GC patients diagnosed in the early stage was lower than advanced GC patients in China in the past[28]. However, the survival time is closely related to the stage at diagnosis for GC patients[19]. Thereafter, China proposed a series of guidelines aimed at improving GC screening, early detection and therapeutic strategies based on the popularity of gastrointestinal endoscopy, and the proportion of early GCs increased in recent years[28,29]. The 5-year OS rate of GC increased from 27.4% in 2003-2005 to 35.1% in 2012-2015 with an ascending trend in China[29], but it is significantly lower than Japan (81.0%) in 2004-2007[19] and South Korea (75.4%) in 2011-2015[20], which may be related to the different preventive, early screening, diagnostic and therapeutic strategies in individual countries[1].

RISK FACTORS

The pathogenesis of GC is complicated, and more attention should be given to individuals with higher GC risks for surveillance. Various factors may synthetically affect GC occurrence and development (Figure 5). Some risk factors are nonmodifiable, such as age, sex, race/ethnicity and genetics[6,28,30,31], and other controllable risk factors may include *Helicobacter pylori* (*H. pylori*) infection, gastrointestinal microbiota, obesity, unhealthy dietary habits and lifestyle, tobacco and alcohol consumption, and chemical, radiation or virus exposure[6,16,28,32-34]. Several relatively rare risk factors may also participate in GC pathogenesis, such as gastroesophageal reflux disease, gastric ulcer or previous gastric surgery[30].

One study reported that 1.8% of GC cases occurred in individuals younger than 34 years, 38.6% occurred in adults between 35 and 64 years old, and 59.6% occurred in elderly individuals over 65 years from 2015 to 2019[35], with a median age at diagnosis of 68 years, which indicate that GC risk increases with aging. Sex differences exist in GC incidence, which is almost twofold higher in males than females [1,35]. These data suggest the protective effect of sex steroid hormones in GC pathogenesis[36]. Males tend to have higher risks of *H. pylori* infection than females, which may also lead to sex differences[37,38]. Approximately 10% of GC cases exhibited familial aggregation, which indicates that a family history of GC may be an independent risk factor[39,40]. A total of 1%-3% of GC patients may have germline mutations, and the underlying molecular mechanisms have not been fully clarified[41].

Chronic *H. pylori* infection is the major confirmed cause of GC, and it may be related to approximately 90% of noncardia GC cases[10,42-44]. *H. pylori* is a Gram-negative pathogenic bacterium and an indigenous member of the gastric microbiota[18,45]. The prevalence of *H. pylori* infection in adults exceeds 50% of the human population with regional variations globally[1,45,46]. *H. pylori* infection is easily acquired during childhood[47], and it is generally carried asymptomatically for a lifetime. Since 1994, *H. pylori* has been classified as a class I carcinogen by the World Health Organization. The long-term colonization of *H. pylori* in the gastric mucosa contributes to the development of various gastric diseases, such as persistent inflammation, chronic gastritis, gastric mucosal atrophy and intestinal metaplasia, with its different genes encoding virulence factors[45,48]. Chronic *H. pylori* infection also induces epigenetic and genetic changes in gastric epithelial cells, which suggests the genetic instability of these cells[48]. Therefore, *H. pylori* infection is etiologically related to GC, and the duration also predisposes individuals toward GC later in life[48]. Because *H. pylori* infection is closely related to noncardia GC, its eradication significantly decreased the incidence of noncardia GC[48]. However, *H. pylori* infection is only necessary, but not sufficient, in the pathogenesis of GC[48]. Although controversial[18], *H. pylori* screening and eradication has been proposed as a preventive strategy for GC[48]. Data from healthy asymptomatic infected Asians showed that eradicating *H. pylori* reduced GC

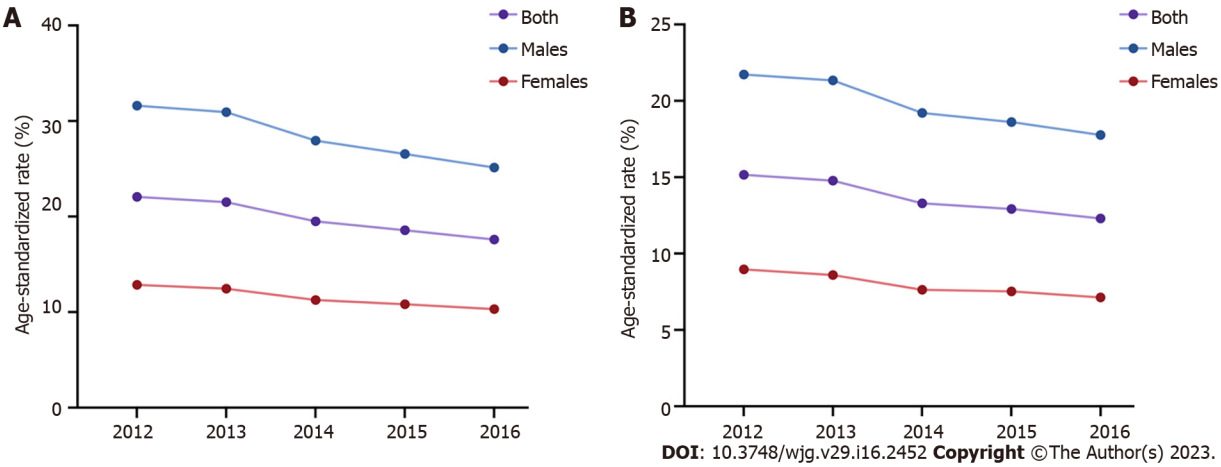

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Figure 4 Trends in age-standardized incidence and mortality rates of gastric cancer according to the National Central Cancer Registry of China in 2012-2016[22-26]. A: Trends in age-standardized incidence rates of gastric cancer (GC) according to the National Central Cancer Registry (NCCR) of China in 2012-2016; B: Trends in age-standardized mortality rates of GC according to the NCCR of China in 2012-2016.

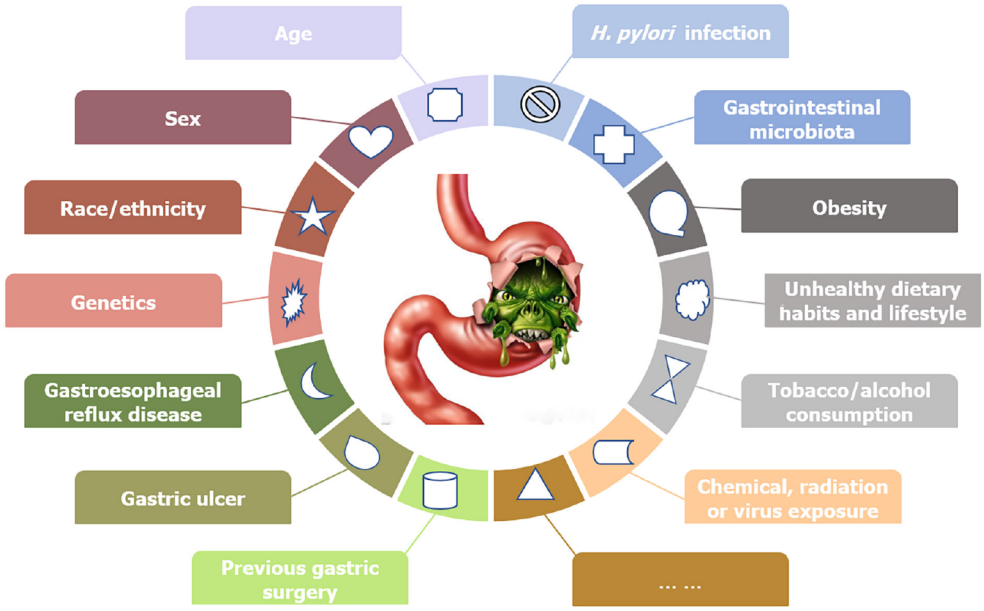

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Figure 5 Summarization of risk factors of gastric cancer. *H. pylori*: *Helicobacter pylori*.

incidence[49], and population-based screening and eradication of *H. pylori* infection was cost-effective [50]. The efficacy of eradicating *H. pylori* to prevent GC also depends on other risk factors, such as the time of *H. pylori* eradication, intragastric acidity, resistance to antimicrobial agents and the compliance of infected patients[48,51,52]. In contrast, a similar screening strategy may not be economical and is generally unwarranted in some countries with low GC incidences[2]. Anderson *et al*[18] once indicated that population-based *H. pylori* eradication in the United States may raise certain unanswered questions about safety, efficacy, unanticipated consequences and failure to reduce the GC burden. Less than 5% of *H. pylori*-infected individuals may develop GC due to the genetics of *H. pylori* and the host, duration of *H. pylori* infection and certain environmental factors[1,53]. The efficacy of *H. pylori* eradication and its cost-effectiveness should be further investigated in different geographic regions[48].

With the progression of molecular biological technologies, such as next-generation sequencing, a series of studies examined the correlations between GC and gastric microbiota other than *H. pylori*[54, 55]. Ferreira *et al*[56] performed 16S rRNA gene sequencing analysis of gastric microbiota for 54 GC patients and 81 gastritis controls, and found that the gastric microbiota of GC had reduced microbial diversities, reduced abundance of *H. pylori*, and the increment of other microbial genera that were similar to intestinal microbiota; besides, the nitrosating functions and genotoxic potential were also increased. He *et al*[57] investigated the characteristics of the intestinal microbiota in fecal samples from GC patients and healthy individuals using 16S rRNA gene sequencing technology, and showed that the

relative abundances of *Faecalibacterium*, *Bifidobacterium*, *Subdoligranulum*, *Enterococcus*, *Streptococcus* and *Bacteroides* were closely associated with GC risk and occurrence. Aziz *et al*[58] found four types of microbial proteins in serum and tissue biopsy specimens of GC patients, including *Acinetobacter baumannii*, *Escherichia coli*, *Fusobacterium nucleatum* and *Bacteroides fragilis*, as well as *H. pylori*. Overall, microbiota-related GC studies indicated an alteration of the gastric microbiota during gastric carcinogenesis, which was distinct from patients with chronic gastritis and healthy individuals[59]. Therefore, it is necessary to further analyze the gastric microorganisms and explore the possible underlying pathogenesis of microbial dysbiosis in the progression to carcinoma to provide guidance for preventative, screening and therapeutic strategies for GC.

The diverse risk and protective factors of GC are dietary or lifestyle-related factors[60], and unhealthy dietary habits and lifestyle factors may account for 33%-50% of all GC cases[10,61]. Lower intake of fruit and vegetables, higher intake of salt or salted/processed food, and tobacco and alcohol consumption are GC risk factors[60,62,63]. For example, a 5 g/day increase in salt intake increased the risk of GC by 12% [53]. Excessive salt intake may destroy the gastric mucosa and increase DNA synthesis and cell proliferation to promote the development of GC[64], and excessive salt intake also acts synergistically with *H. pylori* to increase GC incidence[60,65,66]. However, no scientific evidence has confirmed a definite causal association between excessive salt intake and GC risk[60]. Tobacco consumption is also an important behavioral risk factor for GC and may increase GC risk by approximately 50% in males and 20% in females according to relevant data[32,67,68]. Tobacco consumption may induced chronic inflammation in the gastrointestinal tract, alter mucosal cell proliferation, promote immune dysfunction, and increase the risks of bacterial or viral infections, which leads to the carcinogenesis of GC[69]. Alcohol consumption also positively correlates with GC risk[69-71]. These two factors, tobacco and alcohol consumption, affect GC development independently in high-risk populations, and modification of these unhealthy choices may significantly reduce the incidence and mortality of GC[72]. In contrast, increased fresh fruit and vegetable consumption may inhibit GC development and reduce its risk[53]. Specifically, a systematic review and dose-response meta-analysis found that a 100 g/day increase in fruit consumption inversely associated with a 5% reduction in GC risk[53]. Another meta-analysis also indicated that the relationship between intake of citrus fruit and risk of cardia GC was statistically significant, and daily intake of 100 g citrus fruit reduced GC risk by 40%[73], which suggests that phytochemicals in fruit may have antioxidant effects, prevent or reduce DNA oxidation, and regulate cell proliferation and apoptosis[74]. The history of medication may affect GC pathogenesis, and the use of aspirin and other nonsteroidal anti-inflammatory drugs may lower GC risk[75].

In summary, various known and unknown factors may be related to GC risk, and understanding the underlying mechanisms will facilitate appropriate preventative and screening strategies to reduce GC incidence.

PROGNOSTIC FACTORS

Patient- and tumor-related prognostic factors

Although the incidence of GC in males is significantly higher than females[1], few studies have focused on sex differences in GC prognosis. The relationships between race, gene polymorphism and GC prognosis have been explored in many studies[76,77]. Current data show high GC incidences in Asian populations and better GC prognosis in Asian GC patients than Caucasian populations, even after controlling for other well-known prognostic factors[78-80]. However, whether differences exist due to different management strategies or distinct races and tumor biology is not clear. Increasing evidence indicates that genetic polymorphisms are associated with GC survival, and single nucleotide polymorphisms (SNPs) are novel biomarkers of cancer susceptibility, progression and prognosis[81,82]. For example, Gonzalez-Hormazabal *et al*[83] found that allele A carriers of IL-8 rs4073 were associated with lower OS in GC. The role of ERCC1 SNPs was also extensively studied in patients with gastrointestinal tumors receiving oxaliplatin-based chemotherapy, and ERCC1 rs11615 polymorphisms were closely associated with the clinical outcomes of GC[84]. Wang *et al*[85] showed that lncRNA H19 rs2839698 was also related to the OS of GC patients. However, more prospective studies with larger sample sizes are needed to verify the above conclusions and elucidate the underlying mechanisms of SNPs in GC prognosis.

The Lauren classification[86] is globally recognized as the classification system for GC. According to the Lauren classification[86], GC is classified into intestinal and diffuse types, and diffuse GC generally has a poor prognosis compared to intestinal GC[87-89]. The survival of GC is strongly related to the stage at diagnosis. For patients with early GC, the cancerous area is localized with no local or distant metastasis, and these patients generally have a better prognosis than patients with advanced GC[4]. The cancer site in advanced GC patients is not localized and generally accompanied by metastasis[90]. GC easily recurs even after surgical resection, and it generally has a poor prognosis[91]. Tumor size, depth of invasion, lymph node metastasis (LNM), and tumor-node-metastasis stage are important prognostic factors[5]. For example, the 5-year survival rate of patients with proximal GC is lower than patients with distal GC, which indicated the correlation of tumor location and GC prognosis[5]. Notably, a Chinese

study[92] recruited 611 patients with early GC and showed no significant difference in 5-year survival between mucosal and submucosal cancers. Notably, LNM may be a significant prognostic factor for early GC[92,93], and the 5-year survival rate may be twice as high in patients without LNM than patients with LNM[94].

Treatment-related prognostic factors

A variety of factors affect GC prognosis, including patient-related factors (gender, age, race), tumor-related factors (tumor location, histological type, depth of invasion, and metastasis) and treatment-related factors. We primarily focused on the prognostic factors related to GC treatment.

Surgery is the basis of GC treatment, and it is the only procedure that completely eradicates GC lesions[3,91,95]. Surgical options primarily include endoscopic mucosal resection, distal esophagectomy, subtotal gastrectomy or total gastrectomy[96]. The surgical strategies depend on the tumor location and invasion depth, and may vary in different institutions. For early GC with a low LNM risk, endoscopic treatment or surgery alone may be effective, and patients with advanced GC may benefit from broad lymph node dissection and multimodal therapies[97]. In terms of the extent of lymph node dissection (D), current data indicate that D2 lymph node dissection is the most recommended surgical procedure for advanced GC, and GC patients may have a higher 5-year survival rate after D2 lymph node dissection than after D1 dissection[98]. Accurate preoperative staging and resection evaluation are key factors in successful surgery for GC[95]. The precision medicine of surgical strategies for GC, by the concept of accelerated rehabilitation, includes minimally invasive surgery, precise operation of intraoperative fluorescence navigation and precise perioperative management[95]. Compared to traditional surgery, minimally invasive surgery, including laparoscopy and da Vinci robot-assisted surgery, may reduce surgical trauma and improve recovery after surgery[99]. Although no significant differences in postoperative outcomes were found between laparoscopic and robotic gastrectomies, robotic gastrectomy may require a longer surgical duration and greater financial cost, and is not superior to laparoscopic procedures in perioperative surgical outcomes[99]. Patients with advanced GC need lymph node dissection, which is difficult and complicated. Therefore, whether laparoscopy is suitable for these patients needs further evaluation[95].

Because most GC patients are in advanced stages at initial diagnosis, surgery alone may not be sufficient to treat this malignancy, and therapeutic methods other than surgery should also be performed to improve the survival of operable GC patients[95]. As another important conventional treatment, chemotherapy is classified as perioperative, neoadjuvant or adjuvant, and palliative chemotherapy[28]. The OS of GC patients was significantly improved with perioperative chemotherapy compared to GC patients after surgical resection alone, and perioperative chemotherapy also increased the OS compared to postoperative chemotherapy[100,101]. Specifically, there are several combined options for chemotherapy strategies[28,102,103], including neoadjuvant triple chemotherapy, such as docetaxel, oxaliplatin and S-1 (DOS) or Epirubicin, cisplatin and 5-fluorouracil (ECF), and double chemotherapy, such as capecitabine and oxaliplatin (XELOX), tegafur and cisplatin (SP) or 5-fluorouracil and oxaliplatin (FOLFOX). The ToGA trial[104] showed that trastuzumab in combination with chemotherapy improved survival for HER2-positive GC patients, and an anti-HER2 targeting strategy was proposed as a standard option for HER2-positive GC patients. For example, trastuzumab plus XELOX, trastuzumab in combination with capecitabine, or bevacizumab and trastuzumab combined with docetaxel, oxaliplatin and capecitabine achieved encouraging efficacy and safety for patients with HER2-positive advanced GC, and may be considered first-line treatments for these patients, but further evaluation is warranted[105-107]. However, trastuzumab combined with DOS showed less effectiveness than trastuzumab in combination with XELOX, and further investigation is ongoing[108]. The use of radiotherapy in GC treatment has become more common with the advancement of radiotherapy-related technology[109]. Radiotherapy is generally combined with surgery, chemotherapy, molecular targeted therapy or other treatments, and these combinations eventually benefit GC patients[28]. Radiotherapy may be used as an important adjuvant therapy in the perioperative period for patients with advanced operable GC, especially for some patients after D2 Lymphadenectomy, and it effectively improved progression-free survival time and reduced the local recurrence rate[109]. Notably, perioperative chemotherapy or postoperative chemotherapy plus radiotherapy are listed as preferred strategies in certain guidelines[30]. For GC patients in the early-stage with LNM, OS may be improved after the adoption of adjuvant chemotherapy and radiotherapy, but the benefit was less certain for adequately staged GC patients without LNM[110].

Immune-based therapy for GC

Although surgery, chemotherapy, molecular targeted therapy, radiotherapy or combined modality treatment improved the survival of GC patients[30,91], these treatments have limited efficacies in treating patients with advanced GC, and potential therapeutic strategies are urgently needed for these advanced GC patients. A number of recent studies[111,112] found that immune-based therapy for solid malignancies produced good results and significantly prolonged survival, and immunotherapy showed certain positive efficacies for GC patients compared to other traditional therapies[113-115], which may bring new hope to GC patients[116]. There are three main immunotherapeutic options for GC, including immune checkpoint inhibitors (ICIs), cellular immunotherapy and cancer vaccines.

As a co-suppressor molecule, the immune checkpoint regulates the survival, proliferation, differentiation or response to homologous antigens of T cells *via* the major histocompatibility complex-T-cell receptor, prevent excessive immune responses, and maintain the immune homeostasis in the human body[117,118]. Tumor cells in patients with malignant diseases could inhibit T cells then escape immune responses *via* the mechanisms described above[119]. Immunosuppressants are primarily used to reactivate the immune responses of T cells to tumor cells by blocking immune checkpoints or corresponding ligands/receptors with antibodies[120]. ICIs have been widely investigated[121], and these monoclonal antibodies[30] primarily target programmed cell death protein-1 (PD-1), programmed death ligand-1 (PD-L1) or cytotoxic T-lymphocyte antigen 4 (CTLA-4). PD-1 is a co-inhibitory receptor that is primarily expressed on the surface of activated T cells, Treg cells and monocytes. As the ligand of PD-1, PD-L1 binds to the PD-1 receptor and induces the inhibition or apoptosis of related immune cells, which helps tumor cells escape immune responses[122]. Moreover, PD-L1 is overexpressed in advanced GC, and its expression may relate to the tumor size, depth of invasion and LNM in 25%-65% of GCs[122-124]. Furthermore, clinical trials indicated that anti-PD-1 therapy for GC patients may have certain effectiveness. The first randomized phase III study demonstrated that the anti-PD-1 monoclonal antibody, nivolumab, effectively improved survival of patients with advanced gastric or gastric-oesophageal junction cancer[125]. Another famous phase II clinical KEYNOTE-059 trial recruited 259 patients from 16 countries and showed that the PD-1/PD-L1 inhibitor pembrolizumab also had similar efficacy and safety for advanced GC patients[126]. Researchers in the phase III CheckMate-649 trial found that nivolumab combined with fluorouracil and platinum as first-line medications improved the OS of patients with advanced HER2-negative GC, gastroesophageal junction cancer or esophageal adenocarcinoma[127]. Mid-term analysis of the KEYNOTE-811 test showed that the combination of pembrolizumab and trastuzumab plus chemotherapy improved the overall response rate (ORR) of patients with advanced HER2-positive GC[128]. CTLA-4 is also found on the surface of activated T cells and may interact with B7-1/B7-2 on the surface of antigen-presenting cells, which results in inhibition of the CD28 signaling pathway, and plays a key role in T-cell activation[119,129]. A monoclonal anti-CTLA-4 antibody targets T-cell co-inhibitory receptor and reactivates T-cell anti-tumor immune activity [130]. However, preliminary studies indicated that some GC patients showed ineffectiveness or even remission after treatment with monoclonal anti-CTLA-4 antibodies, and the objective response rate with antibodies alone was not satisfactory because only one of 18 patients (5.5%) reached the primary endpoint[131]. Besides, the monoclonal anti-CTLA-4 antibody ipilimumab alone as the maintenance therapy did not show any improvements in FPS for patients with unresectable locally advanced or metastatic GC compared with the best supportive care in a phase II clinical trial[132]. One study, focused on the therapeutic efficacies of PD-1 and CTLA4 inhibitors, found that the ORR of patients with metastatic GC who received nivolumab plus ipilimumab was higher than patients who received only nivolumab[133]. However, Shitara *et al*[134] recently found that nivolumab plus ipilimumab did not improve the OS of HER2-negative patients compared to the chemotherapy regimen in advanced GC patients.

The 2022 National Comprehensive Cancer Network Guidelines proposed PD-1/PD-L1 inhibitors as first-line/second-line medications for GC treatment, but anti-CTLA4 immunotherapy was not suggested in GC treatment. However, clinical trials on anti-CTLA4 antibodies (ipilimumab and tremelimumab) are being performed[135]. Clinical trials on many other immunosuppressants, such as LAG3, Tim3, TIGIT and OX40, are also being performed. LAG3 and Tim3 are in phase I and II clinical trials, and TIGIT and OX40 are in the early research stage[136]. Although the toxicity of ICIs may limit their efficacy and clinical application[137], current evidence indicates that the combined modality of ICIs with other treatments may be more effective and applicable in GC, especially when combined with chemotherapy for advanced GC patients with drug resistance[116,138].

Cellular immunotherapy uses immune cytotoxic cells to recognize and attack tumor cells, and induce an effective antitumor response[138]. These immune cells may be expanded T cells and nature killer cells *in vitro* or gene-engineered T-cell receptor (TCR) T cells (TCR-Ts) and chimeric antigen receptor (CAR) T cells (CAR-Ts)[138,139]. TCR-T/CAR-T immunotherapies, as modified T-cell-based immunotherapeutic approaches, are targeted cellular therapies that take advantage of the cytotoxic potential of T cells to attack tumor cells in an antigen-specific manner[140]. For TCR-T immunotherapy, the target TCR genes that recognize specific tumor-associated antigens (TAAs) are transduced into peripheral blood T cells collected from patients, and these modified T cells are reinjected into the patients' circulation[138]. TAAs are presented by major histocompatibility complex-I (MHC-I) to TCR-Ts within the patient, and the combination of TAAs with TCR could activate these T cells to release cytokines and attack tumor cells[141]. TCR-Ts expressing KK-LC-1 (encoded by *CT83*) TCR recognized *CT83*$^+$ tumor cells *in vitro*, and KK-LC-1 is frequently expressed in human epithelial tumors, including GC with the highest expression[142]. NY-ESO-1 antibody positivity was also found in GC, which indicated that NY-ESO-1 may be another target for TCR-T immunotherapy of GC[143]. Although TCR-T immunotherapy is being applied in clinical treatment, there are still some challenges. For example, it is difficult to generate a universal TCR for immunotherapy because of the extreme polymorphism of the MHC locus [141]. To resolve these issues, CAR was developed based on antibody recognition specificities[141], and CAR-Ts are considered a promising class of antitumor treatment[144]. T cells are collected from autologous peripheral blood, genetically modified to produce specific CARs, namely CAR-Ts, then

reinjected into the patient's circulation[145-147]. CAR-Ts recognize and combine the specific antigen on the surface of tumor cells by the extracellular single-chain fragment variable domain, which results in the immobilization and clustering of CARs, the formation of nonclassical immune synapses and activation of CAR-Ts[141]. In contrast to MHC-restricted TCR-Ts, CAR-Ts are typically designed and engineered to recognize non-MHC cell surface proteins[141]. The density of the target antigen is particularly important in the modulation of CAR-T-cell signaling compared to TCR-Ts[141]. Only one kind of targeted tumor antigen may not be sufficient to obtain satisfying antitumor responses, and the expression of targeted antigen in other body cells inevitably results in transient and reversible harmful effects, such as cellular toxicity[144]. Several potential targets, such as NKG2D, FOLR1, HER2, MSLN and CLDN18.2, were found, and the real therapeutic efficacies need further evaluation[148-152]. Therefore, targeting GC-specific antigens remains a challenge, and the lack of truly GC-specific antigens limits the clinical application of CAR-T immunotherapy[144].

As an active antitumor immunotherapy, cancer vaccines are designed to enhance body immune function by inducing humoral and/or cellular immune responses[139,153]. Cancer vaccines primarily include autologous tumor cell vaccines, dendritic cell vaccines, peptide vaccines and genetically engineered vaccines[138]. Patients with gastroesophageal adenocarcinoma or untreated metastatic GC may have higher median OS rates after receiving the G17DT (Aphton) vaccine[154], and vaccination improved the OS of GC patients with good safety and tolerance, especially when combined with chemotherapy[116]. The immune responses (*e.g.*, changes in immune milieu and tumor immune escape mechanisms) to cancer vaccines may be rapidly and accurately monitored using molecular sequencing, artificial intelligence or cellular engineering, which could optimize the design of cancer vaccines and facilitate their clinical application[155].

CONCLUSION

The incidence and mortality of GC have shown a downward trend worldwide, which suggests that GC may become a rare disease in the future[16]. However, GC incidence and mortality rank fifth and fourth, respectively, among all cancer types worldwide, and it remains a major health challenge[16]. With regard to the identified GC risk factors, such as *H. pylori* infection and unhealthy dietary habits and lifestyle, preventive strategies could effectively reduce GC incidence. Therefore, more attention should be given to individuals at higher risk, and unified guidelines for GC surveillance should be established. Due to the different staging and therapeutic strategies used in different regions, the prognosis of GC patients varies greatly. Most GC cases are found in advanced stages at diagnosis, which limits the clinical application and efficacy of surgery[156]. Although chemotherapy has significantly improved the prognosis of advanced GC patients, enormous challenges remain, such as drug resistance and toxicity[107]. With the emergence and promising development of immunotherapy, its clinical application and efficacy have been evaluated in GC patients, especially in advanced GC patients. However, due to the complicated tumor microenvironment and the complex interactions between the immune system and tumor cells, more clinical trials on immunotherapy are needed to verify their efficacy and safety in GC patients.

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FOOTNOTES

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ORCID number: Wen-Juan Yang 0000-0002-2495-439X; He-Ping Zhao 0000-0002-7896-6636; Yan Yu 0000-0003-1587-7748; Ji-Han Wang 0000-0003-1925-330X; Lei Guo 0000-0002-5166-5374; Jun-Ye Liu 0000-0001-5893-7785; Jie Pu 0000-0002-3227-8862; Jing Lv 0000-0003-2801-743X.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Older adults with acute severe ulcerative colitis have similar steroid non-response and colectomy rates as younger adults

Deloshaan Subhaharan, Pradeep Kakkadasam Ramaswamy, Laura Willmann, Hadi Moattar, Maneesha Bhullar, Naveed Ishaq, Alexander Dorrington, Dheeraj Shukla, Carolyn McIvor, John Edwards, Waled Mohsen

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Deloshaan Subhaharan, Pradeep Kakkadasam Ramaswamy, Laura Willmann, Hadi Moattar, Maneesha Bhullar, Naveed Ishaq, Alexander Dorrington, Dheeraj Shukla, John Edwards, Waled Mohsen, Department of Digestive Diseases, Gold Coast University Hospital, Gold Coast 4215, Queensland, Australia

Carolyn McIvor, Department of Gastroenterology, Logan Hospital, Logan 4131, Queensland, Australia

Corresponding author: Deloshaan Subhaharan, MD, Doctor, Department of Digestive Diseases, Gold Coast University Hospital, 1 Hospital Boulevard, Southport, Gold Coast 4215, Queensland, Australia. deloshaan.subhaharan@health.qld.gov.au

Abstract

BACKGROUND

There is paucity of data on outcomes of acute severe ulcerative colitis (ASUC) in older adults (≥ 60 years of age).

AIM

To assess steroid non-response rates during the index admission for ASUC in older adults. Secondary outcomes were response to medical rescue therapy and colectomy rates; at index admission, 3 and 12 mo.

METHODS

This retrospective multicentre cohort study included ASUC admissions who received intravenous steroids between January 2013 and July 2020 at two tertiary hospitals. Electronic medical records were reviewed to collect clinical, biochemical, and endoscopic data. A modified Poisson regression model was used for analysis.

RESULTS

Of 226 ASUC episodes, 45 (19.9%) occurred in patients ≥ 60 years of age. Steroid non-response rates were comparable in older adults and patients < 60 years of age [19 (42.2%) *vs* 85 (47%), P = 0.618, crude risk ratio (RR) = 0.89 [95% confidence interval (CI): 0.61-1.30], adjusted RR = 0.99 (0.44-2.21). Rates of response to medical rescue therapy in older adults was comparable to the younger cohort [76.5% *vs* 85.7%, P = 0.46, crude RR = 0.89 (0.67-1.17)]. Index admission colectomy [13.3% *vs* 10.5%, P = 0.598, crude RR = 1.27 (0.53-2.99), adjusted RR = 1.43 (0.34-

6.06)], colectomy at 3 mo [20% *vs* 16.6%, *P* = 0.66, crude RR = 1.18 (0.61-2.3), adjusted RR = 1.31 (0.32-0.53)] and colectomy at 12 mo [20% *vs* 23.2%, *P* = 0.682, crude RR = 0.85 (0.45-1.57), adjusted RR = 1.21 (0.29-4.97)], were similar between the two groups.

CONCLUSION

In older adults with ASUC, the steroid non-response rate, response to medical rescue therapy, and colectomy rate at index admission, 3 and 12 mo is similar to patients less than 60 years of age.

Key Words: Elderly; Ulcerative colitis; Acute severe ulcerative colitis; Colectomy; Rescue therapy; Infliximab

Core Tip: This is a retrospective study to assess the outcomes of older adults (≥ 60 years of age) hospitalised with acute severe ulcerative colitis (ASUC) as per Truelove and Witts' criteria. We identified 45 episodes of ASUC in older adults and compared outcomes with 181 episodes of ASUC in patients < 60 years of age. Older adults with ASUC have similar steroid non-response rate, response to medical rescue therapy and colectomy rates up to 12 mo from index admission, when compared to patients less than 60 years of age.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, relapsing-remitting, inflammatory disorder of the colon, resulting from numerous factors including genetic predisposition, environmental triggers, and gut microbiota[1,2]. Acute severe UC (ASUC), as defined by the Truelove and Witts criteria, occurs in 10%-25% at diagnosis and 20%-30% during the disease course of UC[3-5]. Intravenous corticosteroids (IVCS) remain the first-line therapy for ASUC, however infliximab (IFX) and ciclosporin (CsA) have been used as medical rescue therapy for those who are steroid-refractory[6-9].

Up to 20% of patients with UC have late-onset disease with their first flare occurring after the age of 60[10,11]. The basic principles of management of ASUC in older adults do not differ from younger patients[12]. However, there are unique challenges in managing inflammatory bowel disease (IBD) in older adults, including delay in diagnosis, misdiagnosis, and variable clinical presentations. Older adults may suffer from comorbidities, polypharmacy, complex drug-drug interactions, cognitive dysfunction, post-surgical complications, as well as social factors, which increase complexity in management of older adults with ASUC[13-17]. Studies have demonstrated higher treatment failure rates in elderly IBD patients who are commenced on their first anti-tumour necrosis factor agent[18]. In the setting of these factors, management decisions need to be patient-centred and individualised to minimise morbidity and mortality for older adults with ASUC.

Advanced age has not been shown to predict outcomes in ASUC[19]. However, in routine clinical practice, age is an important factor which is taken into consideration in the decision-making algorithm. As older adults are generally excluded from clinical trials, management decisions for these patients are often made by extrapolating data from a younger cohort of patients[11]. Moreover, short and long-term outcomes of ASUC in this cohort of patients are not well described. The primary outcome of the study was to assess steroid non-response rates during the index admission for ASUC in older adults. The secondary outcomes were response to medical rescue therapy and colectomy rates at index admission, 3 and 12 mo.

MATERIALS AND METHODS

Study population

All consecutive admissions with a diagnosis of UC at two tertiary Australian hospitals, from January 2013 to July 2020 at Gold Coast University Hospital and from January 2018 to July 2020 at Logan

Hospital, were identified using international classification of disease (ICD-10) codes (K51). Retrospective analysis identified adult patients (≥ 18 years of age) admitted for management of ASUC, as identified by Truelove and Witts criteria[3] (Figure 1). The study was approved by the Gold Coast Health Service Human Research Ethics Committee (Ref: LNR/2020/QGC/67173).

Inclusion was limited to patients with ASUC who received at least 3-5 d of IVCS (either hydrocortisone 400 mg/d or methylprednisolone 60 mg/d). Patients with a diagnosis of Crohn's disease or positive stool cultures for other enteric pathogens were excluded. Patients with superimposed Clostridium difficile or cytomegalovirus infection were included in the final analysis. Demographic, clinical and laboratory results were collected. Endoscopic data was collected from procedure reports and images, and scored based on the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) score[20].

Therapeutic management

All patients received IVCS as per international guidelines[21]. The Oxford criteria was used to determine failure of IVCS therapy after 3-5 d[22]. Patients received IFX or CsA for medical rescue therapy at their treating physician's preference. The standard dose IFX induction strategy utilised was 5 mg/kg at week 0, 2 and 6. Accelerated dose of IFX was defined as 10 mg/kg on day 0 followed by 5 mg/kg at week 2 and 6. The dose of IFX was determined by the treating physician based on clinical assessment of disease severity. CsA was dosed at 2 mg/kg body weight with a target trough level of 200-300 ng/mL at 48 h. In patients responding to medical rescue therapy, maintenance therapy was based on disease severity and prior treatment history as per the treating physician's discretion.

Definitions

UC: The diagnosis of UC was based on standard clinical, endoscopic, and histological criteria[23].

ASUC: The diagnosis of ASUC was based on Truelove and Witts criteria; defined as ≥ 6 bloody stool motions per day and one or more of the following: Haemoglobin < 10.5 g/dL, erythrocyte sedimentation rate ≥ 30 mm/hr or C-reactive protein ≥ 30 mg/L, temperature ≥ 37.8 °C, or heart rate ≥ 90 beats/min[3].

Disease extent: The maximum endoscopic extent at index colonoscopy according to the Montreal classification[24]. In patients with ASUC as their first presentation of disease, the extent was determined from the first available colonoscopy after discharge, or the surgical specimen if they underwent colectomy.

Older adults with ASUC: ASUC occurring in patients ≥ 60 years of age (irrespective of the age at diagnosis of UC).

Endoscopic severity: Defined by the UCEIS. The score (0-8) is calculated by the sum of three descriptors: Vascular pattern (scored 0-2), bleeding (scored 0-3), and erosions/ulcers (scored 0-3). It is assessed at the most severely affected area on flexible sigmoidoscopy[20].

Steroid non-response: Defined as failure to respond to IVCS as defined by the Oxford criteria[22], and receiving either medical or surgical rescue therapy.

IFX dosing: Standard dose strategy was defined as IFX 5 mg/kg at week 0, 2 and 6. Accelerated dose was defined as IFX 10 mg/kg on day 0 followed by 5 mg/kg at week 2 and 6.

Responder to medical rescue therapy: Defined as the patient being discharged from hospital on medical therapy after receiving inpatient medical rescue therapy, and avoiding colectomy during the admission.

Outcomes

The primary outcome was to assess steroid non-response rates during the index admission for ASUC in older adults. The secondary outcomes were response to medical rescue therapy and colectomy rates at index admission, 3 and 12 mo.

Statistical analysis

Descriptive statistics were used to describe the study cohort. Results were reported as median with interquartile range (IQR) for continuous variables, and frequencies with percentages for categorical variables. For comparison of variables, Fisher's exact or Chi-square tests were used for categorical variables, and Wilcoxon Ranksum test for continuous variables. Continuous data was tested for normality using the Shapiro-Wilk test and a two-tailed P value of < 0.05 was considered statistically significant. A modified Poisson regression model was used to estimate risk differences (RDs) and RRs to evaluate the difference in clinical outcomes between the two groups. Kaplan-Meier plots and the Cox proportional hazards regression model were also used. A log-rank test was used to compare the curves of the Kaplan-Meier plots. Multiple imputations were performed to account for missing covariates. All analysis was performed using Stata15 (StataCorp LLC, College Station, Texas).

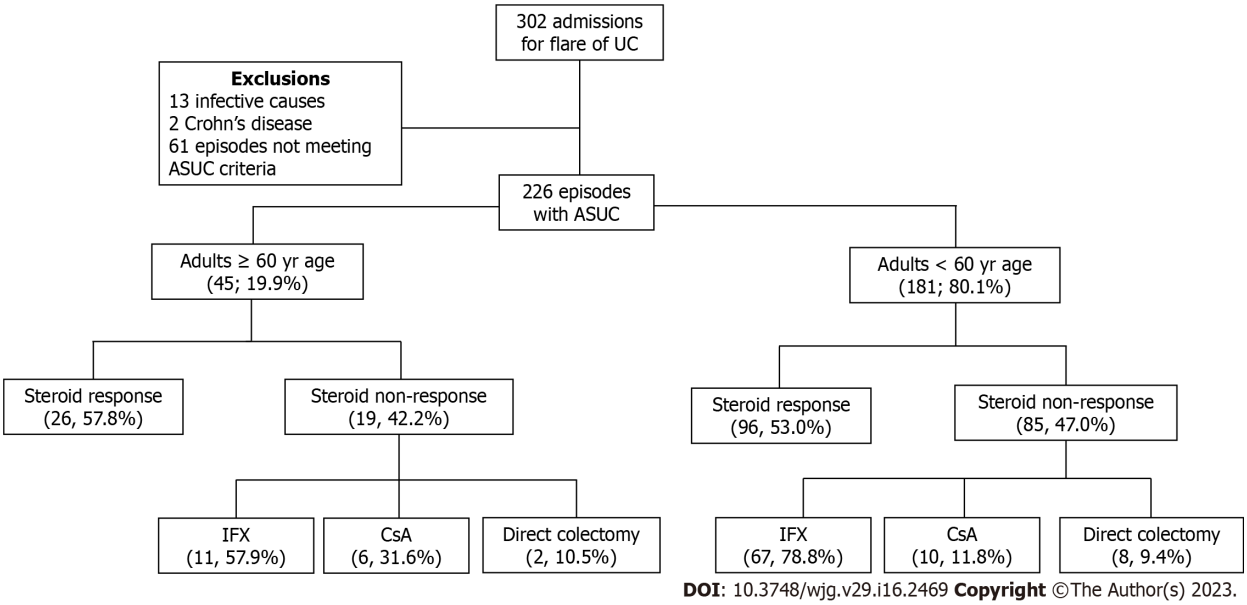

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Figure 1 Patient flow diagram. UC: Ulcerative colitis; ASUC: Acute severe ulcerative colitis; IFX: Infliximab; CsA: Ciclosporin.

RESULTS

A total of 302 admissions for UC who received IVCS were identified, of which 76 were excluded. 226 episodes of ASUC were included in the analysis. 45 (19.9%) episodes of ASUC in older adults ≥ 60 years of age and 181 (80.1%) episodes in younger adults were identified (Figure 1). Median age of disease onset was 66.5 (IQR: 59-76) *vs* 27 (IQR: 21-37), $P < 0.001$. Disease duration was similar between the two groups (2.5 *vs* 2 years, P 0.94). 33 out of 45 (73.3%) episodes had their first presentation of UC after the age of 60 years. Median Charlson Comorbidity Index in older adults was 3 (IQR: 2-4). Smoking status, albumin and platelet count at admission were significantly different between the two groups. Current immunomodulator use, biologic use and oral steroid use at admission were similar between the two groups. Clinical, endoscopic, and biochemical parameters are provided in Table 1. Summary of primary and secondary outcomes are shown in Table 2.

Primary outcome: Steroid non-response during the index admission for ASUC

Failure to IVCS therapy, as defined by the Oxford criteria[22], was similar between older and younger adults [19 (42.2%) *vs* 85 (47%), $P = 0.618$; crude RR = 0.89 (0.61-1.30), $P = 0.34$; adjusted RR = 0.99 (0.34-2.90), $P = 0.175$; odds ratio (OR) = 0.82 (0.43-1.58), $P = 0.344$; crude hazard ratio (HR) = 0.89 (0.556-1.455), $P = 0.674$]. In older adults, of the 19 episodes that failed IVCS, 17 (89.5%) episodes received medical rescue therapy (7 episodes IFX 5 mg/kg, 4 episodes IFX 10 mg/kg, 6 episodes CsA) and 2 (10.5%) patients proceeded directly to colectomy. Median time to initiation of rescue therapy was 4 d (IQR: 3-5 d). In patients < 60 years of age, of the 85 episodes that failed IVCS, 77 (90.6%) episodes received medical rescue therapy (45 episodes IFX 5 mg/kg, 22 episodes IFX 10 mg/kg, 10 episodes CsA) and 8 (9.4%) patients underwent direct colectomy. When the cut-off age was defined as 70 years, a significantly lower proportion of episodes failed IVCS [6/23 (26.1%) in ≥ 70 years *vs* 98/203 (48.3%) in < 70 years, $P = 0.049$; crude RD = -0.22 (-0.41 to -0.03); crude RR = 0.54 (0.27-1.09), $P = 0.034$; adjusted RR = 0.36 (0.08-1.49), $P = 0.897$; crude OR = 0.378 (0.143-1.00), $P = 0.05$].

Secondary outcomes

Response to medical rescue therapy: In older adults, of the 17 episodes who received medical rescue therapy, 4 (23.5%) patients underwent colectomy during the index admission. In the younger cohort, of the 77 episodes who received medical rescue therapy, 10 (13%) patients underwent a colectomy during the index admission. The rates of response to medical rescue therapy in older adults were similar to the younger cohort [76.5% *vs* 85.7%, $P = 0.46$; crude RD = -0.092 (-0.31 to 0.12); crude RR = 0.89 (0.67-1.17), $P = 0.27$; crude OR = 0.54 (0.16-1.85)]. When the cut-off age was defined as 70 years, a lower proportion of episodes responded to medical rescue therapy [4/6 (66.7%) in ≥ 70 years *vs* 75/88 (85.2%) in < 70 years, $P = 0.243$; crude RD = -0.18 (-0.57 to 0.19); crude RR = 0.78 (0.44-1.38); crude OR = 0.34 (0.65-), $P = 0.24$].

Index admission colectomy: In older adults, 6 (13.3%) of 45 patients underwent colectomy during the index admission for ASUC compared to 19 (10.5%) of 191 patients in the younger cohort [crude RD = 0.028 (-0.08 to 0.13); crude RR = 1.27 (0.53-2.99), $P = 0.376$; adjusted RR = 1.43 (0.34-6.06), $P = 0.71$; crude OR = 1.31 (0.50-3.41); crude HR = 1.27 (0.47-3.39), $P = 0.608$]. When the cut-off age was defined as 70

Table 1 Baseline characteristics of patients in the two groups (≥ 60 years and < 60 years)

	≥ 60 yr (*n* = 45)	< 60 yr (*n* = 181)	*P* value
Female, *n* (%)	22 (48.9)	88 (48.6)	1
Median age (yr)	71 (63-77)	32 (24-42)	< 0.001[1]
Median disease duration (yr)	2.5 (0-5)	2 (0.1-6)	0.94[1]
Index presentation of UC as ASUC (*n*, %)	14 (31.1)	45 (24.9)	0.45
Median follow up post admission for ASUC (wk)	104 (20-160)	74 (30-168)	0.97[1]
Median symptom duration before admission (d)	14 (7-24)	14 (5-28)	0.59[1]
Median length of stay (d)	10 (7-19)	9 (7-13.5)	0.22[1]
Disease extent, *n* (%)			0.072
Left-sided colitis	8 (17.8)	54 (29.8)	
Pancolitis	37 (82.2)	127 (70.2)	
Toxic megacolon, *n* (%)	0	4 (2.2)	0.41
Extraintestinal manifestations, *n* (%)	2 (4.4)	31 (17.1)	0.02
Superimposed clostridium difficile, *n* (%)	3 (6.7)	6 (3.3)	0.26
Smoking status, *n* (%)			0.037
Never	21 (46.7)	121 (66.9)	
Current	6 (13.3)	17 (9.4)	
Former	18 (40.0)	43 (23.8)	
5-aminosalicyclate use, *n* (%)			0.86
Current	24 (53.3)	100 (55.2)	
Never	14 (31.1)	49 (27.1)	
Intolerant/ceased	7 (15.6)	32 (17.7)	
Current thiopurine use, *n* (%)	6 (13.3)	29 (16.0)	0.29
Current methotrexate use, *n* (%)	1 (2.2)	2 (1.1)	0.16
Anti-TNF antagonist use, *n* (%)			0.74
Current	6 (13.3)	23 (12.7)	
Never	37 (82.2)	134 (74.0)	
Intolerant	1 (2.2)	6 (3.3)	
Secondary loss of response	1 (2.2)	18 (9.9)	
Vedolizumab use, *n* (%)			0.024
Current	8 (17.8)	9 (5.0)	
Never	35 (77.8)	163 (90.0)	
Intolerant	0	4 (2.2)	
Secondary loss of response	2 (4.4)	5 (2.8)	
Biologics on admission, *n* (%)	14 (31.0)	35 (19.4)	0.11
Oral steroids at admission, *n* (%)	15 (33.3)	78 (43.1)	0.31
Median admission UCEIS	5.5 (5-7)	6 (5-7)	0.82[1]
Median serum albumin on day of admission (g/L)	31 (27-34)	33 (29-38)	0.005[1]
Median haemoglobin on day of admission (g/L)	126 (111-135)	124 (108-139)	0.92[1]
Median platelet count on day of admission (units)	333.5 (277-386)	393 (293-500)	0.006[1]
Median CRP on day of admission (mg/L)	69 (33-121)	54 (30-99)	0.34[1]
Median admission faeces calprotectin (mcg/g)	2400 (1600-4600)	2850 (1400-5300)	0.48[1]

Median stool frequency on day of admission	10 (7-15)	10 (8-18)	0.41[1]

[1]Wilcoxon Ranksum test.

Continuous variables reported as median with interquartile range. UC: Ulcerative colitis; ASUC: Acute severe ulcerative colitis; UCEIS: Ulcerative colitis endoscopic index of severity; CRP: C-reactive protein; TNF: Tumour necrosis factor.

Table 2 Summary of primary and secondary outcomes of older *vs* younger adults with acute severe ulcerative colitis, *n* (%)

	≥ 60 yr, *n* = 45	< 60 yr, *n* = 181	Crude RD (95%CI)	Crude RR (95%CI)	Adjusted RR[1] (95%CI)	OR (95%CI)
Primary outcome: Steroid non-response	19 (42.2%)	85 (47%)	-0.47 (-0.21 to 0.11)	0.89 (0.61-1.30)	0.99 (0.34-2.90)	0.82 (0.43-1.58)
Response to medical rescue therapy	13 (76.5%)	66 (85.7%)	-0.09 (-0.31 to 0.12)	0.89 (0.67-1.17)	-	0.54 (0.16-1.85)
Colectomy same admission	6 (13.3%)	19 (10.5%)	0.028 (-0.08 to 0.13)	1.27 (0.53-2.99)	1.43 (0.34-6.06)	1.31 (0.50-3.41)
Colectomy at 3 mo	9 (20.9%)	30 (17.6%)	-0.03 (-0.10 to 0.16)	1.18 (0.61-2.3)	1.31 (0.32-5.30)	1.23 (0.54-2.80)
Colectomy at 12 mo	9 (24.3%)	42 (28.8%)	-0.04 (-0.20 to 0.11)	0.85 (0.45-1.57)	1.2 (0.29-4.97)	0.79 (0.35-1.80)

[1]Adjusted relative risk for sex, disease duration, smoking status, disease extent, current biologic use.

RD: Risk difference; RR: Relative risk; aRR: Adjusted relative risk; OR: Odds ratio.

years, a similar proportion of episodes underwent colectomy during the index admission [2/25 (8.7%) in ≥ 70 years *vs* 21/201 (8.7%) in < 70 years, P = 0.52; crude RD = -0.026 (-0.15 to 0.09); crude RR = 0.77 (0.19-3.04); adjusted RR = 0.91 (0.16-5.09), P = 0.52; crude OR = 0.74 (0-3.0), P = 0.52].

Colectomy at 3 mo: At 3 mo, 9 (20%) patients ≥ 60 years of age had undergone a colectomy, compared to 30 (17.6%) patients < 60 years of age [crude RD = -0.03 (-0.10 to 0.16); crude RR = 1.18 (0.61-2.3), P = 0.38; adjusted RR = 1.31 (0.32-0.53), P = 0.82; crude OR = 1.23 (0.54-2.80); crude HR = 1.21 (0.55-2.648, P = 0.620]. In older adults, of the 13 episodes which responded to medical rescue therapy, 1 patient with two episodes of ASUC within a three-month period of the index admission underwent a colectomy. When age cut-off was defined as 70 years, a lower proportion of episodes underwent colectomy at 3 mo [2/23 (8.7%) in ≥ 70 years *vs* 37/190 (19.5%) in < 70 years of age, P = 0.264; crude RD = -0.1.09 (-0.235 to 0.02); crude RR = 0.44 (0.11-1.73); adjusted RR = 0.72 (0.14-3.73), crude OR = 0.39 (0-1.58), P = 0.165].

Colectomy at 12 mo: At 12 mo, 9 (24.3%) patients ≥ 60 years of age had undergone a colectomy, compared to 42 (28.8%) patients < 60 years of age [crude RD = -0.04 (-0.20 to 0.11); crude RR = 0.85 (0.45-1.57), P = 0.376; adjusted RR = 1.21 (0.29-4.97), P = 0.88; crude OR = 0.79 (0.35-1.80); crude HR = 0.86 (0.43-1.71), P = 0.69]. The Kaplan-Meier curve for colectomy-free survival is shown in Figure 2. When age cut-off was defined as 70 years, a lower proportion of episodes underwent colectomy at 12 mo [2/23 (8.7%) in ≥ 70 years *vs* 49/203 (24.1%) in < 70 years of age, P = 0.042; crude RD = -0.21 (-0.35 to -0.07); crude RR = 0.29 (0.07-1.14), P = 0.026; adjusted RR = 0.63 (0.11-3.41), P = 0.673; OR = 0.23 (0-0.92)].

DISCUSSION

Although the management of IBD in older adults remains a challenge, the basic treatment paradigms across all age groups are the same. This study is one of the largest studies describing outcomes of ASUC in older adults. It demonstrates that the rates of steroid non-response as well as short and long-term colectomy risk in older adults is comparable to those who are less than 60 years of age.

There is an increasing number of older adults with IBD, correlating with both the rising incidence of IBD and the ageing population[25]. The widely accepted definition of elderly-onset IBD is disease onset at age 60 years or older[25]. Hence, this study used 60 years as the cut-off age to define older adults. In this study, 20% of patients were over 60 years of age at the time of their ASUC presentation; 15% of patients (33 out of 226) had their initial diagnosis of UC after the age of 60. This is comparable to current data showing 10%-25% of IBD patients are diagnosed after the age of 60[25,26]. Previous studies have exhibited that older adults with UC are more likely to present with a severe initial episode, display proctocolitis or limited left-sided colitis, and develop toxic megacolon which is associated with high

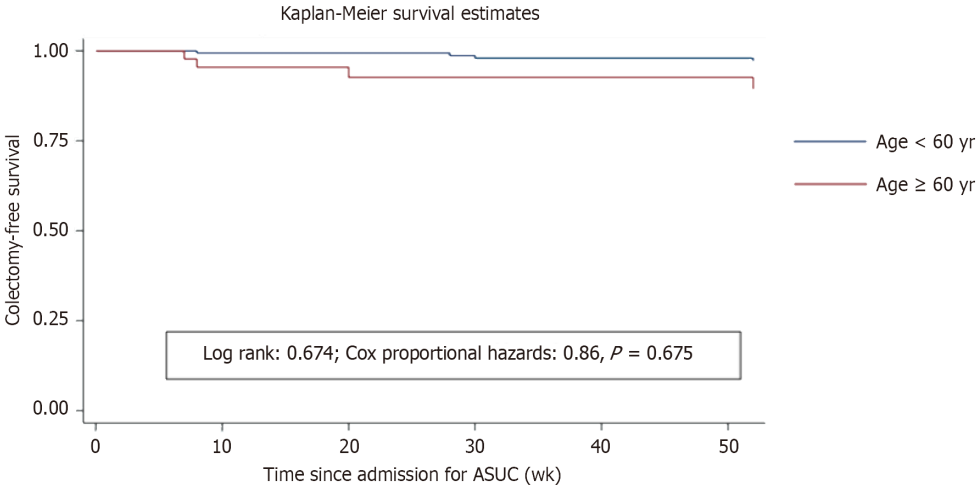

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Figure 2 Kaplan-Meier curve, colectomy-free survival. ASUC: Acute severe ulcerative colitis.

mortality[27,28]. In this study, 13 (28.9%) episodes had proctocolitis or limited left-sided colitis, and there were no episodes of toxic megacolon in older adults.

Traditionally, the Oxford index has been utilised to define steroid failure in patients with ASUC, and in this study the same definitions were applied[22]. Previous studies have shown that about 40% of patients with ASUC fail initial therapy with IVCS[29]. This study reconfirms that the rate of steroid failure is similar between older adults (42.2%) and the younger cohort of patients (47%). This is in contrast to a recently published multicentre Japanese study[30]. IVCS continue to be the first-line treatment option for older adults, although steroid-specific adverse effects are to be taken into consideration. Nevertheless, older adults with ASUC should not be undertreated, as poorly controlled disease and repeated courses of steroids induce undesirable outcomes. In this study, more than 75% of older adults responded to medical rescue therapy and avoided colectomy during admission for ASUC. The effectiveness of medical rescue therapy demonstrated in the current study is comparable to that demonstrated in larger randomised-controlled trials[31,32]. Of the episodes who responded to medical rescue therapy, only 1 patient had undergone a colectomy by 12 mo. Biologic agents in older adults with IBD were recently shown to have similar drug sustainability, effectiveness, and safety[33]. Older adults on IFX also have a similar risk of developing adverse effects and loss of response as younger patients [34]. Thus, medical rescue therapy can be offered judiciously to older adults.

This study has several strengths, foremost that it is one of the largest studies describing outcomes of ASUC in older adults. Although this was not a controlled trial, this cohort of patients was managed through two tertiary IBD subspeciality units which have defined treatment protocols for hospitalised ASUC patients consistent with international guidelines. Results are therefore generalisable to similar real-world clinical settings. The study has a few limitations. Firstly, the study is retrospective. Secondly, long-term safety of IFX and CsA were not studied systematically. The assessment of clinical response after initiation of rescue therapy with the Lichtiger score or Mayo score may have been beneficial. Finally, clinical and biochemical data at 12 mo may have also proved valuable for the analysis of the study.

CONCLUSION

Management of older adults with ASUC remains challenging. This study demonstrates that the rate of IVCS non-response in older adults with ASUC is similar to younger patients, and medical rescue therapy is equally effective. Clinical decisions for older adults with ASUC should still be determined by disease severity rather than chronological age alone.

ARTICLE HIGHLIGHTS

Research background

The management of older adults with acute severe ulcerative colitis (ASUC) is uniquely challenging due to their numerous medical and social factors. Up to 20% of patients with ulcerative colitis have late-onset disease with their first flare occurring after the age of 60.

Research motivation

There is minimal data available on the outcomes of older adults with ASUC. Previous studies report higher treatment failure rates in older adults who are commenced on their first biologic. We planned this study to define both short and long term outcomes for this cohort and determine if they have similar outcomes compared to the younger cohort.

Research objectives

We aimed to determine the steroid non-response rates for older adults with ASUC during index admission. We also aimed to study their response to medical rescue therapy and colectomy rates up to 12 mo from initial presentation.

Research methods

We conducted a retrospective cohort study investigating the short and long term outcomes among 226 ASUC episodes between January 2013 and July 2020 at two tertiary hospitals in Queensland, Australia. Clinical characteristics, laboratory parameters, and disease outcomes, including mortality, were compared between older and younger adults. A modified Poisson regression model was used for analysis.

Research results

The prevalence of older adults with ASUC was 19.9%. Steroid non-response rate in older adults were comparable to younger adults (42.2% *vs* 47%, $P = 0.62$). Response rates to medical rescue therapy was also comparable between the two groups (76.5% *vs* 85.7%, $P = 0.46$). Index admission colectomy (13.3% *vs* 10.5%, $P = 0.60$), colectomy at 3 mo (20% *vs* 16.6%, $P = 0.66$), and colectomy at 12 mo (20% *vs* 23.2%, $P = 0.68$) were also similar between the two groups.

Research conclusions

Older adults with ASUC have similar outcomes compared to younger patients less than 60 years of age for rates of steroid non-response, medical rescue therapy, and colectomy at index admission, 3 and 12 mo.

Research perspectives

Clinical decisions for older adults with ASUC remains to be a challenge however should still be determined by disease severity rather than chronological age alone. Future prospective studies will allow further improvement in their management.

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FOOTNOTES

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ORCID number: Deloshaan Subhaharan 0000-0003-4223-8239; Pradeep Kakkadasam Ramaswamy 0000-0003-3235-763X; Laura Willmann 0000-0001-6852-6082; Hadi Moattar 0000-0001-9359-3199; Maneesha Bhullar 0000-0002-5548-8248; Naveed Ishaq 0000-0002-6002-1255; Alexander Dorrington 0000-0002-9564-6896; Dheeraj Shukla 0000-0001-6239-6942; Carolyn McIvor 0000-0003-4384-3019; John Edwards 0000-0002-9867-0349; Waled Mohsen 0000-0002-5245-8668.

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Retrospective Cohort Study

Liver histopathological lesions is severe in patients with normal alanine transaminase and low to moderate hepatitis B virus DNA replication

Su-Wen Jiang, Xiang Lian, Ai-Rong Hu, Jia-Lin Lu, Zhe-Yun He, Xiao-Jun Shi, De-Dong Zhu, Zong-Yi Wang, Guan-Cheng Huang

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Su-Wen Jiang, Ai-Rong Hu, Zhe-Yun He, Xiao-Jun Shi, De-Dong Zhu, Ningbo Institute of Liver Diseases, Ningbo No. 2 Hospital, Ningbo 315020, Zhejiang Province, China

Xiang Lian, Department of Infectious Diseases, Xiangshan Hospital Affiliated to Wenzhou Medical University, Ningbo 315020, Zhejiang Province, China

Jia-Lin Lu, Zong-Yi Wang, Department of Infectious Diseases, The First Hospital of Ninghai County, Ningbo 315000, Zhejiang Province, China

Guan-Cheng Huang, Department of Infectious Diseases, The Affiliated Yang-Ming Hospital of Ningbo University, Ningbo 315400, Zhejiang Province, China

Corresponding author: Ai-Rong Hu, MD, Doctor, Ningbo Institute of Liver Diseases, Ningbo No. 2 Hospital, No. 41 Xibei Street, Haishu District, Ningbo 315020, Zhejiang Province, China. huairong@ucas.edu.cn

Abstract

BACKGROUND

Chronic hepatitis B virus (HBV) infection remains a major global public health problem. Chronic hepatitis B (CHB) patients can be divided into treatment indication and non-treatment indication individuals according to alanine transaminase (ALT), HBV DNA, serum hepatitis B e antigen status, disease status [liver cirrhosis, hepatocellular carcinoma (HCC), or liver failure], liver necroinflammation or fibrosis, patients' age, and family history of HCC or cirrhosis. For example, normal ALT patients in 'immune-tolerant' phase with HBV DNA higher than 10^7 or 2×10^7 IU/mL, and those in 'inactive-carrier' phase with HBV DNA lower than 2×10^3 IU/mL do not require antiviral therapy. However, is it reasonable to set the defined values of HBV DNA as the fundamental basis to estimate the disease state and to determine whether to start treatment? In fact, we should pay more attention to those who do not match the treatment indications (gray-zone patients both in the indeterminate phase and in the 'inactive-carrier' phase).

AIM

To analyze the correlation of HBV DNA level and liver histopathological severity, and to explore the significance of HBV DNA for CHB with normal ALT.

METHODS

From January 2017 to December 2021, a retrospective cross-sectional set of 1299 patients with chronic HBV infection (HBV DNA > 30 IU/mL) who underwent liver biopsy from four hospitals, including 634 with ALT less than 40 U/L. None of the patients had received anti-HBV treatment. The degrees of liver necroinflammatory activity and liver fibrosis were evaluated according to the Metavir system. On the basis of the HBV DNA level, patients were divided into two groups: Low/moderate replication group, HBV DNA ≤ 10^7 IU/mL [7.00 Log IU/mL, the European Association for the Study of the Liver (EASL) guidelines] or ≤ 2 × 10^7 IU/mL [7.30 Log IU/mL, the Chinese Medical Association (CMA) guidelines]; high replication group, HBV DNA > 10^7 IU/mL or > 2 × 10^7 IU/mL. Relevant factors (demographic characteristics, laboratory parameters and noninvasive models) for liver histopathological severity were analyzed by univariate analysis, logistics analysis and propensity score-matched analysis.

RESULTS

At entry, there were 21.45%, 24.29%, and 30.28% of the patients had liver histopathological severities with ≥ A2, ≥ F2, and ≥ A2 or/and ≥ F2, respectively. HBV DNA level (negative correlation) and noninvasive model liver fibrosis 5 value (positive correlation) were independent risk factors for liver histopathological severities (liver necroinflammation, liver fibrosis, and treatment indication). The AUROCs of the prediction probabilities (PRE_) of the models mentioned above (< A2 *vs* ≥ A2, < F2 *vs* ≥ F2, < A2 and < F2 *vs* ≥ A2 or/and ≥ F2) were 0.814 (95%CI: 0.770-0.859), 0.824 (95%CI: 0.785-0.863), and 0.799 (95%CI: 0.760-0.838), respectively. HBV DNA level (negative correlation) was still an independent risk factor when diagnostic models were excluded, the *P* values (< A2 *vs* ≥ A2, < F2 *vs* ≥ F2, < A2 and < F2 *vs* ≥ A2 or/and ≥ F2) were 0.011, 0.000, and 0.000, respectively. For the propensity score-matched pairs, whether based on EASL guidelines or CMA guidelines, the group with significant liver histology damage (≥ A2 or/and ≥ F2) showed much lower HBV DNA level than the group with non- significant liver histology damage (< A2 and < F2). Patients in the moderate replication group (with indeterminate phase) had the most serious liver disease pathologically and hematologically, followed by patients in the low replication group (with 'inactive-carrier' phase) and then the high replication group (with 'immune-tolerant' phase).

CONCLUSION

HBV DNA level is a negative risk factor for liver disease progression. The phase definition of CHB may be revised by whether the level of HBV DNA exceeds the detection low limit value. Patients who are in the indeterminate phase or 'inactive carriers' should receive antiviral therapy.

Key Words: Chronic hepatitis B; Hepatitis B virus DNA; Histology; Risk factors

Core Tip: According to the guidelines, for patients with normal alanine transaminase (ALT), hepatitis B virus (HBV) DNA levels were defined as ≥ 10^7/2 × 10^7 and < 2 × 10^3 IU/mL in the 'immune-tolerant' and the 'inactive-carrier' phase, respectively. However, it is still controversial. In this study, we analyzed the liver histopathology and the risk factors in 634 cases with positive HBV DNA and normal ALT. We found that patients with low or moderate HBV DNA level had more severe liver diseases. HBV DNA level (negative correlation) was an independent risk factor for liver histopathological severity. Therefore, we consider that the phase definition of chronic hepatitis B may be revised based on whether the level of HBV DNA exceeds the detection low limit value.

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INTRODUCTION

With the promotion of the hepatitis B vaccine or combined hepatitis B immune globulin, new hepatitis B virus (HBV) infections are decreasing. However, there are still about 257 million people infected with

HBV worldwide[1], and about 887000 people died from HBV infection each year, of which liver cirrhosis and hepatocellular carcinoma (HCC) deaths account for 52% and 38%, respectively[2]. It's undeniable that chronic HBV infection is still a major global public health problem. Therefore, the World Health Organization (WHO) has proposed the global health sector strategy of 'eliminating viral hepatitis as a major public health threat by 2030'. One of the goals is to achieve a diagnosis rate to 90% and a treatment rate to 80% of HBV infection by 2030[3]. China had made great progress in reducing HBV infections, but the challenges still remain. Currently, there are still 70 million chronic HBV infections in China[2].

The natural history of chronic HBV infection can be generally divided into four phases: Hepatitis B e antigen (HBeAg) positive chronic HBV infection/'immune-tolerant' phase, HBeAg positive chronic hepatitis B (CHB)/immune-clearance phase, HBeAg negative chronic HBV infection/'inactive-carrier' phase, and HBeAg negative CHB/reactivation phase[2,4,5].

The disease progression and treatment indications judgement is mainly based on serum HBeAg status, HBV DNA level, alanine transaminase (ALT) level, and severity of liver disease, combined with patients' age, family history, and accompanying diseases[2,4-7]. In accordance with the European Association for the Study of the Liver (EASL) guidelines[4] and the CHB treatment algorithm in the United States[5], regardless of HBeAg status, patients with HBV DNA > 2×10^3 IU/mL, ALT > upper limit of normal (ULN), and/or at least moderate liver necrotic inflammation or liver fibrosis should be treated. However, if HBV DNA is less than 2×10^3 IU/mL, how to deal with it clinically becomes an issue. According to the EASL guidelines[4] and the CHB treatment algorithm in the United States[5], CHB patients with normal ALT in the immune tolerant phase refer to HBV DNA > 10^7 IU/mL, in the 'inactive-carrier' phase refer to HBV DNA < 2×10^3, and in the indeterminate phase refer to $2 \times 10^3 \leq$ HBV DNA $\leq 10^7$ IU/mL. According to the Chinese Medical Association (CMA) guidelines[2], patients in the immune tolerant phase refer to HBV DNA > 2×10^7 IU/mL, in the 'inactive-carrier' phase refer to HBV DNA < 2×10^3, and in the indeterminate phase refer to $2 \times 10^3 \leq$ HBV DNA $\leq 2 \times 10^7$ IU/mL.

Although HBV DNA is an important indicator for judging disease progression and treatment indications, the reported conclusions about HBV DNA and disease severity remain controversial[8-14]. Moreover, the 'gray-zone' and/or the indeterminate phase population should not be ignored with the consideration of the guidelines. The aim of this study was to find the correlation of clinical and laboratory parameters with liver histopathological severity in 634 CHB patients with ALT < ULN who required liver biopsy to assess liver inflammation and fibrosis. Studies on liver pathological changes in the 'gray-zone' and/or the indeterminate phase population and the identification of the risk factors for disease progression might be of great significance.

MATERIALS AND METHODS

Data collection

From January 2017 to December 2021, there were 1299 chronic HBV infections (including 634 with ALT < ULN) who underwent liver biopsy were included in this retrospective cross-sectional study conducted in four hospitals. The patients were hospitalized in the Department of Hepatology, Ningbo No. 2 Hospital; the Department of Infectious Diseases, Xiangshan Hospital Affiliated to Wenzhou Medical University; the Department of Infectious Diseases, The First Hospital of Ninghai County; and the Department of Infectious Diseases, the Affiliated Yangming Hospital of Ningbo University, Ningbo, China.

The inclusion criteria were as follows: Patients aged 13-78 years, HBsAg positivity for at least 6 mo, HBV DNA ≥ 30 IU/mL, and no previous anti-HBV treatment. The ULN of ALT was 40 U/L according to the WHO/EASL/Asian Pacific Association for the Study of the Liver guidelines[4,6,7]. The exclusion criteria were as follows: Co-infection with hepatitis C virus, hepatitis D virus, hepatitis E virus, and human immunodeficiency virus; autoimmune hepatitis; Wilson's disease; nonalcoholic fatty liver disease; chronic alcohol consumption (> 30 g/d for men and > 20 g/d for women[15]); and incomplete data.

This study was approved by the ethics committee of Ningbo No. 2 Hospital (PJ-NBEY-KY-2017-069-01, PJ-NBEY-KY-2021-037-02, and PJ-NBEY-KY-2022-138-01). In this study, medical data was obtained from previous clinical diagnosis and treatment, and informed consent was exempted.

The clinical data was collected within one week before liver biopsy. Demographic characteristics and laboratory data, including age, sex, albumin (ALB), globulin (GLB), ALB–GLB ratio (AGR), ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), white blood cell (WBC), neutrophil–lymphocyte ratio (NLR), platelet (PLT), HBeAg, HBV DNA, and noninvasive models such as aspartate transaminase to platelet ratio index (APRI)[16], fibrosis-4 (FIB-4)[17], liver inflammation and fibrosis-5 (LIF-5)[18], were recorded.

Blood test

Blood routine was detected using Sysmex XN-1000 automated hematology analyzer (Sysmex Corporation, Japan). Serum liver function was detected with Simens Advia Chemistry XPT system

analyzer (Siemens Healthcare, Germany). Serum HBV DNA was measured by real-time fluorescence quantitative PCR (ABI7500, Applied Biosystems, CA, USA) and HBV nucleic acid quantitative detection kit (DAAN Gene Co., Ltd. Sun Yat-sen University, China) with the lowest detection value of 30 IU/mL. According to the HBV DNA level, patients were divided into two groups: Low/moderate replication group, HBV DNA ≤ 10^7 IU/mL (7.00 Log IU/mL) or ≤ 2 × 10^7 IU/mL (7.30 Log IU/mL); and high replication group, HBV DNA > 10^7 IU/mL or > 2 × 10^7 IU/mL[2,4,5]. HBsAg and HBeAg were detected by chemiluminescence method (Abbott AxSYM System, IL, United States). In this study, HBeAg was presented as 1 for positive and 0 for negative. The same quality control standards were employed.

Liver histological examination

The biopsy device (BARD Magnum, United States) comprised a biopsy gun (with the tissue length of 22 mm) and a biopsy needle (18G). All patients had no liver biopsy contraindications and signed informed consent forms. Liver biopsy was performed under the guidance of color Doppler ultrasound. Liver tissue samples of more than 2 cm in length and more than 6 intact portal veins were required. The liver specimens were first assessed by two pathology experts from the hospital and then by a senior pathologist from the Department of Pathology, Fudan University, China. The degrees of liver necroinflammatory activity and liver fibrosis were evaluated according to the Metavir system[19]. A Metavir necroinflammatory activity score of ≥ 2 (A2) and ≥ 3 (A3) indicated significant and severe liver inflammation, respectively. A Metavir fibrosis score of ≥ 2 (F2), ≥ 3 (F3), and ≥ 4 (F4) indicated significant liver fibrosis, advanced fibrosis, and cirrhosis, respectively. In accordance with the guidelines, the treatment indications of patients with ALT < ULN were ≥ A2 or/and ≥ F2. Hence, these patients were divided into treatment indication group and nontreatment indication group (< A2 and < F2).

Statistical analysis

Propensity score-matched analysis was used to reduce the effect of selection bias and potential confounding between the two groups. According to the HBV DNA levels of EASL and CMA guidelines, the low/moderate replication group and high replication group were matched at a ratio of 1:1 (nearest neighbor matching within caliper) based on sex, age, ALB, GLB, AGR, ALT, AST, ALP, GGT, WBC, NLR, PLT, APRI, FIB-4, and LIF-5.

The data were analyzed *via* SPSS software version 22.0 (SPSS Inc., IL, United States). HBV DNA levels were expressed as logarithms. The normally distributed variables were presented as means with standard deviations analyzed by using independent-samples *t* test (two datasets). The non-normal distribution variables were expressed as medians (Q1-Q3) analyzed by using nonparametric tests (Mann-Whitney *U* test) for two datasets. The chi-square test was used for categorical data. Ridit analysis and Spearman's rank correlation analysis were used for ranked data. The binary logistic regression analysis was performed taking liver histopathological severity (A and F) as the dependent variables and relevant factors (*P* value < 0.1) as independent variables. The dependent variables were < A2 *vs* ≥ A2, < F2 *vs* ≥ F2, and nontreatment indication *vs* treatment indication. The relevant factors were analyzed, and the diagnostic value was evaluated using receiver operating characteristic (ROC) curve and the area under the ROC curve (AUROC). All tests were two tailed, and statistical significance was set at *P* value < 0.05.

RESULTS

Enrolled patients

There were 50 patients were excluded due to incomplete data of liver pathology, HBeAg, ALB, ALP, WBC, neutrophils, and lymphocytes, and 615 patients were excluded as ALT > 1 × ULN. The flow diagram of the study population is shown in Figure 1. Finally, 634 patients were included in the study, among which 336 (EASL guidelines) and 377 (CMA guidelines) were classified into the low/moderate replication group, including 49 Low-replication (HBV DNA < 2 × 10^3); 298 (EASL guidelines) and 257 (CMA guidelines) were divided into the high replication group.

Baseline characteristics

The baseline characteristics of 634 patients were divided according to liver pathology which are shown in Table 1. The mean age of the participants was 35.61 ± 10.30 years, the mean ALT, AST, and HBV DNA levels were 23.77 ± 8.58 U/L, 24.15 ± 8.91 U/L, and 6.18 ± 1.87 Log IU/mL, respectively. Among these patients, 349 (55.05%) were men and 432 (68.14%) were HBeAg positive.

Patients with liver inflammation A0, A1, A2, and A3 were 117 (18.45%), 381 (60.10%), 97 (15.30%), and 39 (6.15%), with liver fibrosis F0, F1, F2, F3, and F4 were 148 (23.34%), 332 (52.37%), 87 (13.72%), 37 (5.84%), and 30 (4.73%), respectively. Patients with ≥ A2 accounted for 21.45% (136 patients), ≥F2 for 24.29% (154 patients), and treatment indication (≥ A2 or/and ≥ F2) for 30.28% (192 patients).

Table 1 Distribution of baseline characteristics in 634 chronic hepatitis B patients with alanine transaminase < upper limit of normal

Parameters	All patients (*n* = 634)	HBV DNA levels (EASL guidelines)				HBV DNA levels (CMA guidelines)			
		Low/moderate replication (*n* = 336)	High replication (*n* = 298)	$\chi^2/t/Z/u$	*P* value	Low/moderate replication (*n* = 377)	High replication (*n* = 257)	$\chi^2/t/Z/u$	*P* value
Age, mean ± SD, yr	35.61 ± 10.30	38.09 ± 9.99	32.82 ± 9.95	6.640	< 0.001	37.34 ± 10.17	33.07 ± 9.99	5.222	< 0.001
Male, *n* (%)	349 (55.05)	192 (57.14)	157 (52.68)	1.269	0.260	209 (55.44)	140 (54.47)	0.057	0.811
HBeAg positive, *n* (%)	432 (68.14)	142 (42.26)	290 (97.31)	220.486	< 0.001	180 (47.74)	252 (98.05)	178.165	< 0.001
ALB, mean ± SD, g/L	42.72 ± 4.45	42.91 ± 4.97	42.49 ± 3.78	1.189	0.235	42.75 ± 4.88	42.67 ± 3.74	0.220	0.826
GLB, mean ± SD, g/L	27.82 ± 4.20	27.96 ± 4.11	27.66 ± 4.30	0.893	0.372	28.11 ± 4.19	27.40 ± 4.18	2.105	0.036
AGR, mean ± SD	1.57 ± 0.29	1.57 ± 0.30	1.57 ± 0.28	-0.148	0.882	1.56 ± 0.30	1.59 ± 0.28	-1.581	0.114
ALT, mean ± SD, U/L	23.77 ± 8.58	24.84 ± 8.66	22.56 ± 8.34	3.372	0.001	24.38 ± 8.69	22.87 ± 8.35	2.186	0.029
AST, mean ± SD, U/L	24.15 ± 8.91	25.00 ± 7.52	23.20 ± 10.18	2.558	0.011	25.04 ± 10.28	22.85 ± 6.17	3.069	0.002
ALP, mean ± SD, U/L	71.26 ± 26.06	71.52 ± 24.85	70.96 ± 27.39	0.269	0.788	71.21 ± 24.99	71.32 ± 27.60	-0.054	0.957
GGT, median (Q1-Q3), U/L	18.00 (13.00-25.00)	20.00 (15.00-30.00)	16.00 (13.00-23.00)	4.966	< 0.001	19.00 (14.00-29.00)	16.00 (13.00-23.00)	4.069	< 0.001
WBC count, mean ± SD, $\times 10^9$/L	5.39 ± 1.42	5.33 ± 1.39	5.47 ± 1.45	-1.208	0.228	5.33 ± 1.44	5.48 ± 1.39	-1.321	0.187
NLR, mean ± SD	2.03 ± 1.24	2.05 ± 1.37	2.01 ± 1.08	0.443	0.658	2.04 ± 1.33	2.01 ± 1.09	0.298	0.766
PLT count, mean ± SD, $\times 10^9$/L	175.67 ± 48.83	163.93 ± 47.39	188.90 ± 47.09	-6.641	< 0.001	166.86 ± 47.55	188.58 ± 47.90	-5.629	< 0.001
HBV DNA, mean ± SD, log IU/mL	6.18 ± 1.87	4.68 ± 1.26	7.88 ± 0.50	-41.111	< 0.001	4.95 ± 1.41	7.99 ± 0.44	-33.427	< 0.001
APRI, median (Q1–Q3)	0.33 (0.25-0.44)	0.36 (0.28-0.51)	0.29 (0.23-0.38)	6.936	< 0.001	0.36 (0.27-0.48)	0.30 (0.23-0.38)	5.725	< 0.001
FIB-4, median (Q1-Q3)	0.99 (0.69-1.38)	1.13 (0.83-1.61)	0.80 (0.58-1.13)	8.109	< 0.001	1.09 (0.79-1.56)	0.81 (0.59-1.13)	6.877	< 0.001
LIF-5, mean ± SD	0.40 ± 0.15	0.45 ± 0.15	0.36 ± 0.14	7.832	< 0.001	0.44 ± 0.15	0.35 ± 0.14	7.195	< 0.001
Liver inflammatory activity									
A0, *n* (%)	117 (18.45)	58 (17.26)	59 (19.80)	4.189	< 0.001	61 (16.18)	56 (21.79)	4.426	< 0.001
A1, *n* (%)	381 (60.10)	174 (51.79)	207 (69.46)			206 (54.64)	175 (68.09)		
A2, *n* (%)	97 (15.30)	73 (21.73)	24 (8.05)			78 (20.69)	19 (7.39)		
A3, *n* (%)	39 (6.15)	31 (9.23)	8 (2.68)			32 (8.49)	7 (2.72)		
≥ A2, *n* (%)	136 (21.45)	104 (30.95)	32 (10.74)	38.299	< 0.001	110 (29.18)	26 (10.12)	32.952	< 0.001
Liver fibrosis									
F0, *n* (%)	148 (23.34)	61 (18.15)	87 (29.19)	6.382	< 0.001	70 (18.57)	78 (30.35)	6.053	< 0.001
F1, *n* (%)	332 (52.37)	153 (45.54)	179 (60.07)			179 (47.48)	153 (59.53)		
F2, *n* (%)	87 (13.72)	65 (19.35)	22 (7.38)			70 (18.57)	17 (6.61)		
F3, *n* (%)	37 (5.84)	29 (8.63)	8 (2.68)			30 (7.96)	7 (2.72)		

F4, *n* (%)	30 (4.73)	28 (8.33)	2 (0.67)			28 (7.43)	2 (0.78)		
≥ F2, *n* (%)	154 (24.29)	122 (36.31)	32 (10.74)	56.155	< 0.001	128 (33.95)	26 (10.12)	47.212	< 0.001
Treatment indication									
< A2 and < F2, *n* (%)	442 (69.72)	191 (56.85)	251 (84.23)	56.089	< 0.001	224 (59.42)	218 (84.82)	46.730	< 0.001
≥ A2 or/and ≥ F2, *n* (%)	192 (30.28)	145 (43.15)	47 (15.77)			153 (40.58)	39 (15.18)		

Quantitative data of normal distribution were expressed as mean ± standard deviation, non-normal distribution data were expressed as median (Q1-Q3), and categorical data were expressed as frequency and percentage. A: Liver inflammatory activity; AGR: Albumin–globulin ratio; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: Aspartate transaminase to platelet ratio index, APRI = [(AST/ULN)/platelet counts (10^9/L)] × 100[16]; AST: Aspartate aminotransferase; CMA: Chinese Medical Association; EASL: European Association for the Study of the Liver; F: Liver fibrosis; GLB: Globulin; FIB-4: Fibrosis-4, FIB-4 = [age (year) × AST (U/L)]/(platelet count (10^9/L) × [ALT (U/L)$^{1/2}$][17]; GGT: Gamma-glutamyl transpeptidase; HBeAg: Hepatitis B e antigen; LIF-5: Liver inflammation and fibrosis-5, LIF-5 = 0.725 + 0.005 × age (year) + 0.003 × ALT (U/L) + 0.004 × AST (U/L) - 0.201 × (A/G) - 0.002 × PLT (10^9/L)[18]; NLR: Neutrophils lymphocytes ratio; PLT: Platelets; WBC: White blood cell.

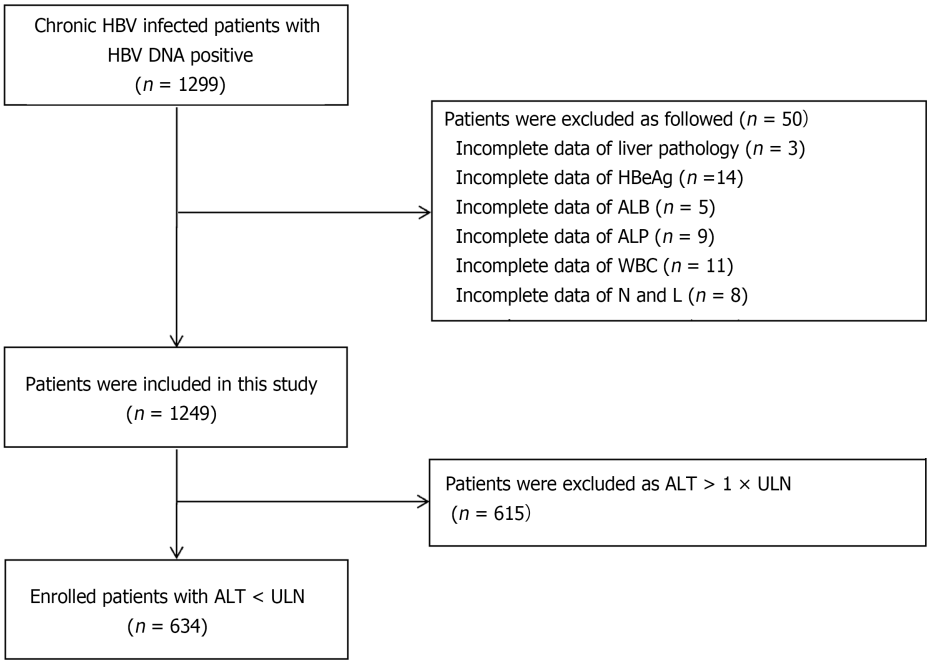

Figure 1 Flow diagram of enrolled patients. ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; L: Lymphocyte; N: Neutrophils; WBC: White blood cell; HBV: Hepatitis B virus; ULN: Upper limit of normal.

Comparisons of clinical indexes and liver pathological changes among 634 patients with different HBV DNA levels (EASL and CMA guidelines)

According to the EASL and CMA guidelines, the low/moderate replication group was comprised of 336 and 298 patients while the high replication group comprised 377 and 257 patients. Patients with high HBV DNA levels had a higher HBeAg-positive composition for both the two guidelines (χ^2 = 220.486 and 178.165, P < 0.001). Compared with the low/moderate replication group, patients in the high replication group had lower age, ALT, AST, GGT, APRI, FIB-4, and LIF-5, and higher PLT. The results were detailed in Table 1.

In general, liver histopathological severity degree in the low/moderate replication group was of the most serious forms regardless of liver inflammation or liver fibrosis. The average Ridit values of necroinflammatory activity grading in the low/moderate replication group, and high replication group were 0.544 and 0.451 (EASL guidelines), 0.541 and 0.440 (CMA guidelines), respectively, with statistically significant differences (u = 4.189, 4.426; r = -0.183, -0.194; P < 0.001). The average Ridit values of liver fibrosis staging in the low/moderate replication group and high replication group were 0.567 and 0.424 (EASL guidelines), 0.556 and 0.418 (CMA guidelines), respectively, with statistically significant differences (u = 6.382, 6.053; r = -0.271, -0.257; P < 0.001). There were 104 (30.95%) and 32 (10.74%) (EASL guidelines), 110 (29.18%) and 26 (10.12%) (CMA guidelines) patients with liver inflammatory activity ≥ 2 (≥ A2) in the two groups, respectively (χ^2 = 38.299, 32.952; P < 0.001). Patients with liver fibrosis ≥ 2 (≥ F2) in the two groups were 122 (36.31%) and 32 (10.74%) (EASL guidelines), 128 (33.95%) and 26

(10.12%) (CMA guidelines), respectively (χ^2 = 56.155, 47.212; P < 0.001). Simultaneously, the number of patients with treatment indication (≥ A2 or/and ≥ F2) in the two groups were 145 (43.15%) and 47 (15.77%) by the EASL guidelines, or 153 (40.58%) and 39 (15.18%) by the CMA guidelines (χ^2 = 56.089, 46.730; P < 0.001). The results are displayed in Table 1 and Figure 2.

In addition, there were 16 (32.65%), 20 (40.82%), and 22 (44.90%) patients with liver inflammatory activity ≥ 2 (≥ A2), liver fibrosis ≥ 2 (≥ F2), and treatment indication (≥ A2 or/and ≥ F2) in the low-replication (HBV DNA < 2 × 10^3, 49 cases), respectively.

Predictors of significant liver histology in 634 patients

The univariate analysis indicated that the statistically significant variables which could affect liver inflammation activity, liver fibrosis, and treatment indications were age, HBeAg, ALB, GLB, AGR, ALT, AST, GGT, PLT, HBV DNA, APRI, FIB-4, and LIF-5. The results are demonstrated in Table 2.

The logistics analysis showed that HBV DNA level (negative correlation), age (negative correlation), GGT level (positive correlation), and LIF-5 value (positive correlation) were independent risk factors for liver inflammation activity; HBV DNA level (negative correlation), GGT level (positive correlation), APRI value (positive correlation), and LIF-5 value (positive correlation) were independent risk factors for liver fibrosis; HBV DNA level (negative correlation), APRI value (positive correlation), and LIF-5 value (positive correlation) were independent risk factors for treatment indications. The AUROC of the prediction probabilities (PRE_) of the abovementioned models (< A2 *vs* ≥ A2, < F2 *vs* ≥ F2, < A2 and < F2 *vs* ≥ A2 or/and ≥ F2) was 0.814 (95%CI: 0.770-0.859), 0.824 (95%CI: 0.785-0.863), and 0.799 (95%CI: 0.760-0.838), respectively. Considering that the diagnostic models such as APRI, FIB-4, and LIF-5 contained some of the indices, the HBV DNA level (negative correlation) was still an independent risk factor for the dependent variables after the diagnostic models were eliminated. The results are listed in Table 3 and Figure 3.

Predictors of significant liver histology in propensity score-matched pairs

To minimize the effect of potential confounders in the comparison of liver histology damages (< A2 and < F2 *vs* ≥ A2 or/and ≥ F2) between the low/moderate replication group and high replication group, we matched 316 pairs (EASL guidelines) and 277 pairs (CMA guidelines) of patients by propensity score-matching. In these pairs, there were no significant differences between the low/moderate replication and high replication groups (P > 0.05) in the baseline characteristics (sex, age, ALB, GLB, AGR, ALT, AST, ALP, GGT, WBC, NLR, PLT, APRI, FIB-4, and LIF-5) (Table 4), hence achieving covariate balance. For the propensity score-matched pairs, both EASL and CMA guidelines, the group of significant liver histology damage (≥ A2 or/and ≥ F2) had much lower HBV DNA levels than that of the non- significant liver histology damage group (< A2 and < F2) (EASL guidelines: 5.81 ± 1.23 Log IU/mL *vs* 7.90 ± 0.49 Log IU/mL, t = -27.967, P < 0.001; CMA guidelines: 5.78 ± 1.42 Log IU/mL *vs* 8.01 ± 0.43 Log IU/mL, t = -24.922, P < 0.001) (Table 4).

DISCUSSION

At present, chronic HBV infections are classified by treatment indications which are based mainly on serum HBV DNA, ALT, and liver disease severity[2,4-7]. The treatment indications were easy to identify clinically. However, we should pay more attention to those not meeting treatment indications (the so-called gray-zone patients), and there is a considerable number of such people. One retrospective cohort study[20] involved 3366 CHB patients came from 5 clinical centers of America and 7 towns of Taiwan, China which were followed up for at least 1 year and the mean time was 12.5 years. Staging of the disease was determined according to the American Association for the Study of Liver Diseases (AASLD) 2018 hepatitis B guidance[21]. The result showed that patients in the indeterminate phase count for 50.9% in American cohort and 31.8% in Taiwan, China with an average of 38.7%. Yao *et al*[22] also adopted the same guidelines (ALT < ULN, male for 35 U/L and female for 25 U/L), and 4759 CHB patients in Nanjing, China were included among which 27.8% were in the indeterminate phase.

According to the guidelines/CHB treatment algorithm in the United States[2,4,5], the 'gray-zone' population is defined as the following: (1) ALT < ULN and HBV DNA < 2 × 10^3 IU/mL (most are HBeAg negative, that is, inactive CHB or HBeAg-negative HBV infection); (2) ALT continues to be normal, 2 × 10^3 ≤ HBV DNA ≤ 10^7 IU/mL (EASL guidelines) or ≤ 2 × 10^7 IU/mL (CMA guidelines), that is, CHB in the indeterminate phase; and (3) immune-tolerant CHB (HBV DNA > 10^7 IU/mL or > 2 × 10^7 IU/mL).

Actually, it is an indisputable fact that a high proportion of the 'gray-zone' population still have disease progression[8,23-30]. A previous study found that in the 'gray-zone' population with ALT < 2 × ULN, 510 of 1148 patients (44.42%) had liver pathological changes ≥ A2 or/and ≥ F2, and in those with ALT < 1 × ULN, nearly 30% had liver pathological changes ≥ A2 or/and ≥ F2[31], regardless of the ULN cutoff of ALT (50 U/L or 30 U/L for men; 40 U/L or 19 U/L for women). In this study, among 634 patients with ALT < ULN, 136 (21.45%) had liver inflammation ≥ A2, 154 (24.29%) had liver fibrosis ≥ F2, and 192 (30.28%) had treatment indications (≥ A2 or/and ≥ F2). The judgment of treatment

Table 2 Comparison of parameters among patients with alanine transaminase < upper limit of normal and different pathological states (Entire cohort)

Parameters	Liver inflammatory activity		Liver fibrosis		Treatment indication		*P* value
	< A2 (*n* = 498)	≥ A2 (*n* = 136)	< F2 (*n* = 480)	≥ F2 (*n* = 154)	< A2 and < F2 (*n* = 442)	≥ A2 or/and ≥ F2 (*n* = 192)	
Age, mean ± SD, yr	34.68 ± 9.88	39.02 ± 11.10	34.36 ± 9.86	39.52 ± 10.71	34.28 ± 9.84	38.69 ± 10.70	< 0.001, < 0.001, < 0.001
Male, *n* (%)	268 (53.82)	81 (59.56)	253 (52.71)	96 (62.34)	234 (52.94)	115 (59.90)	0.232, 0.037, 0.105
HBeAg positive, *n* (%)	354 (71.08)	78 (57.35)	350 (72.92)	82 (53.25)	323 (73.08)	109 (56.77)	0.002, < 0.001, < 0.001
ALB, mean ± SD, g/L	43.06 ± 3.96	41.45 ± 5.75	42.96 ± 4.32	41.95 ± 4.75	43.06 ± 4.01	41.92 ± 5.24	< 0.001, 0.014, 0.003
GLB, mean ± SD, g/L	27.46 ± 4.09	29.14 ± 4.35	27.54 ± 4.06	28.70 ± 4.51	27.49 ± 4.08	28.57 ± 4.38	< 0.001, 0.003, 0.003
AGR, mean ± SD	1.60 ± 0.27	1.46 ± 0.33	1.59 ± 0.28	1.50 ± 0.31	1.60 ± 0.27	1.50 ± 0.32	< 0.001, 0.001, < 0.001
ALT, mean ± SD, U/L	22.81 ± 8.48	27.26 ± 8.07	22.80 ± 8.48	26.80 ± 8.20	22.45 ± 8.43	26.80 ± 8.16	< 0.001, < 0.001, < 0.001
AST, mean ± SD, U/L	22.93 ± 6.24	28.62 ± 14.26	22.79 ± 6.41	28.41 ± 13.26	22.64 ± 6.26	27.63 ± 12.45	< 0.001, < 0.001, < 0.001
ALP, mean ± SD, U/L	68.68 ± 24.45	80.67 ± 29.48	68.62 ± 23.35	79.49 ± 31.80	68.58 ± 23.86	77.41 ± 29.68	< 0.001, < 0.001, < 0.001
GGT, mean ± SD, U/L	20.24 ± 13.58	34.91 ± 33.78	19.87 ± 13.63	34.34 ± 31.87	19.91 ± 13.78	31.38 ± 29.60	< 0.001, < 0.001, < 0.001
WBC count, mean ± SD, × 10^9/L	5.40 ± 1.41	5.33 ± 1.53	5.43 ± 1.44	5.26 ± 1.40	5.41 ± 1.42	5.33 ± 1.46	0.622, 0.210, 0.491
NLR, mean ± SD	2.05 ± 1.21	1.96 ± 1.35	2.07 ± 1.31	1.91 ± 0.99	2.04 ± 1.21	2.02 ± 1.32	0.447, 0.158, 0.824
PLT count, mean ± SD, × 10^9/L	184.72 ± 44.67	142.52 ± 49.26	186.16 ± 44.28	142.94 ± 47.99	187.73 ± 43.49	147.90 ± 49.24	< 0.001, < 0.001, < 0.001
HBV DNA, mean ± SD, log IU/mL	6.35 ± 1.91	5.56 ± 1.59	6.44 ± 1.87	5.39 ± 1.64	6.47 ± 1.88	5.52 ± 1.67	< 0.001, < 0.001, < 0.001
APRI, mean ± SD	0.33 ± 0.14	0.59 ± 0.42	0.32 ± 0.13	0.58 ± 0.39	0.32 ± 0.12	0.54 ± 0.37	< 0.001, < 0.001, < 0.001
FIB-4, mean ± SD	1.01 0.53	1.81 ± 1.34	0.97 ± 0.49	1.81 ± 1.28	0.96 ± 0.49	1.67 ± 1.21	< 0.001, < 0.001, < 0.001
LIF-5, mean ± SD	0.37 ± 0.13	0.54 ± 0.17	0.36 ± 0.12	0.53 ± 0.16	0.36 ± 0.12	0.51 ± 0.16	< 0.001, < 0.001, < 0.001

Quantitative data of normal distribution were expressed as mean ± standard deviation, and categorical data were expressed as frequency and percentage. A: Liver inflammatory activity; AGR: Albumin–globulin ratio; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: Aspartate transaminase to platelet ratio index, APRI = [(AST/ULN)/platelet counts (10^9/L)] × 100[16]; AST: Aspartate aminotransferase; F: Fibrosis; FIB-4: Fibrosis-4, FIB-4 = [age (year) × AST (U/L)]/(platelet count (10^9/L) × [ALT (U/L)$^{1/2}$][17]; GGT: Gamma-glutamyl transpeptidase; HBeAg: Hepatitis B e antigen; GLB, globulin; LIF-5: Liver inflammation and fibrosis-5, LIF-5 = 0.725 + 0.005 × age (year) + 0.003 × ALT (U/L) + 0.004 × AST (U/L) - 0.201 × (A/G) - 0.002 × PLT (10^9/L)[18]; NLR: Neutrophils lymphocytes ratio; PLT: Platelets; WBC: White blood cell.

indications is not only based on ALT level, although ALT is the most commonly used surrogate indicator reflecting liver cell damage. In addition, other surrogate markers including non-invasive tests have been rapidly developed[32-35]. In current, APRI and FIB-4 are the most widely used diagnostic models, but they are not that accurate in assessing the degree of HBV-related liver fibrosis[36]. In a previous study, a linear diagnosis model LIF-5 [LIF-5 = 0.725 + 0.005 × age + 0.003 × ALT + 0.004 × AST - 0.201 × (A/G) - 0.002 × PLT (10^9/L)][18] was constructed for the treatment indication judgment (A≥ 2 and/or F ≥ 2) of CHB patients with ALT < 2 × ULN, which had higher diagnostic value than APRI and FIB-4. This study also confirmed that the LIF-5 value (positive correlation) was an independent risk factor for liver inflammation activity, liver fibrosis, and treatment indication in CHB patients with ALT < ULN.

Table 3 Predictors of significant liver histology in 634 patients with chronic hepatitis B and alanine transaminase < upper limit of normal detected using a step-forward binary logistic regression model

Variables		Regression coefficient	Standard error	Wald's value	*P* value	OR	OR 95%CI	
							Upper limit	Lower limit
< A2 *vs* ≥ A2	Age	-0.039	0.014	7.853	0.005	0.962	0.936	0.988
	GGT	0.015	0.006	5.970	0.015	1.015	1.003	1.027
	HBV DNA	-0.147	0.064	5.306	0.021	0.863	0.761	0.978
	LIF-5	9.616	1.152	69.727	0.000	15002.994	1570.174	143353.477
	Constant	-3.643	0.685	28.240	0.000	0.026		
Eliminate diagnostic models	HBV DNA	-0.161	0.063	6.429	0.011	0.851	0.752	0.964
< F2 *vs* ≥ F2	GGT	0.016	0.006	5.968	0.015	1.016	1.003	1.029
	HBV DNA	-0.229	0.061	14.251	0.000	0.796	0.707	0.896
	APRI	2.747	0.921	8.890	0.003	15.593	2.563	94.859
	LIF-5	4.759	1.285	13.708	0.000	116.591	9.390	1447.723
	Constant	-3.392	0.577	34.608	0.000	0.034		
Eliminate diagnostic models	HBV DNA	-0.247	0.061	16.523	0.000	0.781	0.693	0.880
< A2 and < F2 *vs* ≥ A2 or/and ≥ F2	HBV DNA	-0.199	0.055	13.233	0.000	0.820	0.737	0.913
	APRI	3.124	0.888	12.393	0.000	22.747	3.995	129.530
	LIF-5	4.733	1.182	16.038	0.000	113.618	11.207	1151.897
	Constant	-2.903	0.522	30.931	0.000	0.055		
Eliminate diagnostic models	HBV DNA	-0.221	0.056	15.782	0.000	0.801	0.718	0.894

A: Liver inflammatory activity; APRI: Aspartate transaminase to platelet ratio index, APRI = [(AST/ULN)/platelet counts (10^9/L)] × 100[16]; AST: Aspartate aminotransferase; F: Fibrosis; GGT: Gamma-glutamyl transpeptidase; LIF-5: Liver inflammation and fibrosis-5, LIF-5 = 0.725 + 0.005 × age (year) + 0.003 × ALT (U/L) + 0.004 × AST (U/L) - 0.201 × (A/G) - 0.002 × PLT (10^9/L)[18]; HBV: Hepatitis B virus.

The univariate analysis suggested that the above indexes or diagnostic models, such as gender male, HBeAg negativity, increase in age, GLB, ALT, AST, GGT, APRI, FIB-4, and LIF-5, and decrease in ALB, AGR and PLT were correlated with the liver histopathological severity. However, during the logistic regression analysis, only HBV DNA (negative correlation) and LIF-5 (positive correlation) were independent risk factors for liver histopathological severity (liver inflammatory activity, liver fibrosis, and treatment indication). After excluding the diagnostic models, HBV DNA (negative correlation) was still an independent risk factor for the dependent variables mentioned above. Regardless of ALT level, both entire cohort and propensity score-matched pairs, patients in the low/moderate replication group had more serious liver disease (including liver pathological changes and hematological indicators). And patients with A ≥ 2, F ≥ 2, and treatment indication (≥ A2 or/and ≥ F2) in the low-replication group (HBV DNA < 2 × 10^3) accounted for 32.65%, 40.82%, and 44.90%, respectively, while patients in the high replication group had relatively mild pathological changes. In the entire cohort, the mean HBV DNA levels were 6.35 ± 1.91 Log IU/mL and 5.56 ± 1.59 Log IU/mL for liver inflammatory activity <A2 and ≥ A2; 6.44 ± 1.87 Log IU/mL and 5.39 ± 1.64 Log IU/mL for liver fibrosis < F2 and ≥ F2; and 6.47±1.88 Log IU/mL and 5.52 ± 1.67 Log IU/mL at treatment indication (< A2 and < F2) and (≥ A2 or/and ≥ F2), respectively. In the propensity score-matched pairs, as treatment indication (< A2 and < F2) and (≥ A2 or/and ≥ F2), the mean HBV DNA levels were 7.90 ± 0.49 Log IU/mL and 5.81 ± 1.23 Log IU/mL (EASL guidelines), 8.01 ± 0.43 Log IU/mL and 5.78 ± 1.42 Log IU/mL (CMA guidelines), respectively.

Obviously, it is unreasonable to set a defined value of HBV DNA to judge the state of CHB disease (natural course) and to guide whether to start treatment. First, despite the correlation between the HBV DNA level and the severity of the disease, the results are not consistent[10-13,37-39]. In this study, regardless of ALT values, patients with HBV DNA low/moderate replication had more serious liver disease. The high level of HBV DNA replication causes the deficiency and dysfunction of the HBsAg specific cytotoxic T lymphocytes, leading to the consequent immune tolerance. However, during the prolonged reproduction, HBV interacts with the host immune system, which can induce a cumulative

Table 4 Predictors of significant liver histology in propensity score-matched pairs

Variables	EASL guidelines (316 pairs)				CMA guidelines (277 pairs)			
	≥ A2 or/and ≥ F2	< A2 and < F2	χ^2/t/Z	SMD	≥ A2 or/and ≥ F2	< A2 and < F2	χ^2/t/Z	SMD
Male, *n* (%)	171 (54.11)	168 (53.16)	0.057	0.811	138 (49.82)	153 (55.23)	1.629	0.202
HBeAg positive, *n* (%)	305 (96.52)	306 (96.84)	0.049	0.824	271 (97.83)	271 (97.83)	0	1.000
Age, mean ± SD, yr	32.92 ± 10.13	33.06 ± 9.96	-0.178	0.859	32.54 ± 9.04	33.31 ± 10.07	-0.937	0.349
ALB, mean ± SD, g/L	42.30 ± 4.59	42.65 ± 3.78	-1.046	0.296	42.35 ± 4.81	42.79 ± 3.76	-1.187	0.236
GLB, mean ± SD, g/L	27.74 ± 4.83	27.74 ± 4.26	0.019	0.985	27.23 ± 4.56	27.53 ± 4.25	-0.809	0.419
AGR, mean ± SD	1.57 ± 0.33	1.57 ± 0.28	-0.037	0.971	1.59 ± 0.29	1.59 ± 0.28	0.198	0.843
ALT, mean ± SD, U/L	23.81 ± 8.29	22.78 ± 8.29	1.569	0.117	23.18 ± 9.10	23.00 ± 8.38	0.235	0.814
AST, mean ± SD, U/L	22.13 ± 8.55	22.57 ± 5.76	-0.769	0.442	21.87 ± 7.74	22.72 ± 5.70	-1.465	0.143
ALP, mean ± SD, U/L	71.64 ± 27.61	70.56 ± 27.09	0.495	0.621	72.15 ± 25.92	71.37 ± 27.39	0.345	0.730
GGT, median (Q1-Q3), U/L	16.00 (13.00–23.00)	16.00 (13.00–23.00)	0.780	0.435	16.00 (13.00-24.00)	17.00 (13.00-23.00)	0.590	0.555
WBC count, mean ± SD, $\times 10^9$/L	5.44 ± 1.27	5.47 ± 1.38	-0.276	0.783	5.51 ± 1.31	5.48 ± 1.30	0.274	0.784
NLR, mean ± SD	1.91 ± 0.80	2.01 ± 1.04	-1.431	0.153	1.89 ± 1.01	2.00 ± 1.04	-1.334	0.183
PLT count, mean ± SD, $\times 10^9$/L	183.43 ± 42.01	187.96 ± 47.38	-1.272	0.204	184.38 ± 49.39	187.11 ± 48.08	-0.659	0.510
HBV DNA, mean ± SD, log IU/mL	5.81 ± 1.23	7.90 ± 0.49	-27.967	< 0.001	5.78 ± 1.42	8.01 ± 0.43	-24.922	< 0.001
APRI, median (Q1– Q3)	0.31 (0.23-0.38)	0.29 (0.23-0.38)	0.507	0.612	0.32 (0.22–0.39)	0.30 (0.23-0.38)	-0.147	0.883
FIB-4, median (Q1–Q3)	0.80 (0.49-1.11)	0.80 (0.58-1.12)	1.460	0.144	0.74 (0.50-1.17)	0.82 (0.59-1.13)	-1.110	0.267
LIF-5, mean ± SD	0.37 ± 0.14	0.36 ± 0.14	0.903	0.367	0.35 ± 0.14	0.36± 0.14	-0.187	0.852

Low/moderate replication, hepatitis B virus (HBV) DNA ≤ 10^7 IU/mL; high replication, HBV DNA > 10^7 IU/mL. Quantitative data of normal distribution were expressed as mean ± standard deviation, non-normal distribution data were expressed as median (Q1-Q3), and categorical data were expressed as frequency and percentage. AGR: Albumin–globulin ratio; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: Aspartate transaminase to platelet ratio index, APRI = [(AST/ULN)/platelet counts (10^9/L)] × 100[16]; AST: Aspartate aminotransferase; CMA: Chinese Medical Association; EASL: European Association for the Study of the Liver; F: Liver fibrosis; GLB: Globulin; FIB-4: Fibrosis-4, FIB-4 = [age (year) × AST (U/L)]/(platelet count (10^9/L) × [ALT (U/L)$^{1/2}$][17]; GGT: Gamma-glutamyl transpeptidase; HBeAg: Hepatitis B e antigen; LIF-5: Liver inflammation and fibrosis-5, LIF-5 = 0.725 + 0.005 × age (year) + 0.003 × ALT (U/L) + 0.004 × AST (U/L) - 0.201 × (A/G) - 0.002 × PLT (10^9/L)[18]; NLR: Neutrophils lymphocytes ratio; PLT: Platelets; WBC: White blood cell; HBV: Hepatitis B virus.

immune damage. The hepatocytes suffer occult and persistent pathological apoptosis, with HBV DNA decreases accordingly, while the liver damage continues[40].

Therefore, in the absence of liver pathology, can individuals with HBV DNA < 2 × 10^3 IU/mL be identified as the 'inactive carriers' when ALT or/and transient elastography (TE) and other indicators are normal? However, whether individuals with 2 × 10^3 ≤ HBV DNA ≤ 10^7 IU/mL can be identified as immune tolerant is still unclear (the indeterminate phase). Second, the correlation ship between the HBV DNA level and the progression of end-stage liver disease (such as HCC) is still controversial. Patients in the indeterminate phase without antiviral therapy had significantly higher risk of developing HCC than those in inactive phase[20,41]. Although patients with high HBV DNA level have a heighten risk of HCC progression in the immune tolerant phase, different studies have still held different views [42-44]. This study has shown the same option with the other studies that the proportion of patients with significant liver tissue damage and HCC progression in the immune tolerant phase is relatively low[43,44], and whether conduct antiviral therapy for them has always been a hot controversy topic on

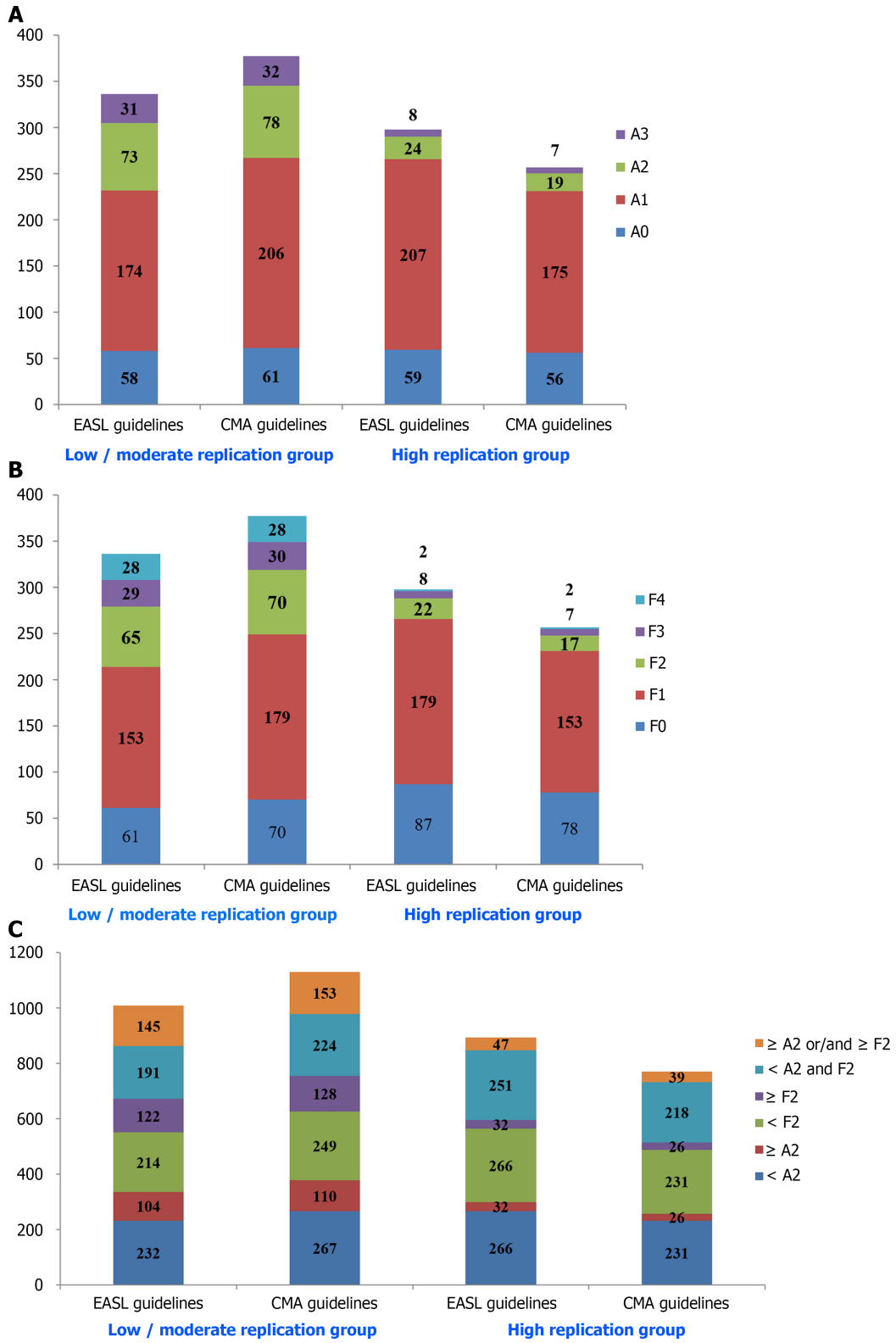

Figure 2 Liver pathological changes among 634 patients with normal alanine transaminase at different hepatitis B virus DNA levels. A: Necroinflammatory activity grading among patients at different hepatitis B virus (HBV) DNA levels; B: Liver fibrosis staging among patients at different HBV DNA levels; C: Liver histopathological severity ≥ A2 or/and ≥ F2 among patients at different HBV DNA levels. A: Liver inflammatory activity; F: Liver fibrosis; CMA: Chinese Medical Association; EASL: European Association for the Study of the Liver.

clinic. The patients with a low HBV DNA level (low-level viremia) still have high risk of disease progression[13,45-48]. Third, for CHB patients in the immune active phase, the defined value of HBV DNA exceeds 2×10^3 IU/mL when HBeAg is negative, while the value of HBV DNA exceeds 10^7 IU/mL or 2×10^7 IU/mL when HBeAg is positive, for which needs further exploration.

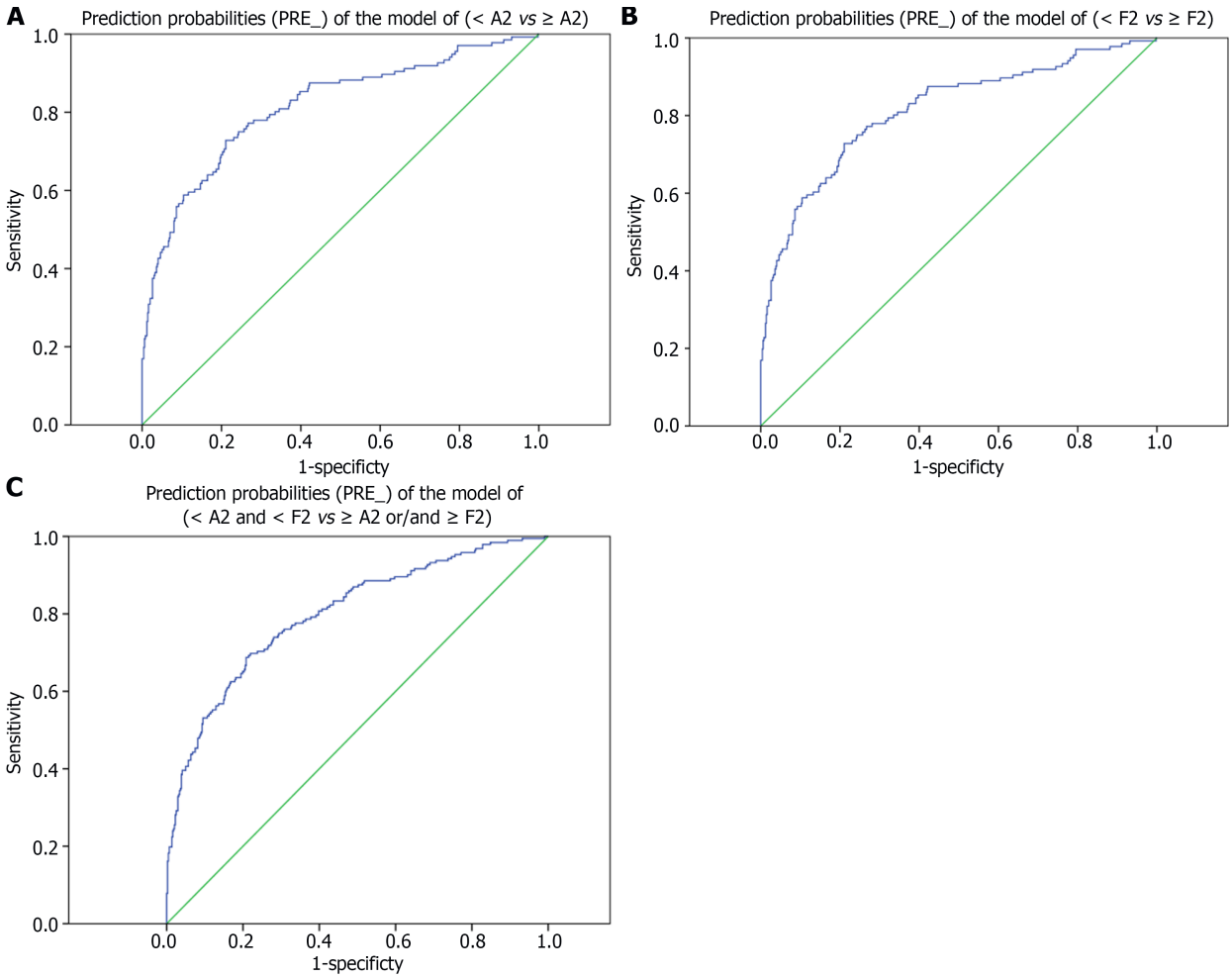

Figure 3 Prediction probabilities (PRE_) of the binary logistic regression model. A: < A2 *vs* ≥ A2; B: < F2 *vs* ≥ F2; C: < A2 and < F2 *vs* ≥ A2 or/and ≥ F2). A: Liver inflammatory activity; F: Liver fibrosis.

There were some limitations in this study. First, the ULN of ALT was 40 U/L, while the AASLD guidelines recommend 35 IU/L for men and 25 IU/L for women[6]. The lower ULN of ALT may help us find more suitable patients needed for treatment. Second, this study had a large time span, the patients enrolled earlier had no TE results due to the absence of the Fibroscan test. Third, it was a cross-sectional study, thus lacking follow-up data. The last, this study didn't determine the HBV genotypes. The dominant genotypes in China are genotype B and C with higher incidence of mother to child transmission, and genotype C infections are more prone to progress to HCC earlier[2,40]. These limitations need to be addressed in the further studies.

CONCLUSION

In summary, this study analyzed the risk factors for liver histopathological severity in CHB patients with normal ALT. It found that HBV DNA (negative correlation) was an independent risk factor for liver disease progression. Because of the widespread use of first-line antiviral drugs and the underlying idea of 'no virus, no disease', it was presumed that the states of CHB disease (natural course) were not that suitable judged by the defined values of HBV DNA level. The classification of CHB may be revised based on whether HBV DNA exceeds the detection value. Patients who are in the indeterminate phase or regarded as the 'inactive carriers' (low HBV DNA, low-level viremia) should receive antiviral therapy.

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis B (CHB) patients can be divided into treatment indication and non-treatment indication individuals. Normal alanine transaminase (ALT) patients in 'immune-tolerant' phase with hepatitis B virus (HBV) DNA higher than 10^7 or 2×10^7 IU/mL and in 'inactive-carrier' phase with HBV DNA lower than 2×10^3 IU/mL do not require antiviral therapy. In fact, we should pay more attention to those who do not match the treatment indications (gray-zone patients both in the indeterminate phase and in the 'inactive-carrier' phase).

Research motivation

In order to analyze the correlation of HBV DNA level and liver histopathological severity, and to explore the significance of HBV DNA for CHB with normal ALT. Patients who are in the indeterminate phase or regarded as the 'inactive carriers' (low HBV DNA, low-level viremia) may have severe liver disease pathologically and hematologically.

Research objectives

The states of CHB disease (natural course) were not that suitable judged by the defined values of HBV DNA level. The classification of CHB may be revised based on whether HBV DNA exceeds the detection value. Patients who are in the indeterminate phase or regarded as the 'inactive carriers' (low HBV DNA, low-level viremia) should receive antiviral therapy.

Research methods

From January 2017 to December 2021, a retrospective cross-sectional set of 1299 patients with chronic HBV infection (HBV DNA > 30 IU/mL) who underwent liver biopsy from four hospitals, including 634 with ALT less than 40 U/L. The degrees of liver necroinflammatory activity and liver fibrosis were evaluated according to the Metavir system. Patients were divided into two groups: Low/moderate replication group, HBV DNA ≤ 10^7 IU/mL [the European Association for the Study of the Liver (EASL) guidelines] or ≤ 2×10^7 IU/mL [the Chinese Medical Association (CMA) guidelines]; high replication group, HBV DNA > 10^7 IU/mL or > 2×10^7 IU/mL. Relevant factors for liver histopathological severity were analyzed by univariate analysis, logistics analysis and propensity score-matched analysis.

Research results

At entry, there were 21.45%, 24.29%, and 30.28% of the patients had liver histopathological severities with ≥ A2, ≥ F2, and ≥ A2 or/and ≥ F2, respectively. HBV DNA level (negative correlation) and noninvasive model liver fibrosis 5 value (positive correlation) were independent risk factors for liver histopathological severities (liver necroinflammation, liver fibrosis, and treatment indication). HBV DNA level (negative correlation) was still an independent risk factor when diagnostic models were excluded. For the propensity score-matched pairs, whether based on EASL guidelines or CMA guidelines, the group with significant liver histology damage (≥ A2 or/and ≥ F2) showed much lower HBV DNA level than the group with non- significant liver histology damage (< A2 and < F2). Patients in the moderate replication group (with indeterminate phase) had the most serious liver disease pathologically and hematologically, followed by patients in the low replication group (with 'inactive-carrier' phase) and then the high replication group (with 'immune-tolerant' phase).

Research conclusions

HBV DNA level is a negative risk factor for liver damage. The phase definition of CHB may be revised by whether the level of HBV DNA exceeds the detection low limit value. Normal ALT patients who are in the indeterminate phase or 'inactive carriers' should receive antiviral therapy.

Research perspectives

How to define the natural history of chronic HBV infection and how to identify the patients with normal ALT who need treatment?

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FOOTNOTES

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Lian X, Shi XJ, Zhu DD, Wang ZY, Huang GC contributed to the provision of study materials or patients; Jiang SW, Lu JL, He ZY contributed to the collection and assembly of data; Jiang SW, Hu AR contributed to the data analysis and interpretation; and all authors final approval of manuscript.

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ORCID number: Su-Wen Jiang 0000-0002-5617-5091; Xiang Lian 0000-0003-4967-6776; Ai-Rong Hu 0000-0002-8176-2714; Jia-Lin Lu 0000-0003-3359-8574; Zhe-Yun He 0000-0001-8831-2866; Xiao-Jun Shi 0000-0002-5904-1494; De-Dong Zhu 0000-0002-9669-0960; Zong-Yi Wang 0000-0002-7393-0060; Guan-Cheng Huang 0000-0002-0319-8804.

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ORIGINAL ARTICLE

Retrospective Study

Cholangioscopy-assisted extraction through novel papillary support for small-calibre and sediment-like common bile duct stones

Wen-Gang Zhang, Ning-Li Chai, Bo Zhang, Xiao Li, Jia-Feng Wang, Hao Dong, Yu-Jie Feng, En-Qiang Linghu

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Wen-Gang Zhang, Ning-Li Chai, Bo Zhang, Xiao Li, Jia-Feng Wang, Hao Dong, Yu-Jie Feng, En-Qiang Linghu, Department of Gastroenterology, The First Medical Center of Chinese PLA General Hospital, Beijing 100853, China

Corresponding author: En-Qiang Linghu, PhD, Chief Physician, Department of Gastroenterology, The First Medical Center of Chinese PLA General Hospital, No. 28 Fuxing Road, Haidian District, Beijing 100853, China. linghuenqiang@vip.sina.com

Abstract

BACKGROUND

To date, endoscopic retrograde cholangiopancreatography has become a well-established treatment for common bile duct (CBD) stones. However, it is not suitable for some special patients, such as pregnant women, children or those who cannot stop taking anti-coagulation/anti-platelet agents because of radiation injury and the risk of postoperative bleeding resulting from endoscopic sphincterotomy. To overcome these two problems, this study introduced cholangioscopy-assisted extraction through a novel papillary support for small-calibre and sediment-like CBD stones.

AIM

To assess the feasibility and safety of cholangioscopy-assisted extraction through a novel papillary support (CEPTS) for small-calibre and sediment-like common bile duct (CBD) stones.

METHODS

This Retrospective study was approved by the Ethics Committee of the Chinese PLA General Hospital. We designed a covered single dumbbell-style papillary support between 2021 and 2022. Between July 2022 and September 2022, 7 consecutive patients with small-calibre (cross diameter ≤ 1.0 cm) or sediment-like CBD stones underwent CETPS procedures in our center. The clinical characteristics and treatment outcomes of these 7 patients were extracted from a prospectively collected database. And the related data were analyzed. Informed consent was obtained from all participating patients.

RESULTS

A total of 2 patients had yellow sediment-like CBD stones, and aspiration extraction was performed after the insertion of papillary support. Of the 5 patients with clumpy CBD stones (0.4-1.0 cm), 2 underwent basket extraction under direct

vision for a single stone (0.5-1.0 cm, black and black grey), 1 underwent balloon plus aspiration extraction under direct vision for 5 stones (0.4-0.6 cm, brown), and 2 underwent aspiration extraction only for a single stone (0.5-0.6 cm, yellow, none). Technical success, namely, no residual stones in the CBD or left and right hepatic ducts, was achieved in all 7 cases (100%). The median operating time was 45.0 minutes (range 13.0–87.0 minutes). Postoperative pancreatitis (PEP) occurred in one case (14.3%). Hyperamylasaemia without abdominal pain was noted in 2 of 7 patients. No residual stones or cholangitis were found during the follow-up.

CONCLUSION

CETPS appeared to be feasible to treat patients with small-calibre or sediment-like CBD stones. Patients, especially pregnant women and those who cannot stop anticoagulation/anti-platelet agents, could benefit from this technique.

Key Words: Cholangioscopy; Common bile duct stones; Papillary support

Core Tip: Endoscopic retrograde cholangiopancreatography (ERCP) has become a well-established treatment for common bile duct (CBD) stones. However, the standard ERCP technique always requires endoscopic sphincterotomy (EST), which might lead to the loss of sphincter of Oddi function and some adverse events, including bleeding and perforation. Moreover, radiation injury makes the standard ERCP technique unsuitable for special patients, such as pregnant women and children. To overcome these problems, the present study introduced cholangioscopy-assisted extraction through a novel papillary support for small-calibre and sediment-like CBD stones, reducing radiation injury and avoiding EST.

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) has become the standard treatment for common bile duct (CBD) stones[1,2]. However, how to reduce radiation injury for special patients, including pregnant women and children, during this technique remains an important issue. Moreover, endoscopic sphincterotomy (EST), which is always necessary during stone extraction procedures, might lead to the loss of sphincter of Oddi (SO) function, some early adverse events, including bleeding and perforation, and some late adverse events, such as cholangitis, malignant degeneration and recurrent CBD stones, owing to free duodenobiliary reflux[3-5]. Importantly, EST was not appropriate for those patients who could not stop taking anti-coagulation/anti-platelet agents.

The emergence of peroral cholangioscopy made it possible to remove CBD stones under direct vison with less radiation injury[6,7]. However, the relatively difficult operation hindered the further development of this technique. In terms of EST, some studies introduce the self-expandable metal stent (SEMS) to avoid EST[8,9]. However, the SEMS was relatively too long and not desirable.

To overcome the problems mentioned above, we optimized the existing metal stent and made a kind of single dumbbell-style papillary support to facilitate cholangioscopy-assisted stone extraction, avoid EST and preserve SO function. In this study, we introduced cholangioscopy-assisted extraction through a novel papillary support (CETPS) for small-calibre and sediment-like CBD stones. This technique combined the advantages of the preservation of SO function and cholangioscopy-assisted stone extraction under direct vision with less radiation injury.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the Ethics Committee of the Chinese PLA General Hospital. Informed consent was obtained from all participating patients. Of note, some instruments, including the cholangioscope, basket and papillary support, were free for patients in this study.

Endoscopists made the clinical decision to perform CETPS for patients diagnosed with small-calibre (cross diameter ≤ 1.0 cm) or sediment-like CBD stones by magnetic resonance cholangiopancreatography (MRCP) and/or endoscopic ultrasound (EUS). Patients undergoing CETPS between July 2022 and September 2022 were enrolled in this study. Related data were extracted from a prospectively collected database.

The stone size was measured under MRCP on the maximal cross section. The definition of operation time was the course between the endoscope inserting into and withdrawing from the body. Technical success was defined as no residual stones in the CBD and left and right hepatic ducts, which was confirmed under cholangioscopy after stone extraction.

CETPS procedure

All procedures were performed by one endoscopist who has experience performing more than 2000 ERCP procedures in total.

First, biliary intubation was conducted. Second, a covered single dumbbell-style support (12 mm in diameter, 25-30 mm in length) was placed in the distal CBD and papilla (Figure 1A). Third, for sediment-like CBD stones, endoscopic aspiration was performed under negative pressure (Figures 1B and 2A); for single clumpy CBD stones, a cholangioscope (Micro-Tech, eyeMax, 9F) was inserted into the CBD, and basket extraction was performed through the working tunnel of the cholangioscope under direct vision (Figures 1C-E, 2B and Video 1); for multiple clumpy CBD stones, a cholangioscope (Micro-Tech, eyeMax, 11F) was inserted into the CBD, and balloon extraction was performed through the working tunnel of the cholangioscope under direct vision (Figures 1F and G, 2C and Video 1). Fourth, the cholangioscope was inserted into the CBD again to confirm whether there were remnant stones. Finally, the papillary support was removed (Figure 1H and I).

Postoperative management

Patients underwent routine blood examination and amylase and lipase tests 24 and 72 h postoperatively. Patients underwent computed tomography (CT) and/or endoscopy if abnormal symptoms or blood test parameters were found postoperatively.

Patients were fasted for 1 day after the procedure, and a liquid diet was followed for an additional 1 day if no adverse events occurred. The diet was gradually restored to normal starting on the third day. Postoperative medications mainly included a double-dose proton pump inhibitor and antibiotics for one day. Somatostatin was used if PEP occurred, and the fasting time was extended accordingly. Of note, of the 7 patients, 3 had gallstones and underwent cholecystectomy within 1 mo after CETPS to avoid the recurrence of CBD stones.

Follow-up with a routine duodenoscope was performed at 2 wk postoperatively to observe the papillary morphology.

Adverse events

In the article, PEP, postoperative bleeding, perforation and cholangitis were regarded as major adverse events. The diagnostic criteria of postoperative bleeding was based on the onset of clinical symptoms, including melena or haematemesis or a decrease > 2 g/dL in haemoglobin level. The present study used the diagnostic and classification criteria of PEP proposed by Cotton *et al*[10]: (1) New or worsened abdominal pain; (2) Serum amylase at least three times the upper limit of normal, measured more than 24 h after the procedure; and (3) New or prolonged hospitalization for at least 2 days.

Statistical analysis

Nonparametric data are expressed as medians.

RESULTS

Between July 2022 and September 2022, 7 patients underwent CETPS at the Chinese PLA General Hospital. Clinical characteristics and treatment outcomes for the 7 patients are shown in Table 1.

A total of 2 patients had yellow sediment-like CBD stones, and aspiration extraction was performed after the insertion of papillary support. Of the 5 patients with clumpy CBD stones (0.4-1.0 cm), 2 underwent basket extraction under direct vision for a single stone (0.5-1.0 cm, black and black grey), 1 underwent balloon plus aspiration extraction under direct vision for 5 stones (0.4-0.6 cm, brown), and 2 underwent aspiration extraction only for a single stone (0.5-0.6 cm, yellow, none).

Technical success, namely, no residual stones in the CBD or left and right hepatic ducts, was achieved in all 7 cases (100%). The median operating time was 45.0 min (range 13.0–87.0 min).

Mild PEP occurred in one case (14.3%). Hyperamylasaemia without abdominal pain was noted in 2 of 7 patients. No residual stones or cholangitis were found during the follow-up.

In one case, the papillary support was dilated by an extraction balloon before the insertion of the cholangioscope, given that the papillary was not dilated enough after the insertion of support.

Table 1 Clinical characteristics and treatment outcomes for 7 patients with choledocholithiasis undergoing cholangioscopy-assisted extraction through novel papillary support

Case No.	Age (yr)/sex	Chief complaint	Stone size (cm)[1]	Stone colour	Number of stones	Operation time (min)	Balloon dilatation	Extraction method	Postoperative adverse events
1	44/female	Abdominal pain	0.6	None	1	64	Yes	Aspiration	Postoperative pancreatitis
2	54/female	Abdominal pain	1.0	Black grey	1	53	No	Basket	Hyperamylasaemia
3	25/female	Abdominal pain	0.4-0.6	Brown	5	45	No	Balloon and aspiration	None
4	68/male	Abdominal pain	0.5	Black	1	87	No	Basket	None
5	29/female	Abdominal pain	Sediment-like	Yellow	Sediment-like	13	No	Aspiration	None
6	83/male	Abdominal pain	Sediment-like	Yellow	Sediment-like	31	No	Aspiration	Hyperamylasaemia
7	32/female	Abdominal pain	0.5 cm	Yellow	1	39	No	Aspiration	None

[1]The stone size was measured under computerized tomography on the maximal cross section.

DISCUSSION

This study showed that CETPS appears to be a feasible and safe treatment option for small-calibre (cross diameter ≤ 1.0 cm) or sediment-like CBD stones with a 100.0% (7/7) technical success rate and a 14.3% (1/7) PEP rate without other adverse events, such perforation, bleeding and cholangitis.

The major advantage of CETPS over ERCP with EST for CBD stones is that SO function is retained, avoiding relevant adverse events, including bleeding, perforation, malignant degeneration and recurrent CBD stones[11-13]. Jun *et al*[8] has confirmed that the use of transpapillary SEMSs was effective in the preservation of SO function. Moreover, Cho *et al*[9] concluded that SEMSs can be used for the extraction of CBD stones in patients on dual antiplatelet agents and do not lead to haemorrhagic or thromboembolic events. Of note, the papillary support introduced in the present study has the following merits compared with the SEMSs used in previous studies[8,9]: (1) The novel papillary support was shorter (25-30 mm) than other SEMSs (40-50 mm); (2) the CBD side of the support had a 70-degree angle; and (3) the papillary side of the support had a single dumbbell-style design. In the case of distal stones, the stones might get stuck in the middle between the relatively long SEMS and CBD wall after stent insertion, so Jun *et al*[8] recommended that the patient's position should be tilted to move the stones from the distal CBD to mid-CBD before stent insertion, although it did not always work. Therefore, we designed a support with a shorter length (25-30 mm) and 70-degree angle on the CBD side (Figure 1H), which could lift the stones into the proximal CBD during the process of stent insertion. On the other hand, the single dumbbell-style design (Figure 1H) on the papillary side could protect the support from entering the CBD due to the persistent friction from the cholangioscope. In terms of the PEP, this study presented a reasonable 14.3% (1/7) rate. Theoretically, the use of support could avoid the unintentional PD insertion of the instructions for stone extraction, which might reduce the PEP rate; however, excessive squeezing from the support would induce PEP. Our next method to improve the support is finding the optimal balance between a sufficient support force and a reasonable PEP rate.

Another important advantage of CETPS over traditional ERCP is how the treatment operation is under direct vision. First, radiation injury for patients and surgeons could be minimized, and special patients, including pregnant women and children, could benefit from this technique. Second, the basket and balloon, designed for cholangioscopy, could be opened in the most appropriate position and frap/hold the stones in a timely manner under direct vison. Moreover, operators could find related adverse events, including CBD perforation, bleeding and injury, in a timely manner.

As mentioned above, CETPS combined the advantages of the SEMS technique with the SO function preservation and the cholangioscopy-assisted treatment operation under direct vision. Moreover, the application of novel papillary support established a smooth passageway for the ingress and egress of the cholangioscope and thus facilitated the operation of cholangioscopy-assisted extraction.

The major limitation of our study was the relatively small sample size. However, this paper introduced a novel papillary support that avoids EST during ERCP. Moreover, cholangioscopy-assisted stone extraction using a balloon under direct vision (Table 1, case 3) has not yet been reported.

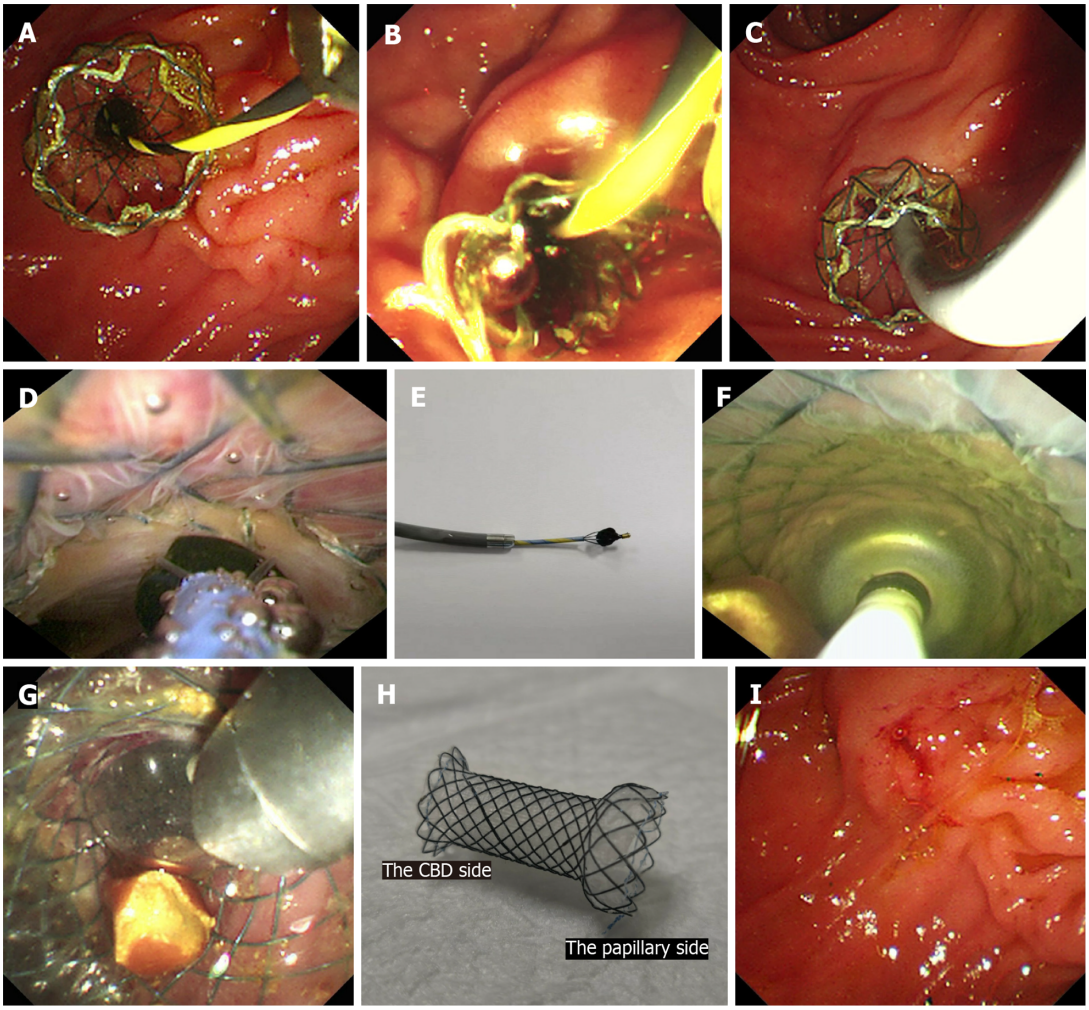

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Figure 1 The procedures of cholangioscopy-assisted extraction through novel papillary support for small-calibre and sediment-like common bile duct stones. A: The novel papillary support was placed in the lower common bile duct (CBD) and papilla; B: Many sediment-like CBD stones flowed from the support under endoscopic aspiration; C: The cholangioscope (Micro-Tech, eyeMax) was inserted into the CBD; D: The single clumpy CBD stone was collected by the basket under cholangioscopy; E: The stone, collected by the basket, was extracted from the CBD and body along with the cholangioscope; F: Multiple clumpy CBD stones were held by the balloon under cholangioscopy; G: One stone, held by the balloon, was extracted from the CBD along with the cholangioscope; H: The novel single dumbbell-style papillary support was removed from the body; I: Papillary morphology immediately after the removal of papillary support.

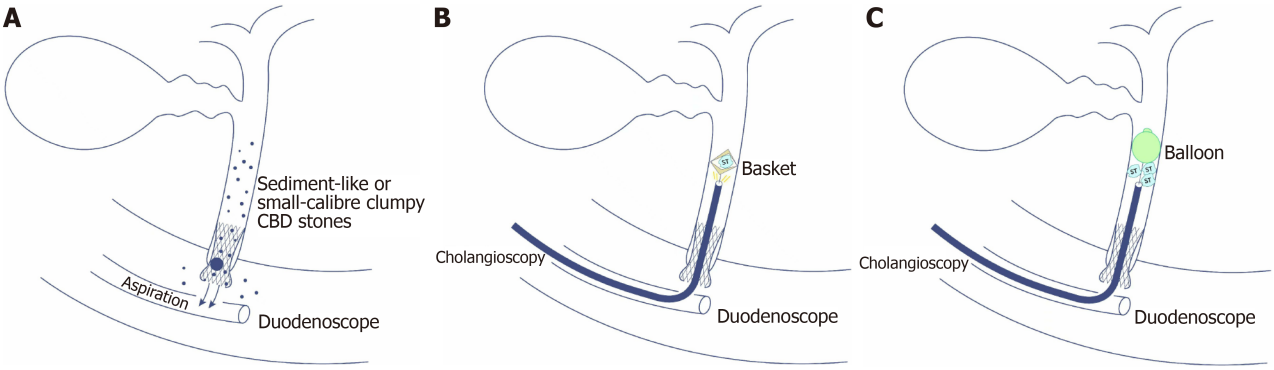

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Figure 2 The sketch map of cholangioscopy-assisted extraction through novel papillary support for small-calibre and sediment-like common bile duct stones. A: The sketch map of cholangioscopy-assisted aspiration extraction through the support for small-calibre or sediment-like common bile duct (CBD) stones; B: The sketch map of cholangioscopy-assisted basket extraction through the support for small-calibre CBD stones; C: The sketch map of cholangioscopy-assisted balloon extraction through the support for multiple small-calibre CBD stones.

CONCLUSION

In conclusion, CETPS seemed to be a new, feasible and safe treatment strategy for small-calibre (cross diameter ≤ 1.0 cm) or sediment-like CBD stones. Patients, especially pregnant women and those who cannot stop taking anti-coagulation/anti-platelet agents, could benefit from this technique because of the visualized operation and absence of EST. However, further prospective studies with larger populations and longer follow-up periods are warranted.

FOOTNOTES

Author contributions: Zhang WG analyzed the data and wrote the manuscript; Zhang WG and Chai NL contributed equally for this study, and considered as co-first author; Linghu EQ and Chai NL performed the procedures; Zhang B, Li X, Wang JF, Dong H, and Feng YJ revised the manuscript.

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ORCID number: Wen-Gang Zhang 0000-0002-3314-0283; Ning-Li Chai 0000-0002-6791-5817; Bo Zhang 0000-0002-4333-0750; Xiao Li 0000-0003-0254-6675; Jia-Feng Wang 0000-0002-7413-770X; Hao Dong 0000-0001-8148-0410; Yu-Jie Feng 0000-0001-9892-9616; En-Qiang Linghu 0000-0003-4506-7877.

Corresponding Author's Membership in Professional Societies: Chinese Society of Digestive Endoscopy, President.

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ORIGINAL ARTICLE

Observational Study

Predictive value of presepsin and acylcarnitines for severity and biliary drainage in acute cholangitis

Han-Yu Zhang, Hong-Li Xiao, Guo-Xing Wang, Zhao-Qing Lu, Miao-Rong Xie, Chun-Sheng Li

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Han-Yu Zhang, Hong-Li Xiao, Guo-Xing Wang, Zhao-Qing Lu, Miao-Rong Xie, Chun-Sheng Li, Department of Emergency Medicine, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

Corresponding author: Chun-Sheng Li, PhD, Professor, Department of Emergency Medicine, Beijing Friendship Hospital, Capital Medical University, No. 95 Yongan Road, Xicheng District, Beijing 100050, China. lcscyyy@163.com

Abstract

BACKGROUND

Bacteremia, which is a major cause of mortality in patients with acute cholangitis, induces hyperactive immune response and mitochondrial dysfunction. Presepsin is responsible for pathogen recognition by innate immunity. Acylcarnitines are established mitochondrial biomarkers.

AIM

To clarify the early predictive value of presepsin and acylcarnitines as biomarkers of severity of acute cholangitis and the need for biliary drainage.

METHODS

Of 280 patients with acute cholangitis were included and the severity was stratified according to the Tokyo Guidelines 2018. Blood presepsin and plasma acylcarnitines were tested at enrollment by chemiluminescent enzyme immunoassay and ultra-high-performance liquid chromatography-mass spectrometry, respectively.

RESULTS

The concentrations of presepsin, procalcitonin, short- and medium-chain acylcarnitines increased, while long-chain acylcarnitines decreased with the severity of acute cholangitis. The areas under the receiver operating characteristic curves (AUC) of presepsin for diagnosing moderate/severe and severe cholangitis (0.823 and 0.801, respectively) were greater than those of conventional markers. The combination of presepsin, direct bilirubin, alanine aminotransferase, temperature, and butyryl-L-carnitine showed good predictive ability for biliary drainage (AUC: 0.723). Presepsin, procalcitonin, acetyl-L-carnitine, hydroxydodecenoyl-L-carnitine, and temperature were independent predictors of bloodstream infection. After adjusting for severity classification, acetyl-L-carnitine was the only acylcarnitine independently associated with 28-d mortality (hazard ratio 14.396; P <

0.001) (AUC: 0.880). Presepsin concentration showed positive correlation with direct bilirubin or acetyl-L-carnitine.

CONCLUSION

Presepsin could serve as a specific biomarker to predict the severity of acute cholangitis and need for biliary drainage. Acetyl-L-carnitine is a potential prognostic factor for patients with acute cholangitis. Innate immune response was associated with mitochondrial metabolic dysfunction in acute cholangitis.

Key Words: Acute cholangitis; Severity; Biliary drainage; Presepsin; Acylcarnitines

Core Tip: Acute cholangitis leads to sepsis and organ dysfunction because of biliary obstruction. Identification of predictive biomarkers for patients who require emergent biliary drainage and patients who may progress to systemic bloodstream infection at an early stage of the disease is a key imperative. Our study suggests that presepsin and acetyl-L-carnitine may serve as biomarkers to predict the severity of acute cholangitis and the need for biliary drainage. Innate immune response was associated with mitochondrial metabolic dysfunction.

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INTRODUCTION

Acute cholangitis refers to bacterial infection of the extra-hepatic biliary tract which typically occurs in association with bile duct obstruction caused by choledocholithiasis, malignant stricture, or primary sclerosing cholangitis. Approximately 20% to 71% of patients with acute cholangitis progress to bacteremia or sepsis, which may lead to life-threatening organ failure and death[1,2]. Early biliary drainage to remove biliary obstruction is one of the main emergency treatments for acute cholangitis. Therefore, the identification of predictive biomarkers for patients who require emergent biliary drainage and patients who maybe progress to systemic bloodstream infection at an early stage of the disease is a key imperative.

Bacteremia induces hyperactive immune response and mitochondrial dysfunction which alter metabolism[3]. Presepsin, a soluble leukocyte differentiation antigen 14 (CD14) subtype, is responsible for pathogen recognition by innate immunity[4]. Presepsin is a biomarker of systemic inflammation that can facilitate early diagnosis, risk-stratification, and prognostic assessment of patients with sepsis[5-7]. Carnitine is responsible for mitochondrial transport and β oxidation of fatty acids. L-carnitine and acylcarnitines are established mitochondrial biomarkers[8]. Preclinical and recent clinical studies have demonstrated the association of plasma carnitine or acylcarnitines with organ dysfunction and poor survival in sepsis[9-11]. However, it is unknown whether presepsin or specific acylcarnitine species can reflect severity of acute cholangitis and the timing of biliary drainage. Therefore, this study aimed to evaluate the value of presepsin as well as specific acylcarnitines as predictors of severity, bloodstream infection, biliary drainage and prognosis in patients with acute cholangitis.

MATERIALS AND METHODS

Study design

A prospective observational study was conducted to clarify the predictive value of presepsin and acylcarnitines for severity and biliary drainage in patients with acute cholangitis. The primary outcomes were the abilities of presepsin and acylcarnitines to predict severe acute cholangitis compared with procalcitonin. The secondary outcomes included the value of presepsin and acylcarnitines to predict emergency biliary drainage, positive bloodstream infection, and prognosis of acute cholangitis. This study complied with the principles of the Declaration of Helsinki. The Beijing Friendship Hospital Ethics Committee approved the study protocol (No. 2018-P2-063-01). Patients were enrolled after providing written informed consent.

Study participants

This was a single-center study conducted at the emergency department and emergency intensive care unit of Beijing Friendship Hospital, a National Clinical Research Center of Digestive Diseases. Between May 2019 and July 2021, consecutive adult patients who fulfilled the acute cholangitis criteria based on the Tokyo Guidelines 2018 (TG18) for acute cholangitis were enrolled[12]. The severity was stratified as mild, moderate, and severe according to TG18[12]. The exclusion criteria were as follows: (1) Patients with chronic kidney or liver disease who may have increased presepsin or acylcarnitine levels at baseline[13]; (2) HIV infection; (3) pregnant and lactating women; (4) patients with abdominal trauma or history of abdominal surgery in the past seven days; (5) incomplete data about the main study indices (presepsin, acylcarnitines, or blood culture results); and (6) patients who declined to participate.

Data collection

We recorded demographic data, comorbidities, clinical and laboratory data, severity grading of acute cholangitis and biliary drainage data within 48 h after admission. Disease severity was assessed using the sequential organ failure assessment (SOFA) scores. The criteria for implementing biliary drainage were based on the American Society for Gastrointestinal Endoscopy (ASGE) guidelines for the management of cholangitis[14]. Blood samples of all patients and bile samples of patients who were subjected to endoscopic or percutaneous biliary drainage were cultured for aerobic and anaerobic bacteria. Pathogens in blood samples were identified by blood culture and metagenomic next generation sequencing (mNGS). Data for 28-d mortality were collected during follow-up.

Sample collection and processing

Blood samples (5 mL) were collected immediately after admission and stored at 4 °C. 200 μL of these blood samples were extracted to detect presepsin concentration. The remaining blood samples were centrifuged for 10 min at 4500 g and the plasma sample was then stored at -80 °C within 24 h. For acylcarnitines detection, 50 μL plasma sample was drawn into a 2 mL centrifuge tube and mixed with 140 μL methanol and 10 μL internal standard (NSK-B-1) and further centrifuged for 5 minutes at 12000 g. 100 μL supernatant was transferred into 200 μL inner liner before analyses. For mNGS detection, 3 mL blood sample was drawn from patients and centrifuged at 4000 g for 10 min within 8 h after collection. DNA was extracted from plasma using a TIANamp Micro DNA Kit (Tiangen Biotech, Beijing, China, No. DP316) according to the manufacturer's operating manual. The extracted DNA specimens were used for the construction of DNA libraries.

Measurements of presepsin

A chemiluminescent enzyme immunoassay was used to test presepsin concentration by a PATHFAST analyzer (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The detection range was 20 pg/mL to 200000 pg/mL. Information regarding conventional inflammatory biomarkers procalcitonin, C-reactive protein (CRP) and other indicators were obtained from the clinical laboratory data.

Determination of acylcarnitines

Plasma acylcarnitines at enrollment were determined by an ultra-high-performance liquid chromatography-mass system (UHPLC-MS, Supplementary file 1) using a Waters XEVO TQ-S Micro triple quadrupole mass spectrometer (Waters Corp, United States). The length of carbon chains was used to define short-chain (C ≤ 5), medium-chain (C6-10) and long-chain acylcarnitines (C ≥ 12) (Supplementary Table 1).

Next-generation sequencing

mNGS testing of the blood samples was performed and analyzed by BGI-Shenzhen, as previously reported[15]. Briefly, the extracted DNA was fragmented to 300 bp. DNA libraries were constructed by end-repair, adapter ligation and PCR amplification using the PMseqTM high throughput gene detection kit for infectious pathogens (combined probe anchored polymerization sequencing method, BGI-Shenzhen, China, No. RM0438), according to the manufacturer's instructions (Supplementary file 2). Using bioinformatics analysis methods and pathogenic microorganism database, the types of pathogenic microorganisms obtained by sequencing were analyzed, and the detection results of each sample were obtained.

Statistical analyses

Factoring a two-sided $\alpha = 0.05$, $\beta = 0.2$, and assuming 50% of patients with mild acute cholangitis[16], it was determined that 268 patients were required for enrollment, *i.e.*, 134 with mild and moderate acute cholangitis and 134 with severe acute cholangitis. This study enrolled 387 patients to account for patients lost to follow-up and patients with incomplete data collection. Continuous variables with non-normal distribution were presented as median (25^{th} to 75^{th} percentile) and compared by Mann-Whitney *U* test or Kruskal-Wallis test. Comparisons between categorical variables were analyzed by Pearson χ^2 test. Significant biomarkers and clinical variables associated with severity, biliary drainage, bloodstream

infection, and 28-d mortality were identified by multivariate logistic regression models. The area under the receiver operating characteristic (ROC) curves were applied to examine the predictive accuracy of presepsin, acylcarnitines, and procalcitonin for severity and biliary drainage. The optimal cutoff levels determined by ROC curves and Youden index were used to dichotomize presepsin, acylcarnitines, procalcitonin, and other independent predictors. The area under the curve (AUC) comparisons were performed using MedCalc Version 13 software (Mariakerke, Belgium). Kaplan-Meier survival curves were established, and between-group differences in 28-d survival were assessed using the log-rank test. The Cox proportional hazard model was used to calculate the hazard ratio (HR) for 28-d mortality. Spearman rank correlation was performed for the correlation analysis. Two-sided P values < 0.05 were considered indicative of statistical significance. SPSS 25.0 software (SPSS, Chicago, IL, United States) was used for statistical analyses.

RESULTS

Characteristics of the study population

From May 2019 through July 2021, 387 patients with acute cholangitis were admitted to the emergency department or EICU. Data from 107 patients were not analyzed because 29 patients did not meet the inclusion criteria, 38 patients refused consent, 35 patients had incomplete main records, and 5 patients were lost to follow-up (Supplementary Figure 1). The remaining 280 patients were enrolled in this study and assigned to the mild group (n = 65), moderate group (n = 84), and severe group (n = 131) based on the TG18 criteria. The age, proportion of patients with biliary drainage, levels of temperature, white blood cell (WBC) count, total bilirubin, direct bilirubin and SOFA score, and 28-d mortality increased with the severity of acute cholangitis, and the differences among the three groups were significant (P < 0.05 for all) (Table 1).

Performance of presepsin to predict the severity of acute cholangitis compared with procalcitonin, CRP, or acylcarnitine

Compared with the mild group, presepsin, procalcitonin, and CRP levels were significantly higher in patients with moderate and severe acute cholangitis (P < 0.001, Table 2). Plasma short chain and medium chain acylcarnitines (C0, C2–C6, C8) increased, while long chain acylcarnitines (C12-C14, C18, C20, C22) decreased with the severity of acute cholangitis (P < 0.05, Table 2). Multivariate logistic regression showed that increased levels of presepsin, WBC, total bilirubin, temperature, and age, and decreased level of tetradecadienyl-L-carnitine (C14:2), but not procalcitonin and CRP levels, were independent predictors of moderate and severe patients, compared with mild patients (Table 3). The AUC of presepsin for predicting moderate/severe cholangitis was 0.823 (sensitivity 0.75; specificity 0.78; cutoff value 1519 pg/mL), which was higher than that of WBC (0.734; P = 0.0189), total bilirubin (0.677; P = 0.0004), temperature (0.598; P < 0.0001), age (0.635; P = 0.0002), and C14:2 (0.678; P = 0.0012) (Figure 1A). Compared with mild/moderate patients, presepsin, procalcitonin and valeryl-L-carnitine (C5) were independently associated with severe cholangitis (Table 3). The AUC of presepsin (0.801; sensitivity 0.82; specificity 0.66; cutoff value 1680 pg/mL) for severe cholangitis was significantly higher than that of procalcitonin (0.696, P = 0.0028) and C5 (0.664, P = 0.0008) (Figure 1B).

Performance of presepsin and acylcarnitines to predict biliary drainage

One hundred and seventy-six of 280 patients underwent biliary drainage. Compared with patients without biliary drainage, patients with biliary drainage had significantly increased temperature, levels of presepsin, total bilirubin, direct bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (P < 0.05 for all), rather than procalcitonin (P = 0.199) and CRP (P = 0.410) levels. On multivariate logistic regression, high presepsin (OR 2.312, P = 0.004), direct bilirubin (OR 1.902, P = 0.027), ALT (OR 1.878, P = 0.022) and temperature (OR 2.108, P = 0.006), and low plasma butyryl-L-carnitine (C4) (OR 3.326, P = 0.001) were identified as independent predictors of biliary drainage (Table 4). The AUC of a combination of these five predictors was 0.723, which was significantly greater compared with presepsin (0.604, P = 0.0001), direct bilirubin (0.600, P < 0.0001), ALT (0.579, P < 0.0001), temperature (0.599, P = 0.0001), and C4 (0.574, P < 0.0001) alone (Figure 1C). In the model composed of these five factors (Table 4), a cutoff score of 3 was associated with 79.6% sensitivity, 54.8% specificity, 63.8% PPV, and 72.9% NPV for predicting biliary drainage (LR + 1.76 and LR– 0.37) (Figure 1D).

The concentrations of presepsin, acylcarnitines, and procalcitonin in patients with positive and negative blood infection

As a substitute for the severity of acute cholangitis, blood infection was identified by blood culture and blood mNGS. Compared to patients with no blood infection (n = 188), patients with blood infection (n = 92) were more likely to require biliary drainage, and had significantly higher temperature, WBC, and SOFA scores (P < 0.05 for all, Supplementary Table 2). The proportion of male patients in the blood infection group was significantly lower than that in the group without blood infection. The positive

Table 1 Clinical characteristics according to severity grading of acute cholangitis

Variables	All cases (*n* = 280)	Mild (*n* = 65)	Moderate (*n* = 84)	Severe (*n* = 131)	*P* value
Demographic data, age (yr)	74 (66, 84)	69 (63, 81)	79.5 (69, 87)	74 (66, 84)	0.001
Male, *n* (%)	166 (59.3)	34 (52.3)	53 (63.1)	79 (60.3)	0.392
Comorbidities, *n* (%)					
CHD	78 (27.9)	19 (29.2)	22 (26.2)	37(28.2)	0.911
Heart failure	23 (8.2)	3 (4.6)	5 (6.0)	15 (11.5)	0.173
Hypertension	87 (31.1)	16 (24.6)	32 (38.1)	39 (29.8)	0.192
CVD	33 (11.8)	4 (6.2)	10 (11.9)	19 (14.5)	0.233
COPD	10 (3.6)	1 (1.5)	2 (2.4)	7 (5.3)	0.313
Diabetes mellitus	64 (22.9)	11 (16.9)	18 (21.4)	35 (26.7)	0.286
Biliary drainage, *n* (%)					0.015
No	104 (37.1)	34 (52.3)	27 (32.1)	43 (35.0)	
ERCP/PTCD	176 (62.9)	31 (47.7)	57 (67.9)	88 (67.2)	
Infection data					
Temperature (℃)	37.5 (36.7, 38.5)	37.2 (36.5, 38.0)	37.5 (36.6, 38.6)	37.8 (36.8, 38.5)	0.044
WBC count (× 10^9/L)	10.73 (7.37, 14.83)	8.10 (6.47, 10.22)	13.00 (8.61, 16.56)	11.35 (7.89, 15.71)	< 0.001
Liver function					
TBIL (μmol/L)	98.69 (62.63, 142.32)	69.26 (48.02, 103.37)	114.40 (77.04, 164.67)	103.99 (74.30, 139.53)	< 0.001
DBIL (μmol/L)	70.29 (42.68, 98.75)	43.20 (26.53, 75.64)	78.90 (58.09, 110.40)	72.07 (44.85, 96.29)	< 0.001
ALT (U/L)	148.00 (80.00, 287.50)	166.00 (63.50, 386.50)	145.00 (84.62, 300.00)	148.00 (77.00, 240.00)	0.626
AST (U/L)	138.05 (82.50, 286.10)	129.50 (69.60, 346.20)	138.80 (96.10, 299.10)	137.85 (72.03, 266.53)	0.744
SOFA score	2 (1, 4)	1 (1, 2)	1 (1, 2)	4 (3, 5)	< 0.001
28-d mortality, *n* (%)	9 (3.2)	0 (0.0)	0 (0.0)	9 (6.9)	0.005

Data expressed as median (P_{25}, P_{75}) or *n* (%). CHD: Coronary heart disease; CVD: Cerebral vascular disease; COPD: Chronic obstructive pulmonary disease; ERCP: Endoscopic retrograde cholangiopancreatography; PTCD: Percutaneous transhepatic biliary drainage; WBC: White blood cell; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SOFA: Sequential organ failure assessment.

rates of blood culture, blood mNGS, and bile culture were 29.3% (82/280), 66.7% (14/21), and 76.7% (135/176), respectively. The most common bacteria identified were *Escherichia coli*, *Klebsiella pneumoniaeleisure* and *Enterococcus faecium*. Blood infection positivity was associated with significantly higher level of presepsin ($P = 0.001$), procalcitonin ($P < 0.001$), acetyl-L-carnitine (C2, $P = 0.009$), propionyl-L-carnitine (C3, $P = 0.035$), hexanoyl-L-carnitine (C6, $P = 0.036$), and hydroxydodecenoyl-L-carnitine (C12:1 OH, $P = 0.018$).

Ability of presepsin, acylcarnitines, and procalcitonin to identify bloodstream infection in acute cholangitis

We dichotomized presepsin, procalcitonin, C2, C3, C6, and C12:1 OH using the optimal cutoff value. After adjusting for sex, severity grading, and SOFA score, presepsin (OR 3.466, $P = 0.018$), procalcitonin (OR 4.054, $P < 0.001$), C2 (OR 3.716, $P = 0.005$), C12:1 OH (OR 3.611, $P = 0.002$), and temperature (OR 1.671, $P = 0.003$) were found to be independent predictors for bloodstream infection (Table 5). The AUC of presepsin for diagnosing blood infection was 0.610 (sensitivity 0.91; specificity 0.32; cut-off 1147.5 pg/mL), but there was no significant difference between presepsin and procalcitonin (AUC: 0.679), C2 (AUC: 0.599), C12:1 OH (AUC: 0.603), and temperature (AUC: 0.639) in this respect (Supplementary Table 3).

Prognostic value of presepsin, acylcarnitines, and procalcitonin for 28-d mortality

The 28-d mortality rate in this study was 3.2%. The characteristics of survivors and non-survivors are illustrated in Supplementary Table 4. Compared with patients who survived, those who died within 28 d had significantly increased presepsin ($P = 0.004$), C2 ($P = 0.001$), and C12:1 OH ($P = 0.004$) (Figure 2A-C), but not increased procalcitonin ($P = 0.591$) and CRP ($P = 0.141$). The AUC of presepsin (0.839), C2

Table 2 Biomarkers according to severity grading of acute cholangitis

Biomarkers	All cases (*n* = 280)	Mild (*n* = 65)	Moderate (*n* = 84)	Severe (*n* = 131)	*P* value
Presepsin (pg/mL)	1864.00 (1169.75, 2765.75)	1053.00 (576.50, 1505.50)	1634.00 (1103.00, 2325.75)	2536.00 (1812.00, 3730.00)	< 0.001
Procalcitonin (ng/mL)	11.32 (2.10, 40.53)	3.57 (0.64, 10.87)	10.70 (1.48, 31.00)	27.43 (4.76, 54.32)	< 0.001
CRP (mg/L)	83.71 (44.23, 153.29)	48.02 (17.00, 103.48)	80.76 (48.39, 143.91)	107.00 (61.18, 174.12)	< 0.001
Acylcarnitines (μmol/L)					
C0	28.54 (19.60, 38.65)	25.39 (18.72, 32.47)	29.65 (19.96, 38.38)	30.00 (21.19, 40.64)	0.042
C2	9.64 (6.16, 14.01)	8.35 (5.23, 12.29)	9.44 (6.67, 12.53)	10.32 (6.43, 15.30)	0.016
C3	0.38 (0.26, 0.60)	0.31 (0.21,0.43)	0.35 (0.26, 0.53)	0.46 (0.29, 0.71)	< 0.001
C4	0.09 (0.06, 0.16)	0.08 (0.06, 0.12)	0.09 (0.05, 0.13)	0.10 (0.07, 0.19)	0.003
C5	0.11 (0.07, 0.18)	0.08 (0.06, 0.18)	0.09 (0.06, 0.14)	0.14 (0.08, 0.24)	< 0.001
C6	0.11 (0.07,0.15)	0.08 (0.06, 0.14)	0.10 (0.06, 0.13)	0.13 (0.08, 0.20)	< 0.001
C8	0.13 (0.10, 0.22)	0.12 (0.08, 0.19)	0.13 (0.10, 0.20)	0.14 (0.11, 0.23)	0.037
C12	0.09 (0.04, 0.18)	0.14 (0.07, 0.28)	0.09 (0.04, 0.17)	0.08 (0.03, 0.13)	< 0.001
C12:1	0.05 (0.03, 0.08)	0.06 (0.04, 0.10)	0.05 (0.03, 0.09)	0.04 (0.02, 0.07)	0.001
C12DC	0.0005 (0.0004, 0.0008)	0.0006 (0.0005, 0.0009)	0.0005 (0.0004, 0.0009)	0.0004 (0.0003, 0.0007)	0.041
C13	0.03 (0.02, 0.07)	0.04 (0.03, 0.07)	0.03 (0.02, 0.08)	0.02 (0.01, 0.05)	0.008
C14	0.02 (0.01, 0.03)	0.03 (0.02, 0.04)	0.02 (0.01, 0.03)	0.02 (0.01, 0.03)	0.001
C14:1	0.09 (0.04, 0.16)	0.16 (0.07, 0.31)	0.09 (0.05, 0.13)	0.06 (0.03, 0.11)	< 0.001
C14:2	0.07 (0.03, 0.14)	0.12 (0.05, 0.22)	0.06 (0.03, 0.13)	0.05 (0.02, 0.10)	< 0.001
C16	0.10 (0.06, 0.15)	0.12 (0.08, 0.17)	0.10 (0.06, 0.14)	0.09 (0.05, 0.13)	0.003
C16:1	0.06 (0.03, 0.12)	0.10 (0.05, 0.16)	0.05 (0.03, 0.10)	0.05 (0.02, 0.12)	0.003
C16:2	0.02 (0.002, 0.06)	0.04 (0.01, 0.09)	0.01 (0.002, 0.05)	0.02 (0.002, 0.05)	0.006
C18:2	0.13 (0.07, 0.21)	0.16 (0.09, 0.24)	0.12 (0.07, 0.18)	0.11 (0.05, 0.22)	0.014
C18 OH	0.0004 (0.0002, 0.0009)	0.004 (0.003, 0.0013)	0.0006 (0.0002, 0.0010)	0.0004 (0.0002, 0.0007)	0.035
C20	0.0010 (0.0004, 0.0018)	0.0013 (0.0009, 0.0029)	0.0013 (0.0005, 0.0020)	0.0007 (0.0003, 0.0014)	0.001
C20:4	0.0013 (0.0007, 0.0023)	0.0018 (0.0009, 0.0021)	0.0018 (0.0008, 0.0037)	0.0012 (0.0005, 0.0020)	0.024
C22	0.0009 (0.0003, 0.0015)	0.0011 (0.0006, 0.0023)	0.0010 (0.0003, 0.0018)	0.0006 (0.0002, 0.0014)	0.009

Data expressed as median (P_{25}, P_{75}). CRP: C-reactive protein; C0: DL-Carnitine; C2: Acetyl-L-carnitine; C3: Propionyl-L-carnitine; C4: Butyryl-L-carnitine; C5: Valeryl-L-carnitine; C6: Hexenoyl-L-carnitine; C8: Octanoyl-L-carnitine; C12: Dodecanoyl-L-carnitine; C12:1: Dodecenoyl-L-carnitine; C12DC: Dodecanedioyl-L-carnitine; C13: Tridecanoyl-L-carnitine; C14: Tetradecanoyl-L-carnitine; C14:1: Tetradecenoyl-L-carnitine; C14:2: Tetradecadienyl-L-carnitine; C16: Hexadecanoyl-L-carnitine; C16:1: Hexadecenoyl-L-carnitine; C16:2: Hexadecadienyl-L-carnitine; C18:2: Octadecadienyl-L-carnitine; C18OH: Hydroxyoctadecanoyl-L-carnitine; C20: Icosyl-L-carnitine; C20:4: Arachidonoyl-L-carnitine; C22: Behenoyl-L-carnitine.

(0.880), and C12:1 OH (0.822) for 28-d mortality was similar to that of SOFA score (0.848, $P > 0.05$ for all) (Figure 2D and Supplementary Table 5). After adjusting for severity of acute cholangitis by multivariate Cox proportional hazard models, high C2 ($P = 0.004$) was the only independent predictor of 28-d mortality, rather than the levels of presepsin (P=0.732), C12: 1 OH ($P = 0.899$), and SOFA score ($P = 0.133$) (Supplementary Table 6). Based on ROC curves for 28-d mortality and Youden index, a cutoff value of 17.07 μmol/L was used to dichotomize C2. Patients with high C2 Level had significantly higher 28-d mortality compared to those with low C2 Level (HR 14.396; 95%CI: 3.599-57.576; $P < 0.001$; Figure 2E).

Correlation of presepsin with acylcarnitines

Increased level of presepsin ($r = 0.424$, $P < 0.001$), procalcitonin ($r = 0.357$, $P < 0.001$), and C2 ($r = 0.208$, $P < 0.001$) showed a significant association with SOFA score. We confirmed that presepsin, but not procalcitonin, showed a significant positive correlation with total bilirubin ($r = 0.290$, $P < 0.001$), direct bilirubin ($r = 0.304$, $P < 0.001$), and C2 ($r = 0.270$, $P < 0.001$) (Supplementary Table 7).

Table 3 Clinical variables and biomarkers associated with severity of acute cholangitis (multivariate logistic regression models)

	Mild *vs* moderate/severe		Mild/moderate *vs* severe	
Variables	**OR (95%CI)**	***P* value**	**OR (95%CI)**	***P* value**
Presepsin (pg/mL)	1.001 (1.000-1.002)	< 0.001	1.000 (1.000-1.001)	< 0.001
Procalcitonin (ng/mL)	1.018 (0.995-1.041)	0.134	1.021 (1.008-1.035)	0.001
WBC (× 10^9/L)	1.149 (1.050-1.258)	0.003	0.990 (0.945-1.037)	0.676
CRP (mg/L)	1.001 (0.995-1.008)	0.642	1.003 (0.999-1.008)	0.104
TBIL(μmol/L)	1.008 (1.003-1.013)	0.003	0.999 (0.996-1.002)	0.504
Temperature (℃)	1.540 (1.046-2.267)	0.029	1.118 (0.859-1.456)	0.407
Age (yr)	1.051 (1.018-1.085)	0.002	1.000 (0.977-1.022)	0.973
C14:2 (μmol/L)	0.036 (0.002-0.663)	0.025	-	-
C5 (μmol/L)	-	-	11.490(2.213-59.656)	0.004

WBC: White blood cell; CRP: C-reactive protein; TBIL: Total bilirubin; C14:2: Tetradecadienyl-L-carnitine; C5: Valeryl-L-carnitine; OR: Odds ratio; CI: Confidence interval.

Table 4 A model for predicting biliary drainage in acute cholangitis multivariate logistic regression models

Variables	B	SE	Wald	df	OR	95%CI	*P* value	Score
Presepsin > 1868 (pg/mL)	0.838	0.291	8.308	1	2.312	1.308-4.087	0.004	1
C4 ≤ 0.1803 (μmol/L)	1.202	0.347	12.017	1	3.326	1.686-6.561	0.001	1
DBIL > 51.18 (μmol/L)	0.643	0.290	4.912	1	1.902	1.077-3.358	0.027	1
ALT > 159 (U/L)	0.630	0.275	5.254	1	1.878	1.096-3.219	0.022	1
Temperature ≥ 37.3 (℃)	0.746	0.270	7.602	1	2.108	1.241-3.581	0.006	1

C4: Butyryl-L-carnitine; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; OR: Odds ratio; CI: Confidence interval.

DISCUSSION

The immunologic profile and mitochondrial function of patients with acute cholangitis are similar to those of septic patients. Thus, in this prospective study, we explored the value of presepsin and carnitine metabolites as biomarkers to predict severity, emergency biliary drainage, and prognosis of patients with acute cholangitis. Our results indicated that the ability of presepsin level to predict moderate/severe and severe cholangitis was superior to that of procalcitonin level. High presepsin, direct bilirubin, ALT, temperature, and low C4 were independent predictors of urgent biliary drainage, and the combination of these five predictors significantly improved the predictive accuracy. As a substitute for severity of acute cholangitis, blood infection was found to be independently associated with the biomarkers of presepsin, procalcitonin, C2, and C12:1 OH. High C2 was identified as the only independent predictor of 28-d mortality. Additionally, the positive correlation between presepsin and C2 reflected the association between innate immune response and mitochondrial fatty acid β-oxidation (FAO) impairment during the progression of acute cholangitis.

CD14 are expressed on the surface of innate immune cells and play a role in the activation of innate immune response after recognition of bacteria[4,17]. Presepsin (soluble CD14) has been confirmed as a marker of host response in sepsis patients. Increased presepsin was demonstrated to be associated with organ dysfunction, positive blood culture and mortality in sepsis[4]. This result was consistent with our finding wherein presepsin level was found to identify severe acute cholangitis and bloodstream infection. Animal model of acute obstructive cholangitis demonstrated infiltration of macrophages and neutrophils into the liver sinusoids and around the bile duct leading to coagulopathy[18]. The study of Guicciardi *et al*[19] revealed that macrophages contributed to the pathogenesis of sclerosing cholangitis. These findings suggested the activation of innate immune response in acute cholangitis. In addition, a recent study showed that the conventional septic biomarker procalcitonin which was produced by C cells of the thyroid gland predicted severe but not moderate/severe acute cholangitis with better accuracy than WBC and CRP[16]. Furthermore, our finding demonstrated the superior ability of presepsin to predict severe or moderate/severe cholangitis compared to procalcitonin and other

Table 5 Variables associated with bloodstream infection multivariate logistic regression models

Variables	B	SE	Wald	df	OR	95%CI	*P* value
Presepsin (High *vs* low[1])	1.243	0.524	5.617	1	3.466	1.240-9.689	0.018
Procalcitonin (High *vs* low[2])	1.400	0.401	12.208	1	4.054	1.849-8.889	< 0.001
C2 (High *vs* low[3])	1.313	0.464	7.998	1	3.716	1.496-9.229	0.005
C3 (High *vs* low[4])	0.319	0.400	0.636	1	1.376	0.628-3.015	0.425
C6 (High *vs* low[5])	-0.450	0.436	1.065	1	0.638	0.271-1.499	0.302
C12:1 OH (High *vs* low[6])	1.284	0.410	9.829	1	3.611	1.618-8.058	0.002
Sex	0.282	0.356	0.628	1	1.326	0.660-2.667	0.428
Temperature (°C)	0.513	0.174	8.714	1	1.671	1.188-2.350	0.003
Severity grading	-0.071	0.285	0.062	1	0.932	0.533-1.628	0.804
SOFA score	-0.062	0.081	0.587	1	0.940	0.802-1.101	0.444

[1]Presepsin (high *vs* low): > 1147.5 pg/mL *vs* ≤ 1147.5 pg/mL.
[2]Procalcitonin (high *vs* low): > 10.83 ng/mL *vs* ≤ 10.83 ng/mL.
[3]C2 (high *vs* low): > 14.59 μmol/L *vs* ≤ 14.59 μmol/L.
[4]C3 (high *vs* low): > 0.53 μmol/L *vs* ≤ 0.53 μmol/L.
[5]C6 (high *vs* low): > 0.12 μmol/L *vs* ≤ 0.12 μmol/L.
[6]C12:1 OH (high *vs* low): > 0.02 μmol/L *vs* ≤ 0.02 μmol/L.
C2: Acetyl-L-carnitine; C3: Propionyl-L-carnitine; C6: Hexanoyl-L-carnitine; C12:1 OH: Hydroxydodecenoyl-L-carnitine; SOFA: Sequential organ failure assessment; OR: Odds ratio; CI: Confidence interval.

markers. The AUC of presepsin was higher than that of other markers in predicting any severity of acute cholangitis. As a surrogate of severe acute cholangitis, predictors for positive bloodstream infection were explored. The most commonly identified bacteria in our study were *Escherichia coli*, *Klebsiella pneumoniaeleisure*, and *Enterococcus faecium,* which is consistent with the findings reported by An *et al*[20]. Similar to the study by Umefune *et al*[16] on the association between procalcitonin and positive blood culture in acute cholangitis, the current study found that presepsin, procalcitonin, C2, and C12:1 OH were independent predictors of positive blood infection.

Additionally, to facilitate early identification of patients who require emergency biliary drainage, we established a predictive model consisting of five factors including presepsin, direct bilirubin, ALT, temperature, and butyryl-L-carnitine (C4). Previous studies suggested that procalcitonin might be a decision-supporting biomarker for urgent biliary decompression even in cases that are not categorized as severe based on TG13[21,22]. However, there was no evidence in this study that procalcitonin, rather than presepsin, could independently predict biliary drainage. The results indicated superior ability of presepsin to reflect the degree of biliary obstruction compared to procalcitonin.

Acylcarnitines are recognized for facilitating FAO for energy production in mitochondria[23]. The blood concentrations of acylcarnitines, which represent a group of mitochondrial-derived metabolites, reflect disorders of long-chain FAO[24]. The production of acetylcarnitine (C2) represents metabolic flexibility in buffering the metabolic status between glucose oxidation and fat oxidation states[25]. Elevation in plasma concentration of C2 is a signal of metabolic inflexibility[8]. Mitochondrial metabolic dysfunction has been implicated as one of the potential causes of organ dysfunction in sepsis[26]. Metabolic flexibility was shown to be an important characteristic of patients with sepsis for survival [27]. Plasma C2 Level was shown to be associated with multiple organ dysfunction, extubation, and freedom from vasopressors, or mortality in patients with sepsis[10,11]. In several studies, plasma short chain and medium chain acylcarnitines (C2, C3, C4, C5, C6, C8, C10) were significantly increased in the non-survivors[11,28-30] and only C2 was associated with all of these indices and 28-d mortality in sepsis [12]. Similar to previous studies, our findings showed that concentrations of short- and medium-chain acylcarnitines increased with the severity and C2 was the only acylcarnitine implicated in 28-d mortality. Increased plasma C2 Level may indicate metabolic inflexibility of nonsurvivors with acute cholangitis. Inconsistent with the absence of long chain acetylcarnitine in sepsis studies, we found that concentrations of long chain acylcarnitines decreased with the severity of acute cholangitis, which might be due to impairment of long-chain FAO with disease progression.

Interestingly, in the current study, reduced butyryl-L-carnitine (C4) was found to be an independent predictor of biliary drainage. Butyrate, a short chain fatty acid, is produced in the bowel by bacterial fermentation of dietary fiber. C4, a butyrate ester of carnitine, is known to help maintain intestinal health and prevent intestinal inflammation[31]. C4 combined with presepsin, direct bilirubin, ALT, and temperature showed better predictive accuracy for emergency biliary drainage. The total score of this model was 5, and 83.2% of patients with score > 4 required biliary drainage (Figure 1D). Moreover, the

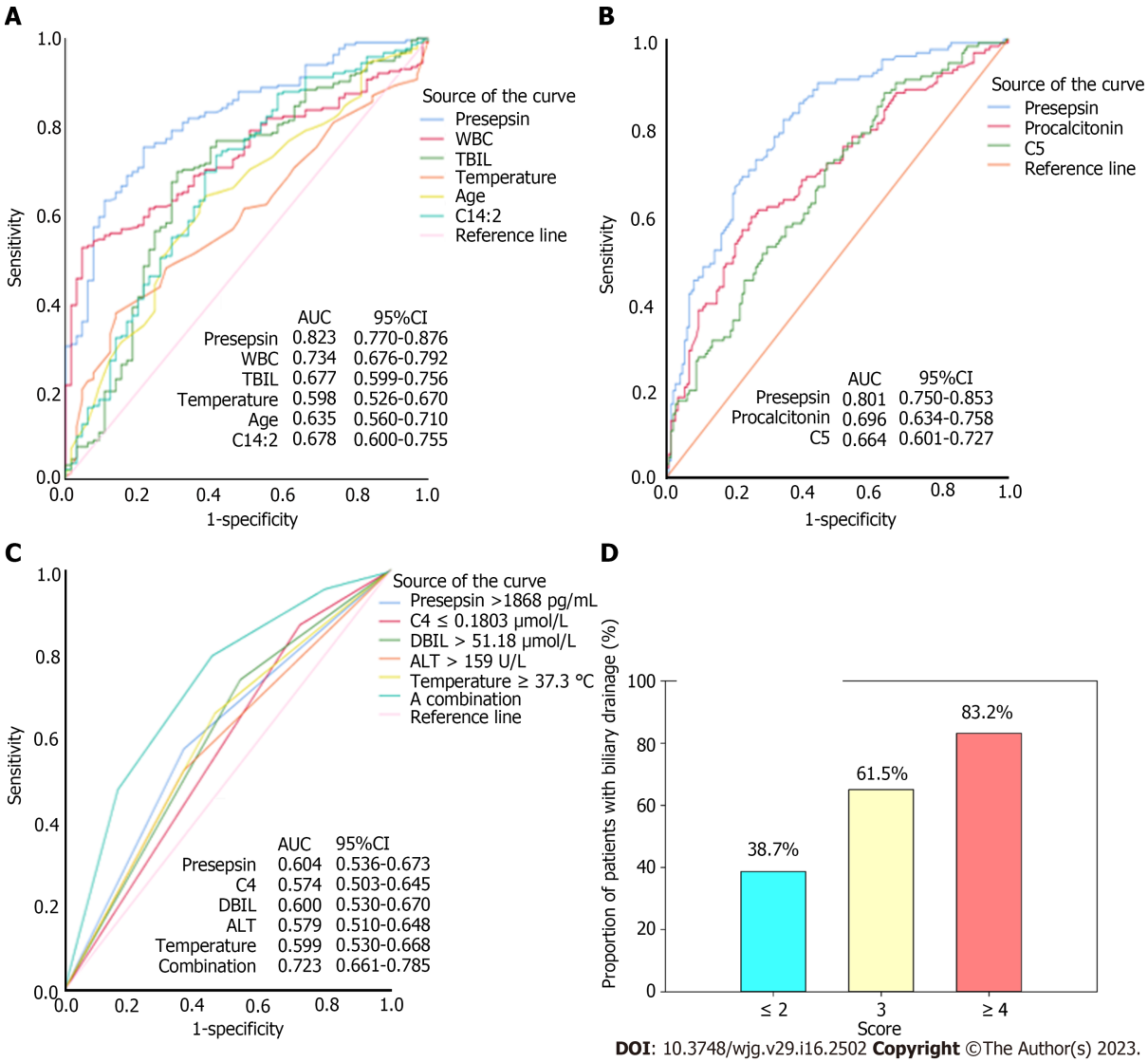

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Figure 1 Receiver operating characteristic curves for biomarkers and clinical parameters for predicting the severity of acute cholangitis and the need for biliary drainage. A: Mild *vs* moderate/severe acute cholangitis; B: Mild/moderate *vs* severe acute cholangitis; C: No biliary drainage *vs* biliary drainage in acute cholangitis; D: Correlation between the score of biliary drainage prediction model and the proportion of patients with biliary drainage. WBC: White blood cell; TBIL: Total bilirubin; C14:2: Tetradecadienyl-L-carnitine; C5: Valeryl-L-carnitine; C4: Butyryl-L-carnitine; ALT: Alanine aminotransferase.

association between C4 and SOFA score, presepsin, and procalcitonin (Supplementary Table 7) may be explained by the compensatory mechanism of intestinal health on intestinal inflammation in acute cholangitis. Furthermore, the association between C2 and inflammation, as well as the hepatic host response to bacteria leading to the accumulation of long-chain acylcarnitines and defective FAO[32], may explain why C2 and hydroxydodecenoyl-L-carnitine (C12:1 OH) were identified as independent predictors of bloodstream infection in acute cholangitis.

The association between innate immunity and FAO may explain the positive correlation between presepsin and acetylcarnitine. Recent evidence suggested that metabolic reprogramming including FAO was a prerequisite for the activation of macrophages and monocytes[33,34]. A study by Zhu *et al*[35] found that the rewiring of metabolic and mitochondrial bioenergetics by monocytes activated, deactivated and resolved acute inflammation in turn. During deactivation, the characteristics of lipid metabolic rewiring included increased acylcarnitines levels. The function of immunocytes depends on specific metabolic programs in mitochondria, including post-translational modifications (*e.g.*, acetylation). In their *in vitro* and *in vivo* studies, Chi *et al*[36] found that histone deacetylase 3 couples mitochondria to deacetylate the FAO enzyme HADHA for NLRP3 inflammasome activation in macrophages.

Some limitations of this study should be considered. First, we did not analyze the dynamic changes in presepsin and acylcarnitines levels over time throughout the disease course. Second, the association of presepsin or acylcarnitines with chronic liver or kidney dysfunction was not assessed in this study. Third, due to the low mortality, a larger sample size was required to verify biomarkers that were associated with death. Fourth, blood mNGS was required for larger population size to improve the

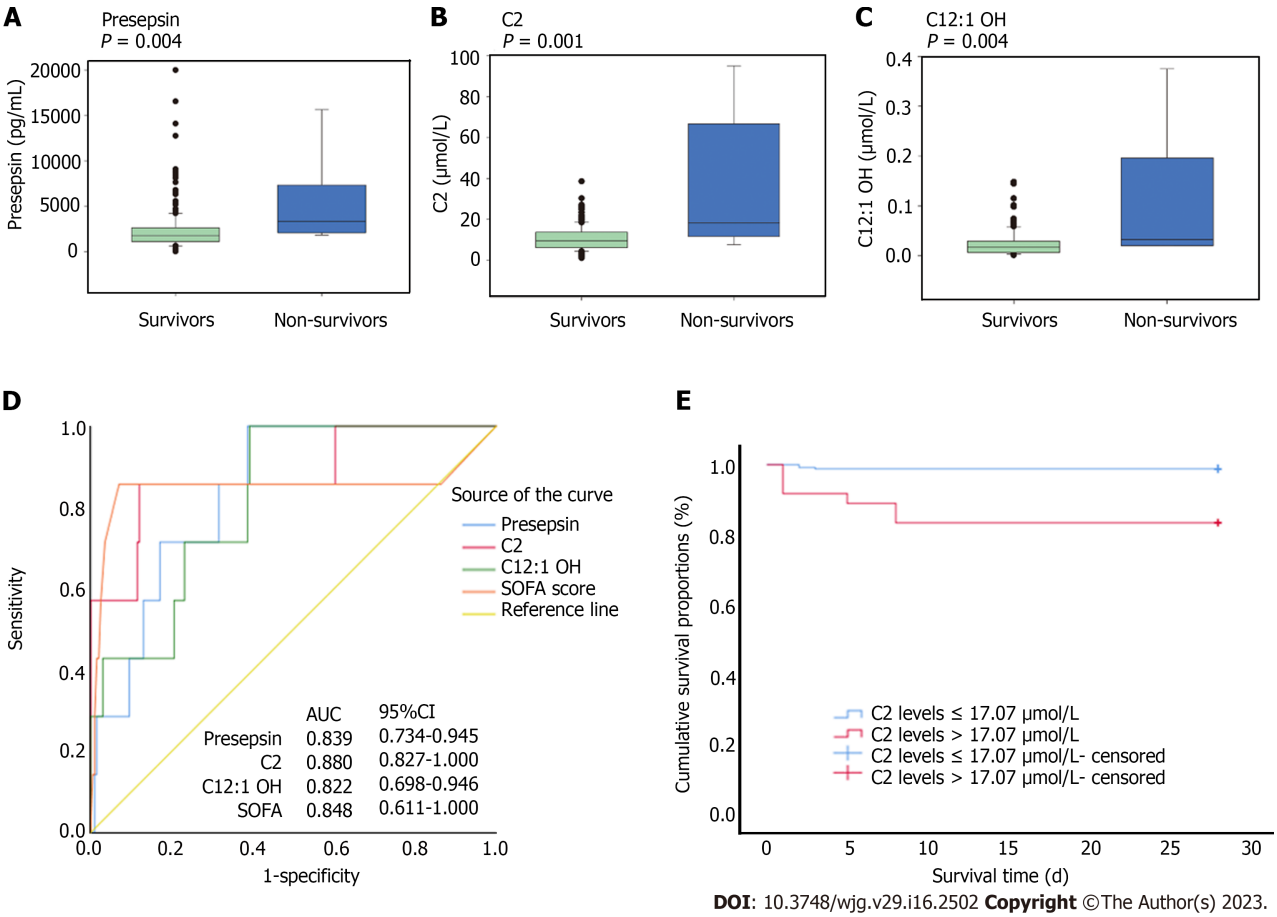

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Figure 2 Association of presepsin and acylcarnitines with 28-d survival in acute cholangitis. A-C: 28-d survival showed significant correlation with presepsin (A), acetyl-L-carnitin (C2; B), and hydroxydodecenoyl-L-carnitine (C12:1 OH; C); D: Receiver operating characteristic curves of presepsin, C2, C12:1 OH and sequential organ failure assessment score for predicting 28-d mortality in patients with acute cholangitis; E: Kaplan–Meier survival curves showed that patients with C2 Levels > 17.07 μmol/L had a lower probability of survival at 28 d (log-rank = 25.01; $P < 0.001$) compared to patients with lower levels in acute cholangitis. C2: Acetyl-L-carnitine; C12:1 OH: Hydroxydodecenoyl-L-carnitine; SOFA: Sequential organ failure assessment; AUC: Area under the curve.

detection rate of positive bloodstream infection.

CONCLUSION

Our study identified presepsin as a specific biomarker to predict the severity and emergency biliary drainage of acute cholangitis compared to procalcitonin and other clinical parameters. Acetyl-L-carnitine might be a promising biomarker for predicting mortality in patients with acute cholangitis. Our findings clarify the association between innate immune responses and mitochondrial FAO impairment in acute cholangitis.

ARTICLE HIGHLIGHTS

Research background

Acute cholangitis is potentially lethal when accompanied by sepsis because of biliary obstruction. It is necessary to identify predictive biomarkers for patients who require emergent biliary drainage and patients who maybe progress to systemic bloodstream infection at an early stage of the disease.

Research motivation

Bacteremia induces hyperactive immune response and mitochondrial dysfunction. Presepsin is responsible for pathogen recognition by innate immunity. Acylcarnitines are established mitochondrial biomarkers. However, it is unknown whether presepsin or specific acylcarnitine species can reflect the severity of acute cholangitis and the timing of biliary drainage.

Research objectives

To clarify the early predictive value of presepsin and acylcarnitines for severity and biliary drainage of acute cholangitis.

Research methods

In this prospective observational study, 280 patients with acute cholangitis were included from May 2019 to July 2021. The severity was stratified as mild, moderate, and severe according to according to the Tokyo Guidelines 2018. Blood presepsin and plasma acylcarnitines were tested at enrollment by chemiluminescent enzyme immunoassay and ultra-high-performance liquid chromatography-mass spec -trometry, respectively. Patients were followed-up for 28 d.

Research results

The concentrations of presepsin, procalcitonin, short- and medium-chain acylcarnitines increased, while long-chain acylcarnitines decreased with the severity of acute cholangitis. The areas under the receiver operating characteristic curves (AUC) of presepsin for diagnosing moderate/severe and severe cholangitis (0.823 and 0.801, respectively) were greater than those of conventional markers. The AUC of a combination of presepsin, direct bilirubin, alanine aminotransferase, temperature, and butyryl-L-carnitine for predicting biliary drainage was 0.723. Presepsin, procalcitonin, acetyl-L-carnitine, hydroxy-dodecenoyl-L-carnitine, and temperature were independent predictors of bloodstream infection. After adjusting for severity classification, acetyl-L-carnitine was the only acylcarnitine independently associated with 28-d mortality (hazard ratio 14.396; $P < 0.001$) (AUC: 0.880). Presepsin concentration showed positive correlation with direct bilirubin and acetyl-L-carnitine.

Research conclusions

Presepsin may serve as a specific biomarker to predict the severity and biliary drainage of acute cholangitis. Acetyl-L-carnitine might be a promising prognostic factor for patients with acute cholangitis. Innate immune response was associated with mitochondrial metabolic dysfunction in acute cholangitis.

Research perspectives

Prospective observational study reports the predictive value of presepsin and acylcarnitines for severity and biliary drainage of acute cholangitis. Future research should focus on the association between acylcarnitines and the changes of intestinal microflora and bacterial translocation in acute cholangitis.

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FOOTNOTES

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ORCID number: Han-Yu Zhang 0000-0002-1865-047X; Hong-Li Xiao 0000-0002-7353-114X; Guo-Xing Wang 0000-0003-1379-5749; Zhao-Qing Lu 0000-0001-8895-1596; Miao-Rong Xie 0000-0003-2479-9470; Chun-Sheng Li 0000-0002-3161-4457.

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