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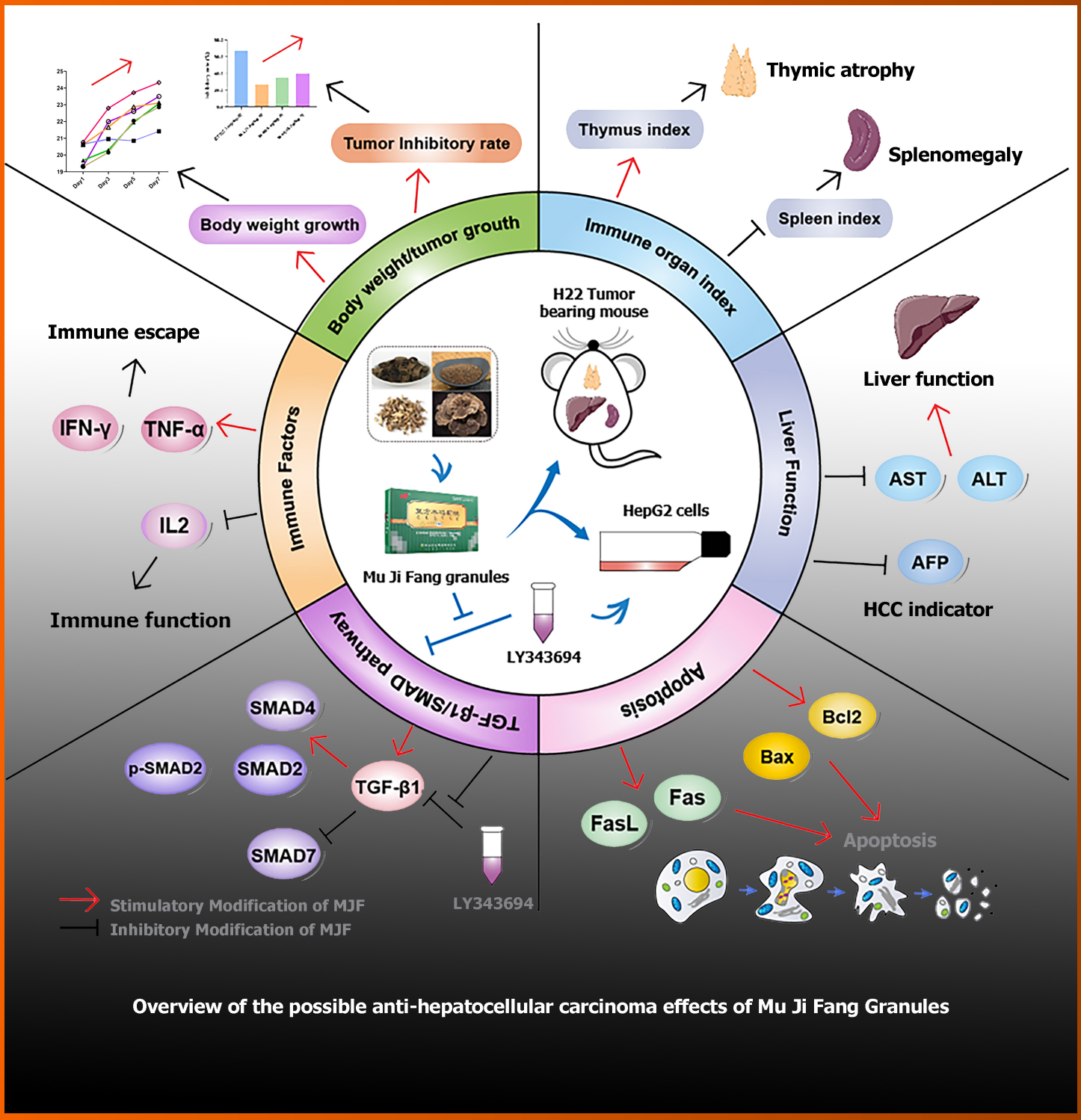

Overview of the possible anti-hepatocellular carcinoma effects of Mu Ji Fang Granules

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World Journal of Gastrointestinal Oncology

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AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

INDEXING/ABSTRACTING

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FIELD OF VISION

Machine learning algorithm to construct cuproptosis- and immune-related prognosis prediction model for colon cancer

Yuan-Yi Huang, Ting-Yu Bao, Xu-Qi Huang, Qi-Wen Lan, Ze-Min Huang, Yu-Han Chen, Zhi-De Hu, Xu-Guang Guo

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Abstract

BACKGROUND

Over the past few years, research into the pathogenesis of colon cancer has progressed rapidly, and cuproptosis is an emerging mode of cellular apoptosis. Exploring the relationship between colon cancer and cuproptosis benefits in identifying novel biomarkers and even improving the outcome of the disease.

AIM

To look at the prognostic relationship between colon cancer and the genes associated with cuproptosis and the immune system in patients. The main purpose was to assess whether reasonable induction of these biomarkers reduces mortality among patients with colon cancers.

METHOD

Data obtained from The Cancer Genome Atlas and Gene Expression Omnibus and the Genotype-Tissue Expression were used in differential analysis to explore differential expression genes associated with cuproptosis and immune activation. The least absolute shrinkage and selection operator and Cox regression algorithm was applied to build a cuproptosis- and immune-related combination model, and the model was utilized for principal component analysis and survival analysis to observe the survival and prognosis of the patients. A series of statistically meaningful transcriptional analysis results demonstrated an intrinsic relationship between cuproptosis and the micro-environment of colon cancer.

RESULTS

Once prognostic characteristics were obtained, the CDKN2A and DLAT genes related to cuproptosis were strongly linked to colon cancer: The first was a risk factor, whereas the second was a protective factor. The finding of the validation analysis showed that the comprehensive model associated with cuproptosis and immunity was statistically significant. Within the component expressions, the expressions of HSPA1A, CDKN2A, and UCN3 differed markedly. Transcription analysis primarily reflects the differential activation of related immune cells and pathways. Furthermore, genes linked to immune checkpoint inhibitors were expressed differently between the subgroups, which may reveal the mechanism of worse prognosis and the different sensitivities of chemotherapy.

CONCLUSION

The prognosis of the high-risk group evaluated in the combined model was poorer, and cuproptosis was highly correlated with the prognosis of colon cancer. It is possible that we may be able to improve patients' prognosis by regulating the gene expression to intervene the risk score.

Key Words: Cuproptosis; Immune; Colon cancer; Prognosis models; Immune infiltration analysis

Core Tip: We comprehensively analyzed the effect of cuproptosis and immunity on the prognosis of colon cancer based on the close association between the three. Scrupulously, a variety of algorithms were applied to analyze and construct a three-gene prognostic model whose efficacy was statistically significant in both the training set and the external validation set. Moreover, in order to provide help for prognosis assessment and personalized treatment of colon cancer, we performed immunoinfiltration analysis and immunocheckpoint inhibitor-related genes expression analysis in different risk groups according to the cuproptosis- and immune-related combination model.

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INTRODUCTION

Colon cancer is among the most serious cancers worldwide and is genetically complex[1,2]. According to the World Health Organization (WHO), over 940000 cases are reported worldwide, and almost 500000 people die annually of colon cancer[3]. As with other solid cancers, colon cancer is infested with various types of immune cells. Among these, factors favoring tumors are the inflammatory reaction of B cells, innate immune cells and numerous T cell subtypes. Unlike other epithelial cancers, many inflammatory T cells that infiltrate colon cancer are specific for the commensal microbiota, rather than specific tumor antigens or autoantigens. Therefore, they do not directly kill cancer cells. However, natural killer (NK) cells, $CD8^+$ T cells and $CD4^+$ T cells are still the main immune monitors[4,5]. At the same time, $CD11b^+Gr1^+$ cells recruited in the late stage contribute to the suppression of the antitumor immune response and tumor angiogenesis, which share characteristics with monocytes, macrophages, neutrophils and dendritic cells[5]. The tumor microenvironment (TME) formed in the tumor development process consists mostly of stroma and immune cells[6,7]. Changes in their composition and proportion will affect the immunosuppressive effect of tumors and subsequently affect pathological state, treatment and prognosis. At present, further research on immune biomarkers is required to provide evidence for immune checkpoint blockade therapy in colon cancer, which is more appropriate for the majority of colon cancers[6-8].

Copper, the micronutrient essential to human health, is an essential cofactor of biological function. However, it becomes toxic when its concentration exceeds the threshold maintained by evolutionary conservative homeostatic mechanisms[9]. Cuproptosis occurs when copper accumulates. Too much copper binds to the lipid components of the tricarboxylic acid (TCA) cycle, resulting in the accumulation of lipoprotein and the loss of iron-sulfur tuft, eventually leading to protein-toxic stress and cell death [10]. Studies have shown that cuproptosis may have a high cytotoxic effect on tumor cells. Abnormal copper levels can affect tumor growth by causing irreversible damage to organelles and consequently inducing tumor cell death through multiple mechanisms, which could be used as new targets for anticancer therapy[11-13]. Meanwhile, to adapt to adverse microenvironments, several copper chaperones are upregulated in cancer cells, which bind to cytoplasmic copper and transfer to SOD1, supporting antioxidant function and indirectly mediating cancer cell metastasis[14]. In the relationship between excessive copper and immunity, excessive copper can induce splenomegaly, disorder of the white medulla in the spleen, expansion of the red medulla and accumulation of dead cells, decrease the number of thymocytes, inhibit the proliferation of lymphocytes in other tissues and induce their necrosis and apoptosis[15]. At the same time, serum Cu and ferroxidase ceruloplasmin were positively correlated with inflammation[16].

To further study the effect of cuproptosis and immunity on colon cancer, we built a prognostic model based on the currently known genes, which is likely to provide guidance for the treatment of colon cancer.

MATERIALS AND METHODS

Study flow chart

The idea of this study is shown in Figure 1 in the form of a flow chart, and it aims to help readers better understand the research process of the article.

Data collection and handling

RNA-seq data of colon cancer patients and 41 normal controls and clinical information from 443 of these patients were downloaded from The Cancer Genome Atlas (TCGA) (https://portal.gdc.com). We acquired the gene profile data of normal tissues from Genotype-Tissue Expression (GTEx) (https://portal.gdc.cancer.gov/), and we obtained differentially expressed genes (DEGs) with the "Limma" package of R software. For external validation purpose, we downloaded the GSE39582 dataset containing 568 colon cancer samples from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Clinical information about the training set and validation set is shown in Table 1.

Screening cuproptosis- and immune-related differentially expressed genes independently associated with prognosis

We collected ten cuproptosis-related genes (CRGs) from existing reports[10], and the differently expressed cuproptosis-related genes (CR-DEGs) were obtained by intersecting them with the DEGs (Fold Change > 1.2 and p.adjust < 0.05) between colon cancer and normal samples from GTEx. Through the GeneCards database (https://www.genecards.org/), 1793 immune-related genes (IRGs) with a score ≥ 7 were obtained[17] and intersected with the DEGs (Fold Change > 2 and p.adjust < 0.05) in TCGA to obtain immune-related differentially expressed genes (IR-DEGs). The "ggplot2" package in R was used to visualize the results of univariate and multivariate Cox regression analyses on CR-DEGs

Table 1 Clinical information of The Cancer Genome Atlas and Gene Expression Omnibus samples

	Charar	TCGA (*n* = 443)	GSE39582 (*n* = 568)
Status	Alive	346	380
	Dead	97	188
Age	Mean (SD)	66.9 (13.1)	66.8 (13.2)
	Median (MIN, MAX)	68 (31,90)	68 (22,97)
Sex	Female	211	257
	Male	232	311
pTNM_stage	I	75	36
	II	176	267
	III	128	205
	IV	64	60

TCGA: The Cancer Genome Atlas.

and IR-DEGs, respectively, to screen out candidate signatures with significant independent predictive values for the prognosis of colon cancer ($P < 0.05$).

Construction and external validation of prognostic risk score models

We utilized the least absolute shrinkage and selection operator (LASSO) regression algorithm to construct three risk scoring models with candidate signatures using 10-fold cross-validation. This method was implemented with the "glmnet" package[18,19]. On the basis of the median risk score, the cancer samples were divided into two groups: The high-risk group was greater than the median, and the low-risk group was less than the median. Then, we applied the "survival" package for survival analysis and to compare overall survival (OS) in different subgroups using the Kaplan-Meier method and the log-rank test. The "timeROC" package was applied for receiver operating characteristic (ROC) analysis to evaluate the performance of the model. Finally, we used the GSE39582 dataset for external validation of the integrated cuproptosis- and immune-related model.

Principal component analysis and construction of nomograms

We performed principal component analysis (PCA) on colon cancer patients based on the cuproptosis- and immune-related model and used the "ggplot2" package for visualization. According to the results of multivariate Cox proportional hazards analysis, nomograms including risk score and other clinical predictors were built using the "rms" package to predict 1-, 3-, and 5-year OS[20].

Functional enrichment and immune profiling

Based on the cuproptosis- and immune-related model, we carried out Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and gene set enrichment analysis (GSEA) for analyses of DEGs between the high- and low-risk groups. To explore the immune profiles of the high- and low-risk groups, we applied the CIBERSORT and EPIC algorithms to calculate the abundance of infiltrating immune cells in separate subgroups. We compared the expression of genes associated with immune checkpoint inhibitors between the subgroups and displayed the results with great significance ($P < 0.05$).

RESULTS

Acquisition of cuproptosis-related and immune-related differentially expressed genes

A heatmap and volcano map present the results of gene differential expression analysis in TCGA (Figure 2A and B). Four CR-DEGs were obtained by intersecting the CRGs and DEGs (Figure 2C), suggesting that these genes may be involved in the development of colon cancer. Similarly, 299 IR-DEGs were obtained (Figure 2D).

Identification of prognostic features and construction of risk scoring models

Univariate analysis of four CR-DEGs showed that two genes were significant ($P < 0.05$). Among them, CDKN2A was a risk factor (HR > 1), while DLAT was a protective factor (HR < 1) (Figure 2E).

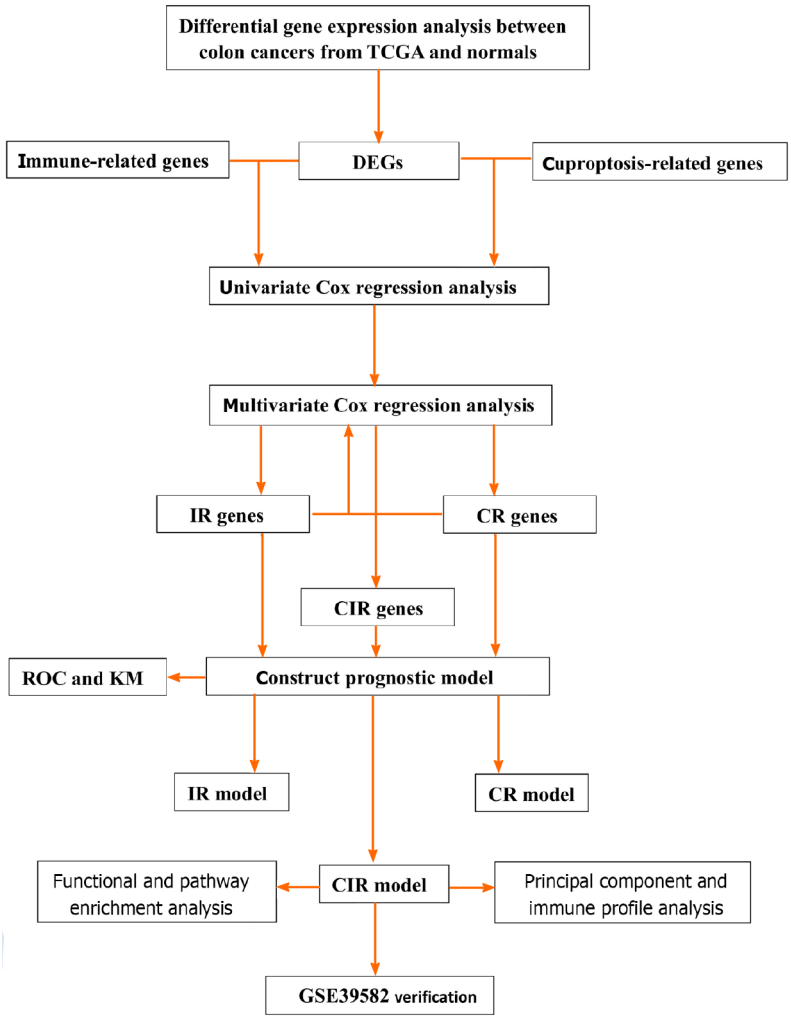

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Figure 1 Brief flowchart of the study. CR: Cuproptosis-related; DEG: Differentially expressed gene; IR: Immune-related; ROC: Receiver operating characteristic; TCGA: The Cancer Genome Atlas.

Multivariate analysis showed that both were still significant (Figure 2F), indicating that they may be independent factors affecting the OS of colon cancer. LASSO regression analysis screened the coefficients of the predictors (Figure 3A), and a colon cancer prognostic risk score model related to copper death was constructed. The model is a formula, and the optimal model is taken when λ_{min}= 0.002: Riskscore = (0.1582) × (expression of CDKN2A) + (-0.3203) × (expression of DLAT).

Analogous results were obtained in the analysis of 299 IR-DEGs. We showed 20 IR-DEGs associated with prognosis at $P < 0.05$, 4 of which had independent effects, except for UCN3 as a protective factor (HR < 1). HSPA1A, ULBP2 and FABP4 were all risk factors (HR > 1). (Figure 3B and C) When λ_{min}= 0.0017 (Figure 3D), the best immune-related colon cancer prognostic risk score model was as follows: Riskscore = (0.2507) × (expression of HSPA1A) + (0.1563) × (expression of ULBP2) + (0.1065) × (expression of FABP4) + (-0.1704) × (expression of UCN3).

After multivariate analysis of CDKN2A, DLAT, UCN3, HSPA1A, ULBP2 and FABP4, the results showed that CDKN2A and HSPA1A may be independent risk factors for colon cancer prognosis (HR > 1), while UCN3 is an independent protective factor (HR < 1) (Figure 3E and F). After coefficient screening, when λ_{min}= 0.0044, the optimal cuproptosis- and immune-related prognostic model was constructed, namely: Riskscore = (0.2391) × (expression of HSPA1A) + (0.1849) × (expression of CDKN2A) + (-0.1784) × (expression of UCN3).

Model evaluation and external validation

Figure 4A shows the risk score and survival time of patients in the model based on two CR-DEGs. The KM curve in Figure 4B shows that patients with lower risk scores had a better prognosis. The ROC curves demonstrated that the area under the curve (AUC) of the model was greater than 0.6 at 1, 3, and 5 years (Figure 4C). This indicates that the model has good predictive performance for prognosis. We performed the same analysis on two other models and obtained similar results (Figures 4D-F and Figures 5A-C). In parallel, the cuproptosis- and immune-related risk scoring model was validated in an

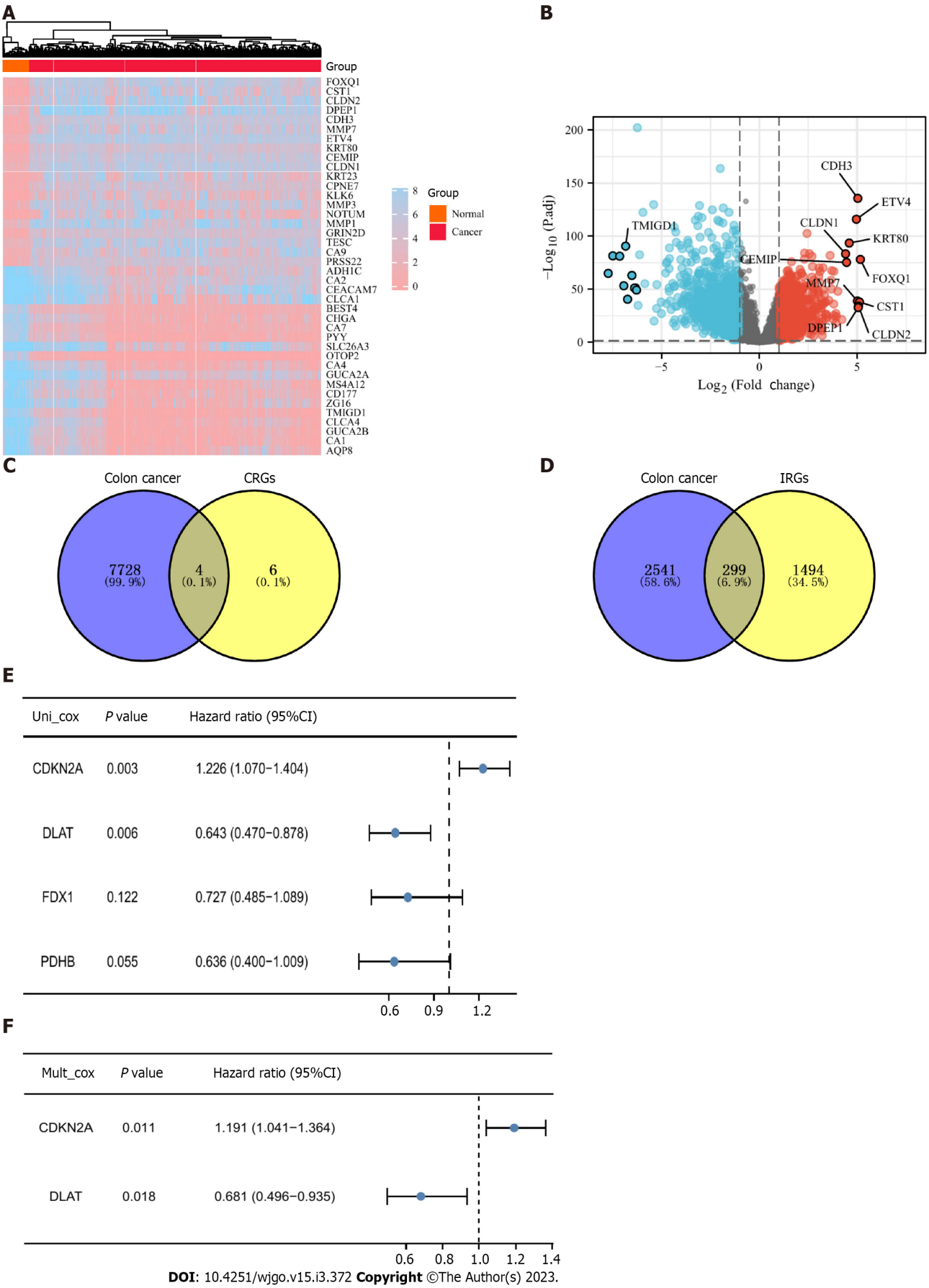

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Figure 2 Acquisition of cuproptosis-related and immune-related differentially expressed genes. A: Heatmap of differentially expressed genes in The Cancer Genome Atlas colon cancer patients compared with normal controls; B: Volcano plot of differentially expressed genes (DEGs), where blue represents downregulated genes and red represents upregulated genes; C: Four cuproptosis-related DEGs (CR-DEGs) were calculated by taking the intersection of DEGs (fold

change > 1.2 and P.adjust < 0.05) and CRGs; D: DEGs with $|log_2FC| > 1$ and p.adjust < 0.05 were intersected with immune-related genes (IRGs) to obtain IR-DEGs; E: Result of univariate Cox regression analysis of CR-DEGs; F: The result of multivariate Cox regression analysis of CR-DEGs.

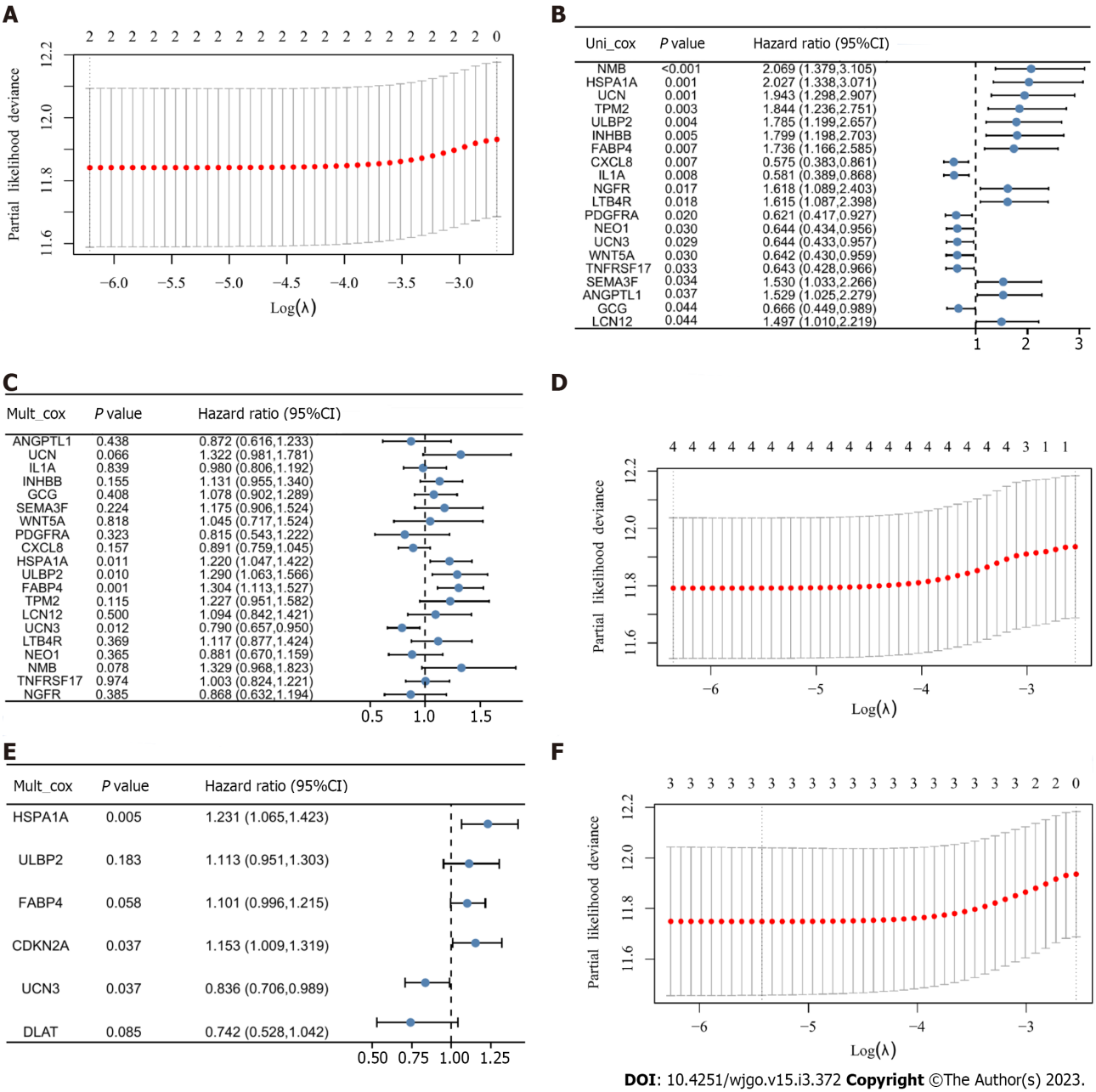

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Figure 3 Identification of prognostic features and construction of risk scoring models. A: Coefficient screening plot generated when the model was constructed using least absolute shrinkage and selection operator (LASSO) regression analysis with CDKN2A and DLAT as variables. When λ takes the minimum value, it is the best model; B: Univariate Cox regression analysis of immune-related differentially expressed genes (IR-DEGs) showed significant results; C: Multivariate Cox regression analysis of IR-DEGs identified four genes independently associated with prognosis; D: Coefficient screening plots generated when the model was constructed using LASSO regression analysis with UCN3, HSPA1A, ULBP2, and FABP4 as variables. When λ takes the minimum value, it is the best model; E: Multivariate Cox regression analysis of two cuproptosis-related (CR)-DEGs and four IR-DEGs. CIR-DEGs with $P < 0.05$ were independently associated with prognosis; F: Coefficient screening plots when the model was constructed with the selected CDKN2A, HSPA1A, and UCN3 variables.

external dataset. Both the KM curve and the ROC curve drawn using the 568 patients with colon cancer in GSE39582 were meaningful (Figure 5D-F). Therefore, we concentrated on the analysis of this model at a later stage.

PCA and the independent predictive value of the risk score

The PCA showed that different subgroups could be clearly distinguished in the cuproptosis- and immune-related models. (Figure 6A and B) Univariate and multivariate Cox regression analyses of the

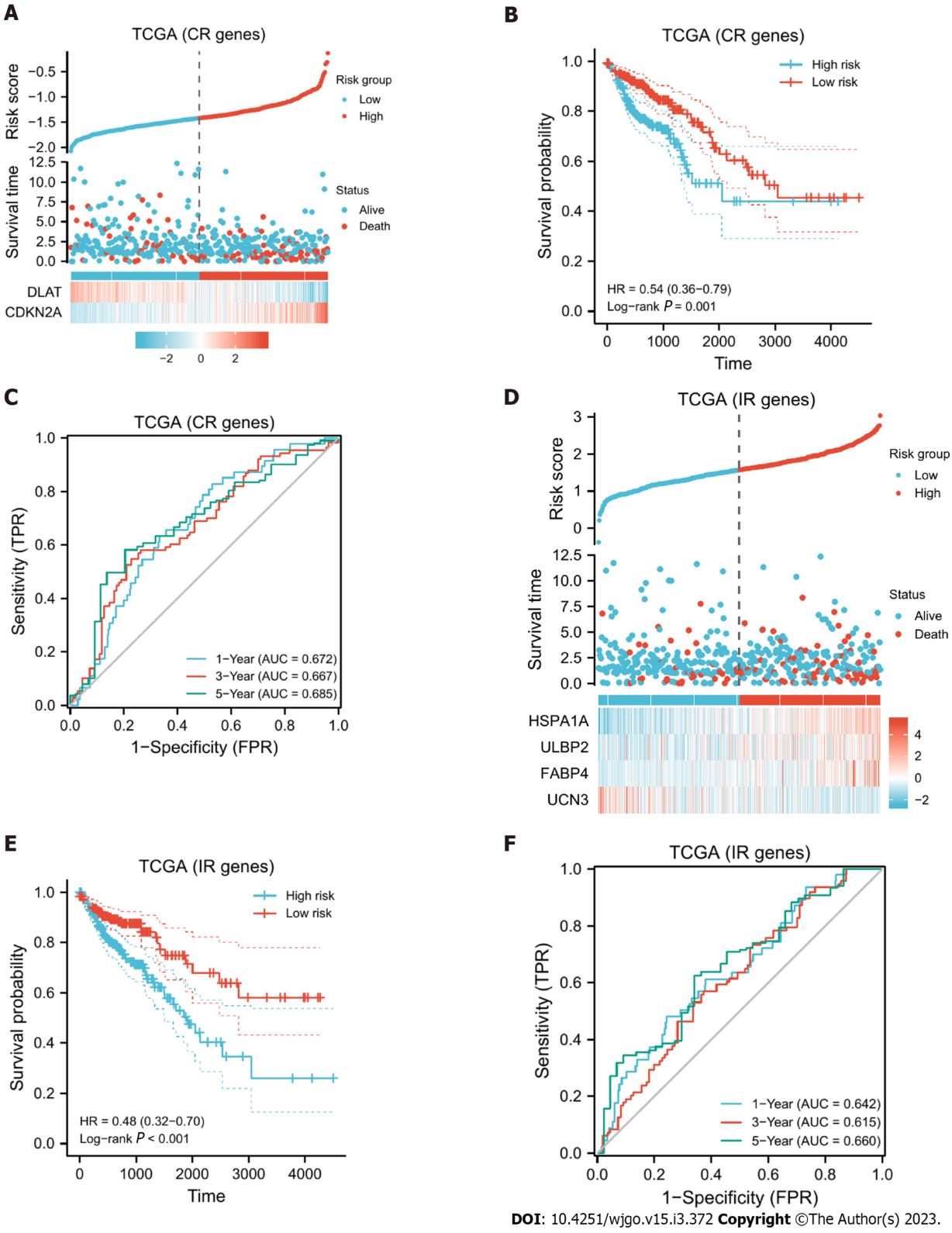

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Figure 4 Model evaluation. A: Expression differences of DLAT and CDKN2A in high- and low-risk groups and the risk scores and survival time of different subgroups are shown; B: KM curve of the cuproptosis-related (CR) model. The higher the CR risk score was, the worse the prognosis. In the figure, high-risk group was taken as the control group, so the low-risk score is a protective factor with the HR < 1; C: Receiver operating characteristic (ROC) curve of the CR model, and the size of the area under the curve can reveal the quality of the model. In the figure, the area under the curve (AUC) values in years 1, 3, and 5 are all greater than 0.6, indicating that the model has strong predictive ability; D: Expression differences of the four immune-related (IR) differentially expressed genes used to construct the model in the high- and low-risk groups and the risk scores and survival times of different subgroups are shown; E: KM curve of the IR model; the higher the risk score was, the worse the prognosis; F: ROC curve of the IR model, and its AUC values at 1, 3, and 5 years were all greater than 0.6. TCGA: The Cancer Genome Atlas database.

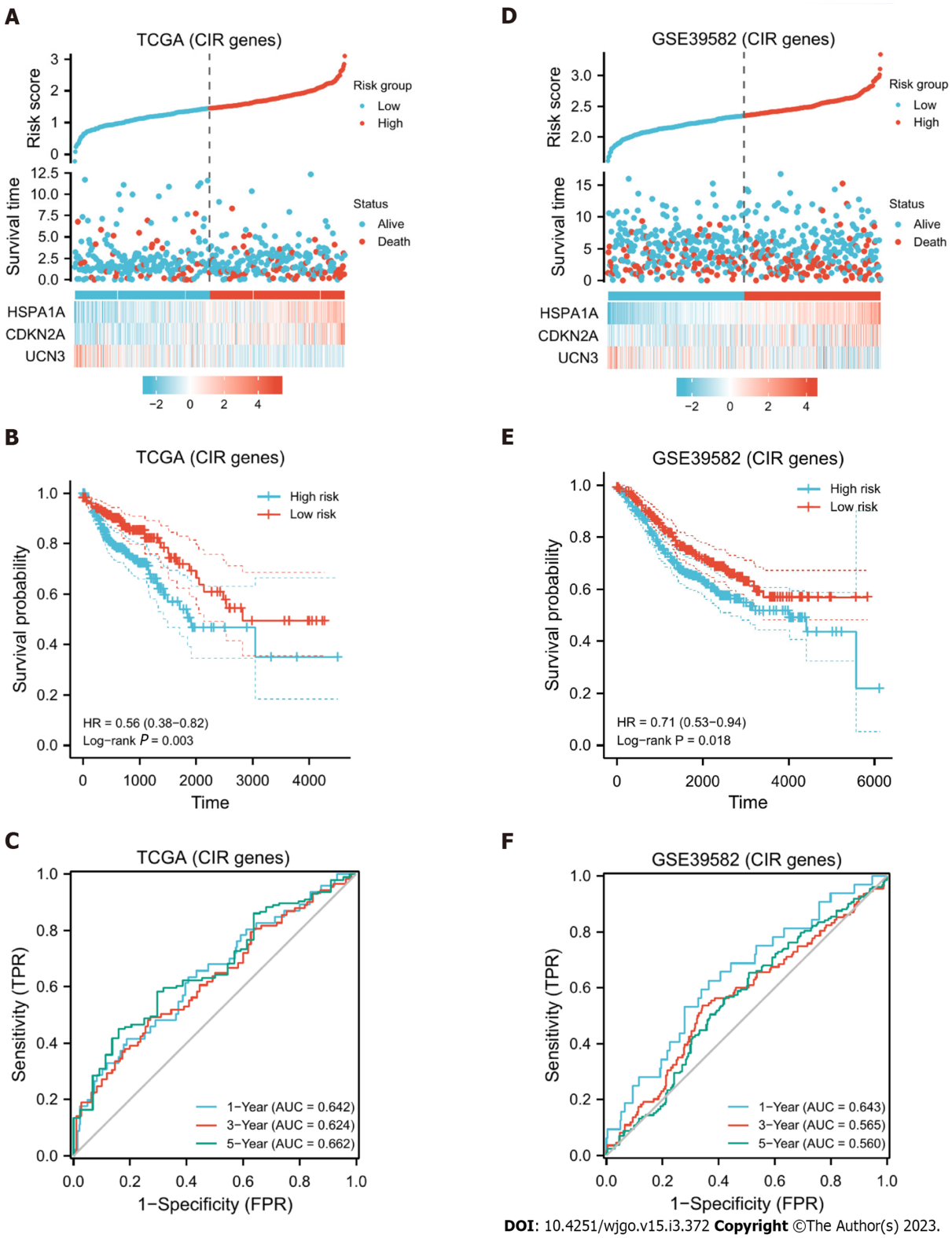

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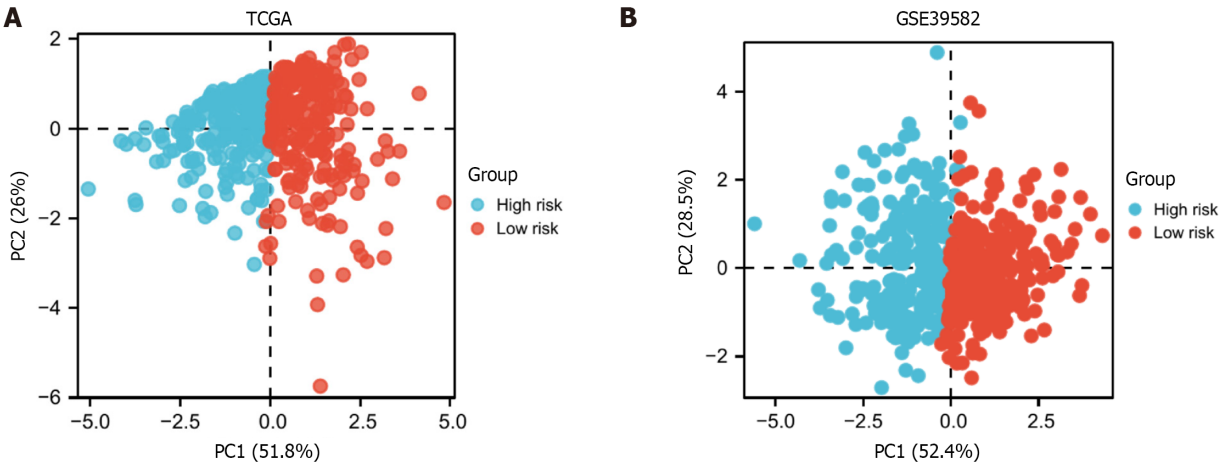
Figure 5 External validation. Based on CDKN2A, HSPA1A, and UCN3, a cuproptosis- and immune-related prognostic model was constructed. In samples of The Cancer Genome Atlas (TCGA) database. A: Expression differences of these three genes in high- and low-risk groups and the risk score and survival time of different subgroups; B: KM curve indicates that the higher the score is, the worse the prognosis; C: Receiver operating characteristic curve shows that the model has area under the curve values greater than 0.6 in years 1, 3, and 5; D-F: In the Gene Expression Omnibus database, the model obtained similar results, and $P < 0.05$.

risk score and other clinical features, such as age and pTNM stage, were carried out. Cuproptosis- and immune-related risk scores were independently related to the OS of patients with colon cancer, indicating that they can serve as independent prognostic predictors. (Figure 6C and D) Afterwards, nomograms were drawn to make it possible to derive a score for each factor from information about a specific patient and finally to estimate the probability of survival for that patient based on the total score (Figure 6E).



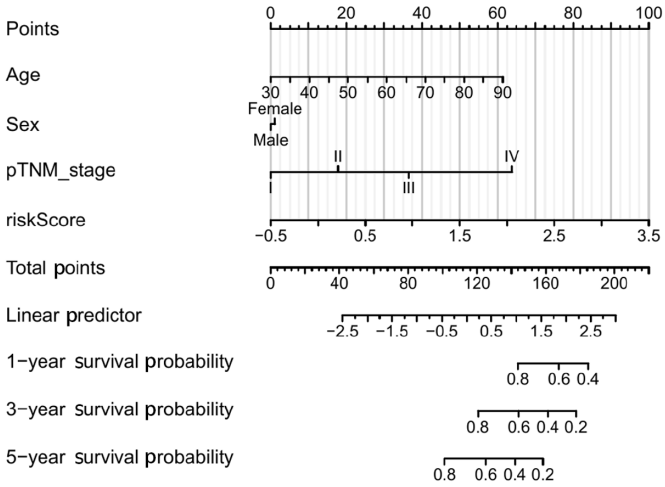
C

Uni_cox	*P* value	Hazard ratio (95%CI)
Age	0.01	1.022 (1.005–1.039)
Sex	0.551	
Male		Reference
Female	0.551	0.885 (0.593–1.322)
pTNM_stage	<0.001	
I		Reference
II	0.169	1.952 (0.753–5.058)
III	0.011	3.402 (1.324–8.743)
IV	<0.001	9.014 (3.500–23.211)
riskScore	<0.001	2.887 (1.912–4.358)

D

Mult_cox	*P* value	Hazard ratio (95%CI)
Age	<0.001	1.036 (1.018–1.054)
pTNM_stage		
I		
II	0.202	1.856 (0.717–4.805)
III	0.009	3.536 (1.366–9.151)
IV	<0.001	9.073 (3.464–23.763)
riskScore	<0.001	2.380 (1.553–3.647)

E



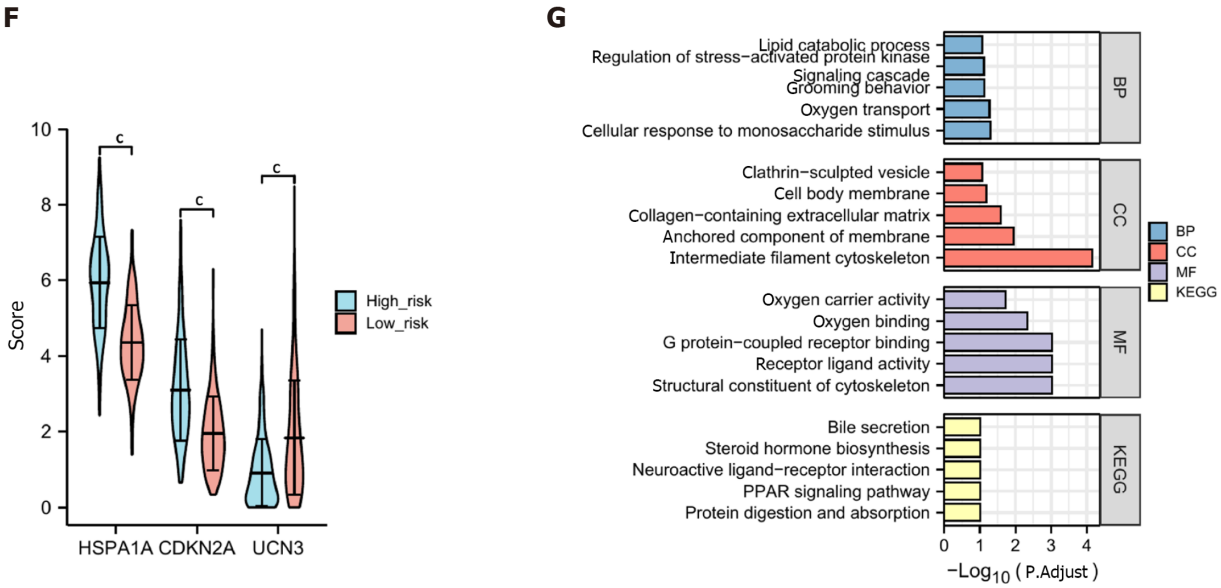


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Figure 6 Principal component analysis and the independent predictive value of the risk score. A and B: The principal component analysis plots in The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus, respectively, and both high- and low-risk groups can be obviously distinguished; C and D: Univariate and multivariate Cox regression analyses of the cuproptosis- and immune-related risk score and other clinical features to screen for factors independently associated with prognosis; E: A nomogram helps predict a patient's prognosis based on the patient's information; F: Differential expression of CDKN2A, HSPA1A, and UCN3 in the high- and low-risk groups; G: Functional enrichment consequences of differentially expressed genes in the high- and low-risk groups. $^{c}P < 0.001$.

Functional enrichment and immune profiling

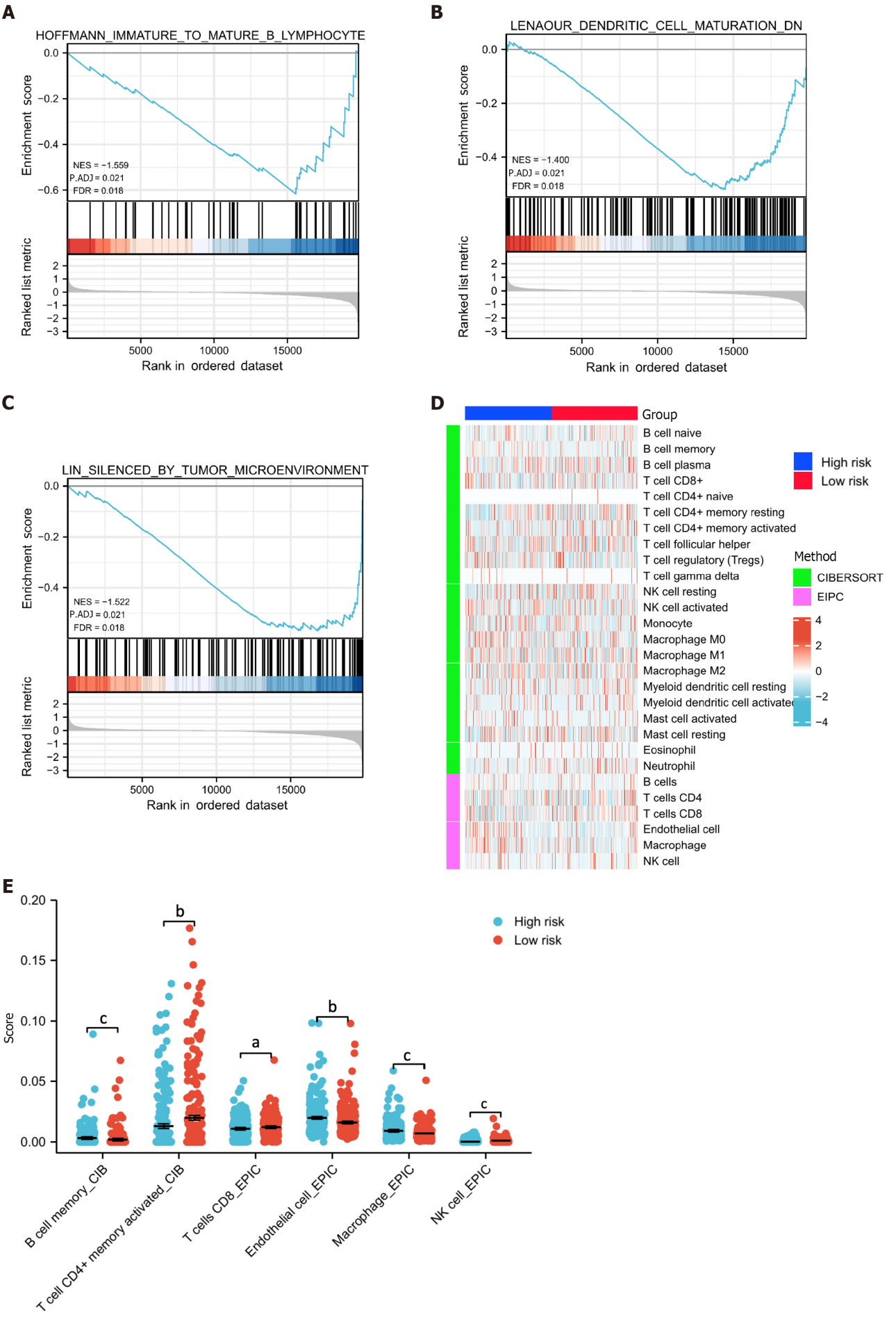
The differential expression of the three genes is presented in Figure 6F. Among them, CDKN2A and HSPA1A were upregulated, while UCN3 was downregulated in the high-risk group. Functional enrichment revealed that the DEGs between the two distinct groups were enhanced in the tumor immune microenvironment (Figures 6G and 7A-7A Therefore, we performed immune cell infiltration analysis. The heatmap in Figure 7D shows the infiltration abundance of different immune cells in different subgroups, among which memory B cells, endothelial cells, NK cells and so on were significantly different (Figure 7D and E). The differential expression results of genes related to immune checkpoint inhibitors (ICIs) showed that the expression of mRNA expression of perforin (PRF1) and TBX2 in the high-risk group was higher than that in the low-risk group, which may be associated with stronger tumor immune escape in the high-risk group (Figure 7F and G).

DISCUSSION

Colon cancer is the third most common cancer and the second most common cause of global cancer mortality[21-23]. During the past decades, the incidence and mortality of this cancer have progressively increased[21], making it imperative and urgent to identify potential biomarkers to evaluate its prognosis and treatment.

A recent study reported that cuproptosis, as a novel type of cell death, is relevant to the development and progression of malignancy[10]. Cuproptosis may have a high cytotoxic effect on tumor cells, and abnormal copper levels can affect tumor growth by causing irreversible damage to organelles and consequently inducing tumor cell death through multiple mechanisms, which could be used as new targets for anticancer therapy[11-13]. Another study found that immune cell infiltration was associated with a variety of reactions to the prognosis and treatment of colon cancer[24].

In our research, we constructed a cuproptosis- and immune-related prognostic risk model, combined with HSPA1A, CDKN2A and UCN3, which significantly differed between the low-risk and high-risk groups for colon cancer. HSPA1A and CDKN2A exhibited carcinogenesis, and their overexpression was associated with poorer prognosis and survival in colon cancer patients, whereas high expression of the UCN3 gene showed a tumor suppressor profile, which was associated with better prognosis and survival. As one of the HSP70 family members, extracellular HSPA1A is thought to elicit antitumor immune responses[25]. Previous studies have shown that the extracellular environment Hsp70 acts as a risk-associated molecular pattern (DAMP) and increases cytokine production, which can form an inflammatory microenvironment conducive to tumor progression[26]. In addition, Hsp70 promotes tumor invasion by binding to Toll-like receptors 2 and 4[27]. CDKN2A has been declared to be associated with gene mutation and cancer progression, such as colon and breast cancers[28]. CDKN2A


A
HOFFMANN_IMMATURE_TO_MATURE_B_LYMPHOCYTE
Enrichment score
NES = −1.559
P.ADJ = 0.021
FDR = 0.018
Ranked list metric
Rank in ordered dataset
B
LENAOUR_DENDRITIC_CELL_MATURATION_DN
Enrichment score
NES = −1.400
P.ADJ = 0.021
FDR = 0.018
Ranked list metric
Rank in ordered dataset
C
LIN_SILENCED_BY_TUMOR_MICROENVIRONMENT
Enrichment score
NES = −1.522
P.ADJ = 0.021
FDR = 0.018
Ranked list metric
Rank in ordered dataset
D
Group
B cell naive
B cell memory
B cell plasma
T cell CD8+
T cell CD4+ naive
T cell CD4+ memory resting
T cell CD4+ memory activated
T cell follicular helper
T cell regulatory (Tregs)
T cell gamma delta
NK cell resting
NK cell activated
Monocyte
Macrophage M0
Macrophage M1
Macrophage M2
Myeloid dendritic cell resting
Myeloid dendritic cell activated
Mast cell activated
Mast cell resting
Eosinophil
Neutrophil
B cells
T cells CD4
T cells CD8
Endothelial cell
Macrophage
NK cell
High risk
Low risk
Method
CIBERSORT
EIPC
E
Score
High risk
Low risk
B cell memory_CIB
T cell CD4+ memory activated_CIB
T cells CD8_EPIC
Endothelial cell_EPIC
Macrophage_EPIC
NK cell_EPIC

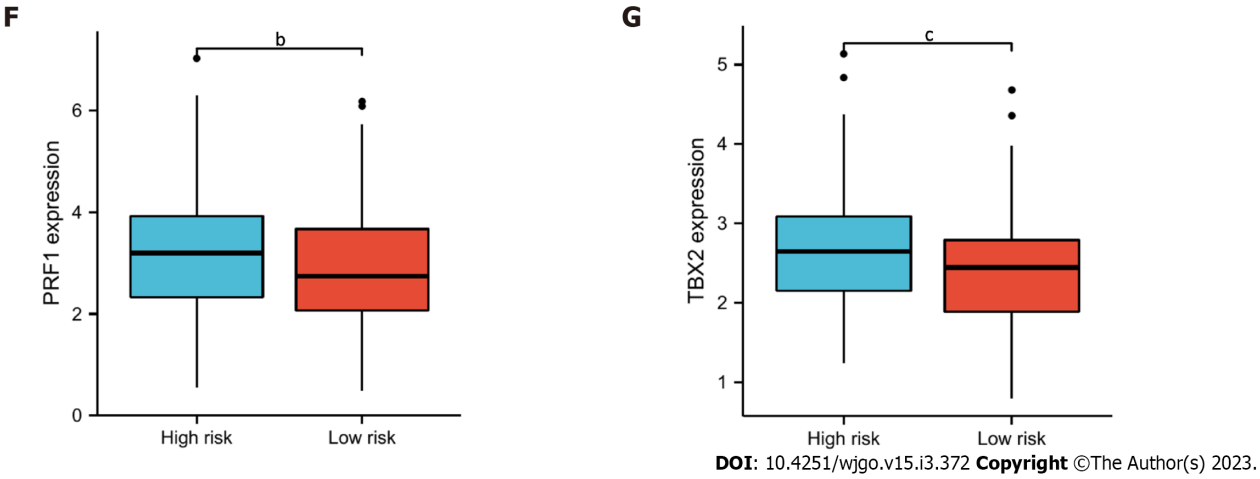


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Figure 7 Functional enrichment and immune profiling. A-C: Gene set enrichment analysis functional enrichment results of differentially expressed genes in the high- and low-risk groups; D: Heatmap showing the infiltrating abundance of different immune cells in different groups analyzed by CIBERSORT and EPIC algorithms; E: Significant ($P < 0.05$) results presented in immune infiltration analysis; F and G: Differential expression of genes related to immune checkpoint inhibitors in the high- and low-risk groups. Both were highly expressed in the high-risk group. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

is upregulated in colon cancer through two potential mechanisms: Immunosuppression and progression of epithelial-mesenchymal transition (EMT), leading to poor prognosis[29]. UCN3 might participate in local inflammatory stress response pathways to assist in the maintenance of tissue integrity and homeostasis[30]. In undifferentiated epithelial cells of colonic crypts and colon cancer cell lines, corticotropin-releasing factor 2 (CRF2) protein is expressed preferentially. UCN3-induced CRF2 signaling decreases the expression of transcription factors involved in intestinal epithelial cell differentiation and modifies the cytoplasmic and transcellular permeability of related cells[31].

Functional enrichment analysis between the high- and low-risk groups were enriched in oxygen transport, cellular response to monosaccharide stimulus, intermediate filament cytoskeleton, G protein-coupled receptor binding, receptor-ligand activity, and structural constituent of cytoskeleton. It seems that the cellular response may be altered in different risk groups[32]. In a previous study, the basal epithelial intermediate filament cytoskeleton was confirmed to be necessary for mass invasion of cancer [33]. G protein-coupled receptor binding has been implicated in the tumorigenesis, progression, and metastasis of cancers[34]. Of the many factors, mediating disease ligands and receptor activity may be implicated in cancers[35]. Through research on multiple cancer datasets, the regulation of the actin cytoskeleton signaling pathways has been identified as a cancer-related factor[36]. KEGG enrichment analysis showed that bile secretion, neuroactive ligand-receptor interaction and the PPAR signaling pathway were significantly enriched. Several studies have investigated whether the secretion of bile might be linked to the pathogenesis of colon cancers[37]. Recent studies have reported that neuroactive ligand-receptor interaction pathways are relevant to colorectal cancers[38]. GSEA showed that immature to mature B lymphocytes, dendritic cell maturation, and silenced by the TME were mainly enriched. However, as antigen-presenting cells (APCs), both dendritic cells and B-lymphocytes can deliver antigens to T-lymphocytes, thus activating immature T-lymphocytes to induce an antitumor immunization effect, which can consequently affect the prognosis of tumors[39-41]. Consequently, the prognosis for colon cancer may be regarded as being related to changes in immune cells and the TME. Meanwhile, a growing number of studies on the TME have shown that tumor-infiltrating immune cells play a vital role in cancer progression and aggressiveness, and their molecular quantitative traits are increasingly considered to have predictive value and correlate with the prognosis of colon cancer, so we followed up with an immune infiltration analysis for high-risk and low-risk groups[42,43].

B-cell memory, endothelial cells, and macrophage infiltration were higher in the high-risk group, and since it has been shown that tumor tissue infiltrating B cells are mainly B-cell memory and that macrophages and endothelial cells can contribute to cancer development and malignant progression by stimulating angiogenesis, tumor cell invasion, and intravascular infiltration, such infiltration is related to poor prognosis and reduced survival in most cancers[44-47]. The low-risk group, on the other hand, had more activated $CD4^+$ memory T cells, $CD8^+$ T cells, and NK cells. $CD4^+$ T cells can recognize tumor antigens presented by APCs and secrete specific cytokines and costimulatory signaling molecules to stimulate $CD8^+$ T cells to proliferate and differentiate into tumor effector T cells or memory T cells, which have strong tumor cytotoxic and immunosurveillance functions[48,49]. In addition, NK cells can produce a large number of cytotoxic granule proteins and cytokines without prestimulation to induce apoptosis, which can rapidly exert a tumor-cytotoxicity effect at the early stage of lesions and can complement and coordinate with the immunosurveillance function of cytotoxic T lymphocytes to inhibit the development of tumor cells[50,51], so their infiltration is mostly associated with a better prognosis.

Genes related to immune checkpoint inhibitors, such as PRF1 and TBX2, suggest that patients in the high-risk score group are more likely to experience immune escape and may be more sensitive to the immunotherapy[52]. It has been shown that immune cytolytic activity (CYT) is an immune indicator of cancer that is based on the PRF1 and granzyme A[53]. Tumors with high CYT have a high mutational load, which predisposes them to microsatellite instability. Unstable microsatellite colorectal tumors can overexpress several immune checkpoint molecules triggering immune evasion and immunosuppression, which are associated with poorer prognosis[54]. There is a significant correlation between tumor TBX2 overexpression and distant tumor metastasis as well as late-stage and recurrence[54,55]. However, the specific mechanisms by which TBX2 promotes tumorigenesis and progression are currently unclear. According to the available studies, TBX2 may inhibit the cellular senescence program by participating in the regulation of cyclin kinase inhibitor p19 ARF and p21, as well as providing cells with the ability to bypass the cytokinesis block, which leads to increased genomic instability[56-58]. Therefore, TBX2 may act as an immortalizing gene that avoids cellular senescence to participate in the regulation of cyclin and thus influence tumor progression and prognosis[54].

In this study, based on cuproptosis- and immune-related genes, we identified prognostic signatures for colon cancer and validated them by using an external dataset. Nevertheless, additional confirmation and experimental validation is necessary.

CONCLUSION

The prognosis of the high-risk group evaluated in the combined model was poorer, and cuproptosis was highly correlated with the prognosis of colon cancer. It is possible that we may be able to improve patients' prognosis by regulating the gene expression to intervene the risk score.

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FOOTNOTES

Author contributions: Huang YY collected and assessed the data; Huang YY, Bao TY, and Huang XQ wrote the manuscript; Lan QW and Huang ZM participated in revising the manuscript; Chen YH and Hu ZD worked together to finalize the submission; Guo XG proposed and designed the study; All authors contributed to the article and approved the submitted version.

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REVIEW

Prevention of malignant digestive system tumors should focus on the control of chronic inflammation

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Abstract

Chronic inflammation, through a variety of mechanisms, plays a key role in the occurrence and development of digestive system malignant tumors (DSMTs). In this study, we feature and provide a comprehensive understanding of DSMT prevention strategies based on preventing or controlling chronic inflammation. The development and evaluation of cancer prevention strategies is a longstanding process. Cancer prevention, especially in the early stage of life, should be emphasized throughout the whole life course. Issues such as the time interval for colon cancer screening, the development of direct-acting antiviral drugs for liver cancer, and the *Helicobacter pylori* vaccine all need to be explored in long-term, large-scale experiments in the future.

Key Words: Chronic inflammation; Digestive system malignant tumors; Prevention; Screening; Life course; Gastrointestinal cancer

Core Tip: Chronic inflammation plays an important role in the development of digestive system malignant tumors (DSMTs). The prevention and control of chronic inflammation is the key process of pre-disease prevention of DSMTs. This article summarizes the current prevention strategies of DSMTs based on chronic inflammation control. Health management throughout the life course significantly reduces the risk of cancer, especially in the early stages of life.

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INTRODUCTION

Cancer is the leading cause of death and an important obstacle to increasing world life expectancy. According to the latest global report, the global cancer burden is projected to increase by 47% in 2040 over 2020, reaching 28.4 million cases. Global cancer morbidity and mortality are increasing rapidly[1]. Digestive system malignant tumors (DSMTs) account for more than one-third of all cancer deaths, and mainly include colorectal (9.4%), liver (8.3%), gastric (7.7%), esophageal (5.5%) and pancreatic (4.7%) cancer[1].

Inflammation represents the host's immune response to destructive stimuli caused by irritants or pathogens. While most pathogens stir up an acute inflammatory response that completely clears irritants from suitable hosts, insufficient resolution of inflammation and an unrestricted inflammatory response can trigger chronic inflammation, destroy host immunity, and predispose the host to various diseases, including cancer[2]. The link between chronic inflammation and DSMTs has been established for more than a century[3,4]. Approximately 25% of cancer cases have been estimated to be related to chronic inflammation[5]. In fact, chronic inflammation tends to lead to tumor formation in various gastrointestinal organs, including hepatocellular carcinoma (HCC) caused by chronic hepatitis induced by hepatitis B virus (HBV) or hepatitis C virus (HCV), gastric cancer (GC) caused by chronic gastritis associated with *Helicobacter pylori* (*H. pylori*), and colorectal cancer (CRC) caused by inflammatory bowel disease (IBD).

Much research evidence suggests that chronic inflammation contributes greatly to tumorigenesis, but the underlying molecular mechanisms are intricate. The innate immune system has pro-tumor and anti-tumor effects on tumorigenesis. The innate immune response can protect the host from tumors induced by viruses by inhibiting or eliminating viral infections. On the other hand, swift removal of pathogens and suppression of inflammation will establish an appropriate inflammatory microenvironment for tumor formation[6-8]. Recent studies have found that when persistent inflammation occurs, the inflammasome complex begins to activate, causing it to assemble and further activate caspase, produce pro-inflammatory cytokines, and induce pyroptosis[9]. Apoptosis-associated speck-like protein containing a CARD promotes tumors through the nuclear factor kappa B (NF-κB) signaling pathway in GC[10]. Nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing protein have the ability to support rapid migration of cancer cells *in vitro* and metastasis *in vivo* in CRC[11].

Because DSMT diagnosis usually occurs in the late stages of disease development, more work is needed to realize the enormous potential of primary prevention and early detection. This article summarizes the latest progress in DSMT prevention strategies based on chronic inflammation.

EPIDEMIOLOGY

The global incidence of DSMTs has decreased gradually, while DSMT mortality has remained steadily at 35.6%. Major DSMTs are colorectal (1.14 million new cases in 2020), gastric (1.08 million), and liver (906577) cancers. In the early stage, most DSMT patients are asymptomatic or have chronic inflammation, which is not easy to detect. When clinical symptoms appear, they are usually in the late stage of the disease. Due to the late diagnosis of DSMTs, the 5-year survival rate is usually very low[12].

Chronic inflammation caused by infection with potential carcinogens is a major risk factor for DSMTs (Figure 1). Parkin DM's study showed that more than one-quarter of cancers were attributable to infection[13]. In 2018, an estimated 2.2 million diagnosed cancer cases were attributed to infections worldwide[12]. Primary causes were *H. pylori* (810000 cases), HBV (360000), and HCV (160000). The highest infection-attributable age-standardized incidence rates (ASIR) was in eastern Asia (37.9 cases per 100000 person-years) and sub-Saharan Africa (33.1), and the lowest ASIR was in northern Europe (13.6) and western Asia (13.8). China accounts for one-third of worldwide cancer cases attributable to

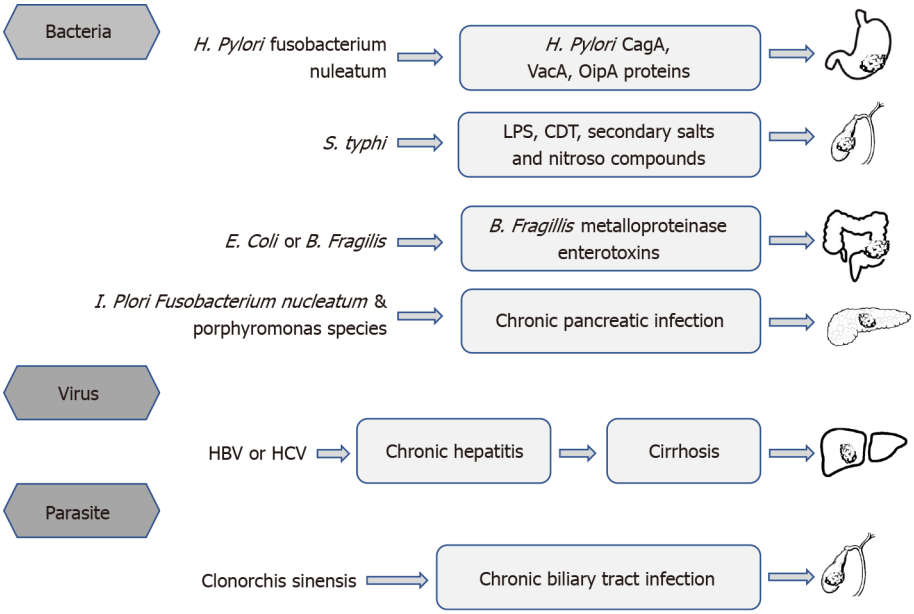

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Figure 1 Carcinogenic mechanism of malignant digestive system tumors based on chronic inflammation. *H. pylori: Helicobacter pylori*; CagA: Cytotoxin-associated gene A; VacA: Vacuolating cytotoxin A; OipA: Outer inflammatory protein A; *S. typhi: Salmonella Typhi*; LPS: Lipopolysaccharide; CDT: Cytolethal distending toxin; *E. coli: Escherichia coli*; *B. fragilis: Bacteroides fragilis*; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

infection, driven by the high ASIR of *H. pylori* (15.6) and HBV (11.7) infection[12]. The overall burden of cancer caused by infection is the same in men and women. In Asia, among six major infectious agents, new cancer cases were mostly attributed to *H. pylori* (31.5%), followed by HBV (28.6%), and HPV (22.0%).

More than half of all DSMTs were caused by modifiable risk factors, including alcohol consumption and smoking, chronic inflammation caused by infection, diet, and obesity[14]. The marked temporal variations in the main DSMT incidence over the past decades are largely ascribed to changes in the prevalence of these risk factors[15,16]. Since most DSMT diagnoses[17,18] occur at an advanced stage with poor prognosis, mortality trends at specific sites usually reflect incidence trends. In 2040, the number of new DSMT cases and deaths worldwide are expected to increase by 58% and 73%, respectively[12]. This estimated burden level highlights the necessity of identifying the best clinical approaches for DSMTs, as well as the need to prioritize and implement preventative strategies. DSMT incidence is declining, while mortality and the number of cancer deaths are still high.

MECHANISM OF CHRONIC INFLAMMATION IN THE OCCURRENCE OF DSMTS

To date, many studies have linked chronic inflammation to DSMTs, including tumors in the colon, liver, stomach, and other parts of the digestive tract[19-22]. Chronic inflammation can generate the production of chemokines, growth factors, reactive oxygen species (ROS), and reactive nitrogen species (RNS). These mediators trigger inflammatory pathways in digestive system epithelial cells including the cyclooxygenase-2 (COX-2), NF-κB, signal transducer and activator of transcription 3 (STAT3), and inducible nitric oxide synthase pathways. Subsequently, activation promotes tumor initiation by increasing cell circulation, inhibiting tumor inhibitory pathways, and activating oncogenes[3]. As soon as tumors are established, inflammation promotes tumor progression by suppressing apoptosis, promoting cell proliferation and angiogenesis, regulating cellular adhesion, and promoting metastasis [23]. Due to their high activity against cancer and inflammation, natural products and their derivatives have been studied, such as berberine[24]. Chronic inflammation caused by exposure to long-term environmental irritants or associated with infection or autoimmune disease precedes tumor development and can promote it through oncogene mutations, genomic instability, early tumor promotion, and heightened angiogenesis[25]. Tumor-associated inflammation develops with tumor development. These inflammatory responses can heighten neoangiogenesis[26], promote tumor development and metastasis, cause local immunosuppression[25], and further increase genomic instability[27]. By causing necrosis and tissue injury, tumor therapy can also trigger an inflammatory response that promotes tumor reemergence and resistance to therapy. This article focuses on chronic inflammation precedes tumor development.

The gastrointestinal tract not only has the function of digesting food and absorbing nutrients but also provides a physical barrier for the body to resist a large number of pathogenic or commensal microorganisms in the gastrointestinal cavity[28]. Through many innate immune mechanisms, the gastrointestinal tract prevents and clears pathogenic microbial infections of the intestine[29]. Host inflammation affects gut microbiome composition and functional capacity[30]. Chronic inflammation can target and induce the expansion of gut microbes[31]. Gut microbiota forms the host immune system and is essential for human health[32]. The appearance of gut inflammatory diseases, such as colitis, is associated with changes in the gut microbes[33]. Colitis occurs when microbes change from a "eubiotic" to a "dysbiotic" state, which is a risk factor of CRC development[34]. Compelling evidence are supporting that alteration of gut microbiota, particularly the dysbiosis condition might produce enrichment in pro-inflammatory opportunistic pathogens and a decrease in butyrate-producing bacteria, which may lead to an imbalance in intestinal homeostasis that could ultimately lead to tumor formation[23,35,36]. The relationship between *H. pylori* and GC is the most iconic relationship between individual microbial species and cancer[37]. When *H. pylori* is present, making up 40%-90% of the stomach microbiome, it becomes the richest organism in the stomach microbiome[38]. *H. pylori* contains toxins like cytotoxin-associated gene A (CagA) and cytotoxin-associated gene A pathogenicity island (VacA), which can manipulate cell survival and chronic inflammation that leads to cancer[39]. Many mechanisms could explain how microbes influence colorectal oncogenesis. Mucosa-associated *Escherichia coli* and *Bacteroides fragilis* (*B. fragilis*) are often found in tissues of patients with Crohn's disease and CRC[40]. *B. fragilis* produces a zinc-dependent metalloproteinase toxin called *Bifidobacterium fragilis* toxin, which cleaves the extracellular domain of the cell surface protein E-cadherin, resulting in the complete degradation of E-cadherin[41]. Long-term colonization of the colonic epithelium by *B. fragilis* increases the risk of CRC. Loss of surface barrier function in CRC triggers commensal bacteria-induced tumors that promote inflammation. The commensal bacteria themselves can also invade tumor tissue, induce tumor-infiltrating myeloid cells to produce inflammatory cytokines, and promote CRC oncogenesis[23]. The resistance response induced by *Fusobacterium nucleatum* (*F. nucleatum*) in the host induces an inflammatory environment and promotes the recruitment of inflammatory cells as well as the secretion of inflammatory factors[42]. This effect of *F. nucleatum* creates a microenvironment conducive to tumor growth. About 3%-5% of acute *Salmonella Typhi* (*S. Typhi*) infected people become chronic carriers. Since *S. Typhi* infection persists in the biliary system, leading to chronic infection of the gallbladder[43]. A case-control study showed that chronic typhoid carriers died from hepatobiliary cancer six times more frequently than in the control group[44]. A study conducted in Chile, a country with the highest infection rate in the world, showed that early detection of *S. Typhi* is critical for the development of gallbladder cancer prevention strategies[45].

Many studies have shown that several chronic diseases, such as cancer, which are more common in later adulthood, are influenced by social and psychological environments at birth, during childhood, in adolescence, and during early adulthood[46]. Studies have shown that an accumulation of damage over the course of life is the cause of disease, not simply what happens at a certain point in life. DSMT is a multifactorial complex systemic chronic disease. Most of the underlying exposures cannot be considered individual factors, and exposures cannot be treated as separate or isolated factors because of their role at different levels, which vary from time to time. Life-course epidemiology[47] attempts to combine the entire biological and social risk processes leading to chronic disease. It studies how exposure to social patterns at various stages of life (childhood, adolescence, and early adulthood) affects disease risk and socioeconomic status in adulthood, which may lead to social inequality in adult health and mortality [48]. Current research in life course epidemiology has focused on chronic infectious diseases, psychological diseases, cardiovascular diseases, and other aspects. Cancer is also regarded as a noninfectious chronic disease, and many related life course epidemiological studies have been carried out on cancer, such as aerodigestive cancer[49], HCC[50], and CRC[51]. Most of these studies focus on early life variable risk factors such as chronic inflammation and diet. The time from susceptibility to disability, death, or recovery is variable. In many nonpersistent infections, symptoms of the disease occur within days (more over a longer period) of the initial infection. For example, HBV and HCV persist and replicate in the body, and may not cause disease (primary liver cancer in this case) until 50 years after infection. Age at the time of infection also affects the severity and progression of the disease. Epstein-Barr virus infection hardly causes any symptoms in childhood, whereas it results in glandular fever in adolescence. With increasing age, hepatitis A has become an increasingly serious disease. In terms of the origin of diseases (such as cancer), the core of the life course health development model is to find the causes from the life course framework, focus on identifying high-risk phenotypes and risk markers in early life, and then take measures to prevent diseases and promote health. We should look at the healthy development trajectory from the perspective of the whole life course (Figure 2). Disease prevention and health promotion are important throughout the whole life course, but in the "window of opportunity" period, such as fetal development, childhood, and adolescence, the intervention is more effective[52]. The earlier an intervention occurs, the better the effect of disease prevention.

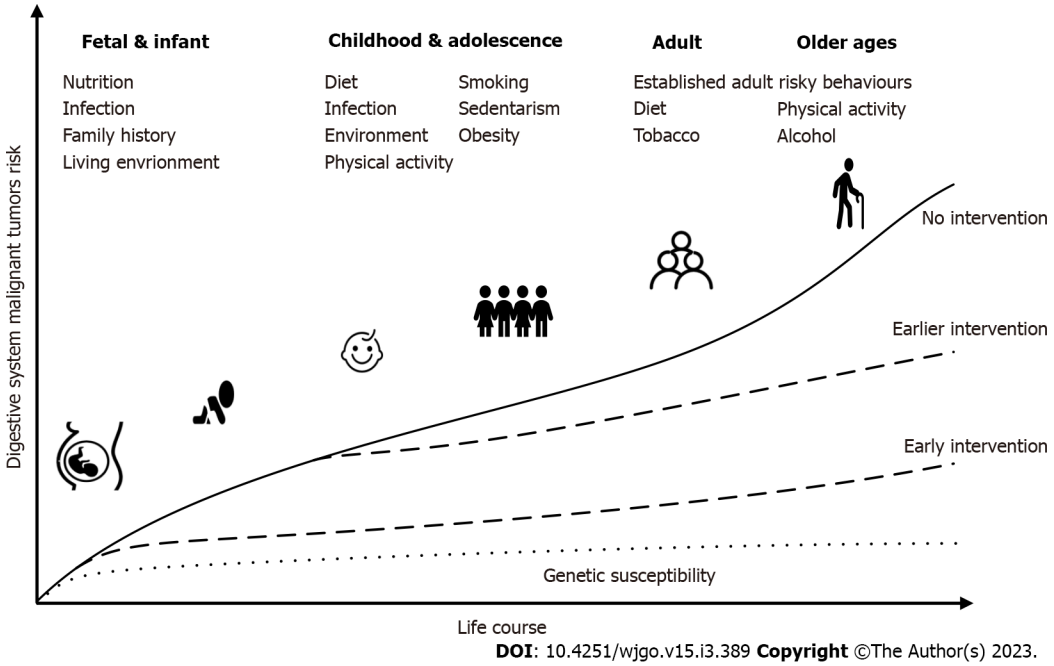

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Figure 2 Human life course and the risk of digestive system malignant tumors. Risk factors at different stages of the life course from the fetal and birth stages affect the occurrence and development of cancer. The potential genetic susceptibility to cancer is changed by chronic inflammation, diet, nutrition and other carcinogens in the environment, which determines the cumulative risk of cancer. Cancer prevention should include actions at each stage of the life course, of which the early stage is the most important. The greatest potential for an effective fight against cancer is to reduce the cumulative risk throughout the life course.

STRATEGIES FOR POPULATION-WIDE AND TARGETED SCREENING OF MALIGNANT TUMOR BASED ON COUNTRACTING CHRONIC INFLAMMATION

Population-wide prevention includes avoidance of known carcinogens, enhancement of host defense mechanisms, lifestyle changes, and chemoprevention. In cancers associated with chronic inflammation, eradication of the relevant pathogen must be considered a population-wide preventative measure. Target population prevention includes screening and treatment of precancerous lesions or early cancers.

Colorectal cancer

According to the Global Cancer Statistics 2020 report, CRC is the third most common cancer worldwide, and its incidence has been increasing despite some advances in screening and treatment[53]. Due to the lack of effective treatment, the 5-year survival rate of stage four CRC patients at diagnosis is less than 10%[23]. Although developed countries have the highest CRC incidence and mortality around the world, CRC incidence has recently shown an increasing trend in low-income and middle-income nations[54]. This trend reflects changes in lifestyle factors and diet: increased intake of animal-derived foods and sedentary lifestyles lead to reduced physical activity and a higher prevalence of excess body weight[1]. However, complex reasons behind this reflect both population ageing and global population growth, as well as changes in the prevalence and distribution of major cancer risk factors. The study found that in countries undergoing major transitions, such as Eastern Europe, Southern Europe, Central and South Asia, and South America, incidence tends to increase consistently with the human development index[55].

CRC has long been considered to be closely related to chronic inflammation, which can occur in the early stages of tumor onset. Studies have shown that[56] IBD, including Crohn's disease and ulcerative colitis (UC), dramatically increases the risk of colitis-associated CRC (CAC). UC patients have a 2.4-fold increased risk of CAC[57]. Currently, the relationship between IBD patients and CRC has been widely confirmed. Compared with the general population, patients with long-term colonic IBD have a higher risk of CRC[58]. Any event that promotes and maintains inflammation may be considered a potential carcinogen[59]. Under healthy conditions, there is a strictly controlled interaction between enterocytes and intestinal immune system cells to maintain the balance between proinflammatory mediators [*e.g.*, tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6] and anti-inflammatory signals (*e.g.*, IL-10 and transforming growth factor-β)[60]. In IBD, an imbalance leads to changes in cell behavior. In CAC, various inflammatory mediators (*e.g.*, TNF, IL-17A, and IL-23) and genotoxic substances (*i.e.*, ROS and RNS) generated by different cellular systems (immune cells and mesenchymal cells) synergistically introduce genetic and epigenetic modifications, eventually leading to tumorgenesis[61]. The genetic mechanisms of tumorigenesis in CAC are similar to that in sporadic CRC, including chromosomal

instability, microsatellite instability, mutations in pivotal tumor suppressor genes, and aneuploidy. But the timing and frequency of these conditions are different between CAC and sporadic CRC[62]; *TP53* variants and aneuploidy were detected earlier in CAC, than sporadic CRCs, while *KRAS* and *APC* variants showed a lower prevalence at later stages of tumorigenesis[60]. Recently, some scholars[42] have found that *F. nucleatum* promotes the occurrence of CRC through several virulence mechanisms: adhesion to the intestinal epithelium or induction of host inflammation and host immune response.

Population-wide prevention: Population-wide prevention is an important supplement to CRC screening and prevention. In addition to some inherent risk or protective factors, such as sex, age, family history, and genetic predisposition, epidemiological studies have revealed some potentially modifiable factors related to the increase or decrease in CRC risk, which all point out the challenges and potential opportunities for prevention.

Various nutrients with proinflammatory or anti-inflammatory activity may affect CRC risk through intertwined pathways, such as intestinal microbial metabolism[63]. In recent years, there have been many studies on the prevention measures of CRC based on the prevention and control of chronic inflammation. Smoking, excessive drinking, being overweight or obese, Western diets and processed meats, and inflammatory eating patterns are risk factors that have been recognized in recent years. On the other hand, physical activity, regular use of aspirin and hormone replacement therapy, non-steroidal anti-inflammatory drugs (NSAIDs), probiotics, and the Mediterranean diet were found to be associated with decreased CRC risk. There are signs that consuming milk and whole grains may also protect against CRC[64]. Inflammation may be a potential mechanism linking dietary patterns to CRC development[65]. A large cohort study of 121050 adults who were followed up for 26 years found that the risk of CRC increased significantly with the intake of a proinflammatory diet. Reducing the adverse effects of an inflammatory diet may reduce the risk of CRC[66]. Physical activity can reduce systemic inflammation, reduce the level of proinflammatory cytokines related to CRC, or affect the inflammatory microenvironment, which may play a crucial role in reducing the risk of CRC[67]. With respect to the use of aspirin in the general population, the incidence of CRC decreased by 26% after 23 years of follow-up. The use of NSAIDs, such as aspirin and celecoxib (COX-2 inhibitors), has a protective effect on the occurrence of CRC[68]. Studies have shown that the potential mechanisms related to the use of probiotics are alterations in the gut microbiota and physicochemical conditions, the production of antioxidant metabolites, a reduction in intestinal inflammation, and the production of harmful enzymes to support the prevention of colon cancer[69].

Targeted screening: CRC usually develops slowly over a period of several decades after normal colonic epithelium is transformed into an adenoma, providing ample time for intervention to prevent CRC[70]. In 1980, the American Cancer Society recommended the use of fecal occult blood tests for early diagnosis of CRC every year for people over 50 years old[71]. Since then, inspection technology has developed rapidly[72] and the screening strategy has been continuously updated[73-76]. Currently, common screening techniques include colonoscopy, rectal endoscopy, and fecal occult blood tests. At the same time, many new technologies, such as color endoscopy, are being studied as complementary technologies to improve the early detection of dysplasia and cancer in high-risk populations[77]. One study evaluated colonoscopy and regular endoscopic follow-up of patients who had undergone precursor resection. Compared with the external control group, the incidence, and mortality of rectal cancer decreased by 31% and 18%, respectively, after 20 years of follow-up[78]. Researchers at the Harvard School of Public Health (HPFS) found that 40% of CRC can be prevented if people undergo colonoscopies regularly[79]. Target population screening can effectively reduce the incidence and mortality of CRC. The incidence and mortality of screening are reduced by approximately 50% and 53%, respectively, but the gap can be filled by modifying the prevention strategy of attributable risk factors in the whole population. However, there are several aspects of CRC screening problems in clinical practice that require specific attention: Who should be provided with CRC screening? When should the first screening test be given? Is the screening interval the same for everyone? How screening strategies be developed? The burden of disease and the overall socioeconomic situation vary from region to region, and each region should propose screening recommendations suitable for its population based on various international standards and consensus.

Population-wide prevention requires a lifelong perspective and may have benefits in the long run. Since CRC and other common chronic diseases have many of the same risk and protective factors, long-term universal prevention efforts aimed at reducing CRC risk factors may provide far more than just the expected benefits[80]. Changing dietary and lifestyle factors may have a significant overall impact on the risk of CRC. In previous HPFS studies, up to 70% of the total burden of colon cancer in the United States population could be prevented by moderately changing diet and lifestyle[81]. To further integrate the combined effects of modifiable risk factors, a comprehensive model of colon cancer incidence was developed that took into account the changes in risk factors throughout the life course. The study found that women with "high-risk" lifestyle factors were nearly four times more likely to develop colon cancer than women in the "low-risk" group[82]. Although endoscopic screening can reduce the incidence of cancer in these high-risk women, the CRC risk after this reduction is still significantly higher than that of medium- and low-risk women. Therefore, a population-wide prevention strategy of changing

lifestyle in early life is an important supplement to CRC screening.

HCC

HCC is the most common form of primary liver cancer (75%-85% of cases). The incidence and mortality of liver cancer have declined in many high-risk countries in eastern and southeastern Asia since the 1970s and in Japan since the 1990s. Vaccination against HBV had been a major public health success. It was first introduced to high-risk countries in East Asia in the early 1980s and greatly reduced the prevalence of HBV infection and the incidence of HCC[83]. It is believed that the aetiology of HCC is mainly related to cirrhosis, viral hepatitis, alcoholic liver disease, metabolic-related fatty liver disease, aflatoxin infection, heavy drinking, being overweight, type 2 diabetes, and smoking[1]. HBV infection and HCV infection account for 56% and 20% of global liver cancer deaths, respectively. Although nonviral risk factors are increasingly important for the burden of liver cancer, the elimination of viral hepatitis is still a key strategy for the primary prevention of liver cancer worldwide[84]. Viral hepatitis is the main pathogenic factor, the most common of which is chronic HBV and HCV infection. Compared with HCV infection alone, cirrhosis and HCC are more likely to occur in patients with HBV/HCV coinfection[85]. Because the chronic infection is usually asymptomatic, many infected people are not diagnosed[86]. As of 2015, an estimated 290 million people worldwide remained undiagnosed, and more than 80% of liver cancer patients are diagnosed at an advanced stage[87].

HCC usually occurs in tissues that experience chronic inflammation[88]. Although the underlying molecular mechanisms of the aetiology are different, in most cases, chronic liver inflammation and the resulting cirrhotic microenvironment promote the initiation and development of HCC[89]. Repeated liver inflammatory injury can lead to liver cell damage, cirrhosis, and ultimately hepatocellular carcinoma[90]. The microbiome profile, consuming a high-fat Western diet, and a high intake of alcohol [91] are associated with various forms of inflammations, which will promote the onset of HCC. The main trigger of inflammation associated with liver cancer is epithelial cell death. Pathways contributing to inflammation-mediated hepatocarcinogenesis mainly include cytokine signaling (TNF-α, IL-6, NF-κB, JNK, and STAT3), innate immune signaling, and adaptive immunity[89]. Experimental evidence indicates that HCV may also contribute to hepatocarcinogenesis directly through the interference of viral proteins with host cell signaling pathways involved in cell survival, transformation, proliferation, and angiogenesis[92]. Benkheil's studies have shown that activation of the epidermal growth factor receptor (EGFR) and downstream signaling through mitogen-activated protein kinase contribute to the expression of various proinflammatory and angiogenic proteins involved in the pathogenesis of liver cancer. These data suggest that sustained activation of EGFR in patients with chronic HCV infection may be a mechanism by which HCV contributes to the pathogenesis of liver cancer[93].

To reduce the burden of global HCC, the global HCC management strategy[94] points out that four major areas need to be improved: prevention of HBV and HCV infection; treatment of chronic hepatitis B, hepatitis C, and liver disease; reduction in exposure to dietary and metabolic risk factors; and improvement in the detection, diagnosis, and treatment of liver cancer. Three of them are related to the prevention and treatment mechanisms of chronic hepatitis.

Population-wide prevention: Population-wide prevention includes universal coverage of the hepatitis B vaccine, control of chronic viral hepatitis through antiviral therapy, and a reduction in environmental and lifestyle-related risk factors[94]. Since the World Health Organization proposed a hepatitis B immunization mid-course target trial for liver cancer prevention in 1983, 189 countries had introduced hepatitis B vaccines into their national infant immunization programs by the end of 2019, with an estimated 85% global coverage of three doses[1]. Increasing evidence showed that this large-scale vaccination greatly reduces the burden of hepatitis B virus-related diseases. This campaign in China has reduced the prevalence of new HBV infections by 90%. It will prevent approximately 2.8 to 3.5 million HBV-related deaths in the future[95]. Mother-to-child transmission (MTCT) is the main route of HBV transmission and its prevention is very important to eliminate HBV. Strengthening the standardized management of pregnant women and their infants with chronic HBV infection is an effective measure to eliminate HBV-MTCT[96]. There is evidence that antiviral therapy for pregnant women with a high HBV load in late pregnancy can reduce the risk of MTCT[97]. A population-based study in Taiwan showed that the HCC incidence in a birth cohort without the hepatitis B vaccine was four times higher than that in a birth cohort with the hepatitis B vaccine[98].

In addition to vaccination against HBV, screening for HBV and HCV in high-risk populations, and universal access to medication for chronic hepatitis B and hepatitis C infection in infected patients will reduce the burden of global liver cancer. Currently, there is no vaccine available to prevent HCV infection. Direct-acting antiviral agents (DAAs) are short-course (8-12 wk) oral drugs[94,99]. The emergence of DAAs as a new HCV drug with a high cure rate (> 95%) offers hope for the treatment of chronic hepatitis C. At present, the impact of DAA treatment of HCV infection on HCC, tumor recurrence, and progression has become a hot topic. Successful DAA treatment does seem to reduce the risk of HCC, but studies have shown that DAAs may increase the risk of HCC recurrence after treatment. Therefore, well-designed prospective multicenter studies are needed to fully characterize the clinical effect of DAA treatment on the risk of HCC recurrence. The countries with the highest prevalence of HCV are mainly low-income and middle-income countries, where a large proportion of

infections occur in healthcare settings through unsafe injections and other invasive procedures. Strengthening infection control through safety measures, such as screening blood transfusions, preventing MTCT, and providing clean needles and medical facilities, is a key aspect of HCV control [100]. Although viral therapy has been improved through DAA therapy, cases of HCV-induced HCC are expected to increase at least until 2030[101]. The increase is mainly attributed to the increase in chronic HCV infection before 1992 when HCV screening was implemented and the disease progressed slowly[93]. People with known risk factors must be regularly monitored to detect early cancer lesions (monitoring and final treatment). Early detection and diagnosis of HCC can significantly improve the survival rate of patients.

For 400 million chronic HBV-infected patients, the hepatitis B vaccine is ineffective in preventing HCC[102]. Increasing evidence shows that persistent HBV replication is an important risk factor for HCC. For CHB patients, antiviral therapy to control viral replication may reduce the risk of HCC. Interferon or nucleoside analogues are effective antiviral drugs to prevent disease progression to cirrhosis and HCC[102]. Additionally, promoting a healthy diet and physical activity, reducing environmental and lifestyle exposure, and preventing metabolic syndrome, nonalcoholic fatty liver disease, and nonalcoholic steatohepatitis are ways to prevent HCC.

Targeted screening: Targeted screening includes early detection through HCC surveillance programs. The current practice guidelines recommend regular HCC screening by ultrasonography every two years for α-fetoprotein (AFP) in people with or without a clinically identifiable HCC risk above a certain threshold[103]. A series of cohort studies and model-based simulation studies have shown that HCC screening is cost-effective when more than 34% of high-risk patients can be screened for HCC, and is associated with improved early cancer detection rates, cure rates, and survival rates[104,105]. International and national liver cancer management guidelines have also been developed[106]. Most liver cancers found in China are advanced. Chinese experts have developed consensus guidelines for secondary prevention[107] that provide guidelines for the prevention, monitoring, and early diagnosis of primary liver cancer in patients with chronic liver disease. To improve the early diagnosis of liver cancer. With the rise in big data and bioinformatics, predicting individual liver cancer risk is critical to implementing effective and feasible liver cancer screening. At present, some studies have evaluated the combination of existing clinical symptoms and laboratory variables to develop HCC risk prediction scores. However, due to its limited performance, it has not been used in clinical practice[101]. New serum/plasma biomarkers (such as tumor-associated antigens)[108,109] have been explored as possible alternatives to AFP. To improve diagnostic performance, a comprehensive score combining serum biomarkers and clinical variables has been proposed and is awaiting clinical validation for further development and application.

Only a few people infected with HBV will continue to be infected, and the main determinant is the age of first contact with the virus[109]. Approximately 90% of children born to carrier mothers are infected, and perinatal infections are associated with about 90% of the risk of becoming carriers[110]. Infections are most common in children in Asia and many other parts of the Sahara and South Africa. For example, in Gambia, 35 to 70% of children were found to be infected at the age of five[111]. The prevalence among infected mothers in this population is much lower than that in China, but the chronic infection rate in this population remains high because children are approximately 20-30% more likely to become carriers after early infection. In contrast, the risk of becoming a carrier during adolescence or adulthood is less than 10%. This model makes the choice of prevention more clearer, and primary prevention of persistent infection must be carried out in the early life course[112].

Gastric cancer

GC remains a globally important malignant tumor, with more than one million new cases in 2020 and an estimated one GC death in every 13 deaths[12]. Although GC is often reported as a single entity, it can generally be divided into two topographical subsites: cardia GC and non-cardia GC. Since *H. pylori* was found in 1982, it has been closely related to a variety of digestive system diseases[113]. *H. pylori* infection is regarded as a risk factor for GC and is classified as a human class I carcinogen[114]. The prevalence of *H. pylori* infection is extraordinarily high, infecting 50% of the world's population[115], and it mainly occurs in developing countries[116]. Moreover, less than 5% of infected hosts will develop cancer, likely due to differences in bacterial genetics, host genetics, age of infection acquisition, and environmental factors[117]. Based on world population data, the attributable fraction of noncardiac GC attributable to *H. pylori* infection is 89%[12,118], and the burden varies widely among regions[119].

H. pylori infection can cause the gastric mucosa to be susceptible to atrophic gastritis, intestinal metaplasia, dysplasia, and ultimately GC[120]. It is usually acquired in early life, followed by a long quiescent period, when chronic gastritis of different intensities is present, and the symptoms are not obvious[121]. Only 10%-15% of individuals with *H. pylori* infection will develop peptic ulcers, and it is estimated that the risk of GC is approximately 5%[1]. Peptic ulcers tend to be chronic infections in 20 to 30 years old patients, and GC occurs decades later. Currently, studies on the pathogenesis of *H. pylori* infection and GC are mainly classified into two main categories. One study found that *H. pylori* act on gastric epithelial cells by releasing virulence factors, namely, CagA and its pathogenicity island and VacA, which deregulates host intracellular signaling pathways and lowers the threshold for neoplastic

transformation[116]. However, other related research has concentrated on *H. pylori* inducing inflammatory responses by recruiting circulating immune cells to the site of infection, which results in an active inflammatory microenvironment. Tumor-infiltrating lymphocytes interact with tumor cells *via* chemokines (IL-1, IL-6, IL-8, TNF-α, and TNF-β), inflammatory molecules, and matrix metalloproteinases to form an inflammatory network[122]. Overactivation of the NF-κB transcription factor and dysregulation of JAK/STAT pathway is considered to be the classical pathways[123] in the progression of *H. pylori* infection and GC-related research. These processes may play an important role in the progression of gastritis and GC, but their key regulators are not fully defined.

A continuous decline in noncardia GC incidence and mortality worldwide has been observed in the last half-century. However, due to the expected growing elderly population, the absolute number of patients is increasing and remains an important global health problem[124]. After *H. pylori* infection in early life, chronic gastritis develops slowly after the Correa cascade, *i.e.*, chronic gastritis, atrophy, intestinal metaplasia, intraepithelial neoplasia, and GC[125]. The extremely long interval (approx. decades) of the developmental process offers us the chance to interrupt the carcinogenic cascade to prevent GC. Multimodal primary secondary GC prevention relies on 4 main pillars: the eradication of *H. pylori* infection, excellent endoscopies as "digitized eyes", diagnostic reliability among pathologists, a structured health care system, and clinical specialists familiar with the management of high-risk patients [126].

Population-wide prevention: Prevention of GC includes the prevention and eradication of *H. pylori* infection, lifestyle modification, and chemoprevention. Clinical studies have shown that children aged 6-15 years provided with an oral *H. pylori* vaccine compared to those one year after vaccination had good protection against *H. pylori* infection with 71.8% prophylactic protection. From vaccine experiments to clinical application, there is still a long way to go, and longer follow-up studies are still needed to confirm its immune competence in the future[127]. Studies have suggested that the risk of recurrent peptic ulcer, peptic ulcer-related adverse events, and GC increases significantly with increasing latency to *H. pylori* eradication. All patients with peptic ulcers and confirmed *H. pylori* infection should receive eradication therapy as soon as possible[128]. To reduce the incidence of GC and related diseases, *H. pylori* eradication has begun nationwide in Japan and South Korea to save future medical burdens[129]. The 2016 Japanese guidelines for the management of *H. pylori* infections suggest the eradication of *H. pylori* in adolescence to control infections in the next generation[130]. Following *H. pylori* eradication, genetic and epigenetic markers have shown promise in GC risk stratification, but require further validation in prospective studies[124,131]. *H. pylori* eradication cannot regress all precancerous lesions, which may depend on the extent and extent of precancerous lesions at the time of eradication[132]. Once people are diagnosed with *H. pylori* infection, untreated infection will persist throughout the patient's lifetime[133]. Lifestyle changes early in life or the establishment of good lifestyle habits and diets are adequate for low-risk individuals. Since the implementation of lifestyle prevention recommendations such as improvements in the preservation and storage of food, adequate hygienic housing, and the consumption of fresh vegetables and fruits, GC incidence has decreased substantially[134]. *H. pylori* eradication has been evaluated as a form of chemoprevention of GC by antimicrobial therapy with additional administration of NSAIDs, such as aspirin[135]. Chronic inflammatory mediators may serve as potential therapeutic targets for the prevention of GC[121]. Currently, there are no global guidelines for the management of *H. pylori* infection, but consensus guidelines for the management of *H. pylori* have been successively developed from region to region[136-139].

Targeted screening: Targeted screening includes the screening and treatment of precancerous lesions. Screening for timely detection and treatment of these epithelial tissue changes is equally important to prevent[123] in addition to the eradication of *H. pylori* infection. Selecting the most effective timing of screening and intervention has important effects on tissue gastric carcinogenesis[140]. Recently, guidelines related to GC screening have been developed in some countries such as China and the United Kingdom[124,141,142]. The statement of the Kyoto Consensus report[130] suggested that after the age of 12 in the infected area, the screening and treatment of infection should start to prevent the subsequent occurrence of precancerous lesions such as atrophy and intestinal metaplasia. The latest guidelines in China set the age for the start of screening at 45 for high-risk populations[142]. The screening interval should be formulated according to the disease burden, medical level, and social and economic conditions of GC in each region. The screening and treatment of *H. pylori* infection have potential cost-effectiveness to prevent GC, especially in the target high-risk population[143].

Studies need to explore effective interventions to eliminate infection and inflammation in pediatric populations. Some studies have introduced a third new family-based *H. pylori* eradication strategy[130, 144] and this method can control *H. pylori* infection in family members and reduce the long-term complications by screening, identifying, treating, and tracking all *H. pylori* infected persons in the whole family[145]. In the long run, it is difficult to control *H. pylori* infection from the source due to the dynamic and gradual nature of the infection, thus increasing the medical burden in the later stage of the disease. However, a family-based eradication strategy will help solve the above problems. The 2016 *H. pylori* infection management guidelines revised in Japan (2019) suggested that individuals should receive eradication treatment before becoming parents to prevent infection within the family and transmission to the next generation[130]. The 2018 Bangkok Consensus report[144] states that it is

recommended to receive screening and treatment for families of GC patients. These guidelines reflect that control of family infection is important to prevent *H. pylori*-induced diseases. Regular follow-up home treatment should be used to detect the infection status of children as soon as possible once parents are diagnosed with infection. Eliminating the risk factors or early life infection plays a key role in preventing gastric tumor genesis.

CONCLUSION

The prevention and control of chronic inflammation is count for much of the malignant digestive system tumor genesis. The whole life course management of human health is of great significance in combating chronic inflammation to prevent the occurrence of DSMTs. This article summarized the prevention strategies for DSMTs and their overall implementation based on the prevention or control of chronic inflammation. Although we have an understanding of the research status of chronic inflammation and DSMTs, we still have some questions to answer in the future. For example, how long is the interval between colonoscopy and screening, and how can interval cancer be prevented? Does DAA therapy play a role in the prevention of HCC? How can drug resistance and reinfection be avoided after eradication of *H. pylori* infection? Health education on cancer prevention knowledge still needs to be continued. The higher the disease awareness rate of people is, the greater the success in disease control. Cancer prevention should be given attention throughout the life course, especially in early life. Control and intervention related to infection and modifiable lifestyle changes in the early life course play an important role in cancer prevention.

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REVIEW

Immunotherapy for advanced or recurrent hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is associated with high morbidity and mortality, and is prone to intra- and extrahepatic metastasis due to the anatomical and functional characteristics of the liver. Due to the complexity and high relapse rate associated with radical surgery or radiofrequency ablation, immune checkpoint inhibitors (ICIs) are increasingly being used to treat HCC. Several immunotherapeutic agents, along with their combinations, have been clinically approved to treat advanced or recurrent HCC. This review discusses the leading ICIs in practice and those currently undergoing randomized phase 1–3 trials as monotherapy or combination therapy. Furthermore, we summarize the rapidly developing alternative strategies such as chimeric antigen receptor-engineered T cell therapy and tumor vaccines. Combination therapy is a promising potential treatment option. These immunotherapies are also summarized in this review, which provides insights into the advantages, limitations, and novel angles for future research in establishing viable and alternative therapies against HCC.

Key Words: Recurrent hepatocellular carcinoma; Immunotherapy; Immune checkpoint inhibitor; Chimeric antigen receptor-engineered T cell; Oncolytic virus; Tumor vaccine

Core Tip: The high recurrence rate of hepatocellular carcinoma (HCC) following radical treatment remains challenging; therefore, immune checkpoint inhibitors (ICIs) are increasingly being used to treat HCC. Herein, we discuss the ICIs in practice and those undergoing trials, and summarize the alternative strategies such as chimeric antigen receptor-engineered T cell therapy and tumor vaccines. Combination therapy is also a promising potential treatment option. We believe our study significantly contributes to the literature as it addresses the current state of immunotherapy against HCC and provides insights into the advantages and limitations, thereby facilitating future research.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy (75%-85% of cases), sixth most diagnosed cancer, and the third most common cause of cancer-related deaths worldwide in 2020[1]. The incidence and main risk factors for HCC vary from area to area. Traditionally, the highest epidemic of HCC is mainly in East and South-East Asia; however, the incidence of HCC has increased in the United States and Europe[2]. The key risk factors of HCC include chronic hepatitis B virus or hepatitis C virus (HCV) infection, aflatoxin-contaminated foods, excessive drinking, obesity, and smoking[3,4].

Hepatic resection is the best method for treating early-stage HCC[5-7]. Radiofrequency ablation (RFA) is also considered a radical treatment in many patients with small HCC and is the recommended treatment for patients with a single tumor < 2 cm or 2–3 nodules of ≤ 3 cm[5-8]. Less than 30% of patients with HCC can be treated with surgery and RFA due to distant metastases, anatomical location limitations, hepatic insufficiency, and neurovascular invasion[9,10]. Besides, patients with HCC who receive radical treatment have a high recurrence rate, typically manifesting as recurrence in liver remnants[11]. The recurrence rate in early HCC patients remains high at 5 years post curable excision [11-13]. Most HCCs (> 70%) are diagnosed at an advanced stage[14]. Radical treatment of recurrent HCC includes repeated hepatic resection and liver transplantation; these radical treatments are complex due to the shortage of donors, small residual areas of liver after hepatectomy, hepatic dysfunction, and multiple metastases. Due to the particularity of advanced and recurrent HCC, radiotherapy alone is not recommended. Systematic chemotherapy is also rarely recommended due to resistance to multiple cytotoxic drugs and abnormal liver function[15]. Therefore, local interventional therapies have been developed to treat recurrence, including transcatheter arterial chemoembolization (TACE)[16] and hepatic artery infusion chemotherapy (HAIC)[17]. Locoregional therapy is, for the most part, not a radical treatment, with recurrence and local disease progression being typical. For patients undergoing these, there is an urgent need to explore new therapies to treat recurrent HCC.

Sorafenib, which was been recommended as a first-line treatment for liver cancer with Child-Pugh type A liver function and Barcelona Clinic Liver Cancer-C in 2007, is a multi- tyrosine kinase inhibitor (TKI) that can extend median overall survival (mOS) and the time to radiologic progression by 3 mo [18]. Lenvatinib, which is an alternative first-line treatment for advanced HCC[19], is not inferior to sorafenib. However, lenvatinib is associated with significant improvements compared with sorafenib in terms of higher objective response rate (ORR), prolonged progression-free survival (PFS), and prolonged time to progression[20,21]. Regorafenib[22], cabozantinib[23], and ramucirumab[24,25] are recommended as second-line treatments for advanced HCC[26]. These licensed systemic multi-TKIs may be poorly tolerated due to their significant side effects, drug resistance, and modest benefits in mOS[21,27-29]. Since nivolumab was approved as a second-line treatment for advanced HCC in 2017, immunotherapy for recurrent or advanced HCC has witnessed rapid development. Nivolumab, pembrolizumab, atezolizumab, durvalumab, ipilimumab, tremelimumab, tislelizumab, sintilimab, and camrelizumab and their combinations have been approved in succession for HCC treatment[8]. The advent of cancer immunotherapy has completely changed the traditional treatment concept for HCC by stimulating the immune system of individuals to kill tumor cells selectively. Other immunotherapy strategies, such as chimeric antigen receptor-engineered T cells (CAR-Ts) and therapeutic cancer vaccines, have matured to the stage of clinical trials, offering new hope for HCC patients[30-32]. This article reviews approved immunotherapies and those in clinical development for HCC treatment.

LIVER AND HCC IMMUNITY

The liver, which receives arterial and venous blood is exposed to pathogens in the systemic circulation (mainly from the gut). Liver immunosurveillance is one of the most critical lines of defense. The liver contains a variety of immune cells, some of which are innate immune cells including neutrophils, macrophages, natural killer cells (NKs), NK T cells (NKTs), dendritic cells (DCs), and Kupffer cells, all of which are essential immune sentinels and antigen-presenting cells (APCs)[33-37]. Kupffer cells can capture antigens under flowing conditions, whereas NKs and NKTs can be activated upon detection of antigens and directly release granulosin and perforase to act on target cells or release large amounts of cytokines (*e.g.*, interferon gamma [IFN-g]) to direct the immune response[38-40]. DCs are the most potent APCs, which can effectively take up, process, and present antigens. As important immune cells, DCs can participate in the development and activation of T and B cells. DCs can also secrete a variety of cytokines (interleukin [IL], IFN, and tumor necrosis factor) and chemokines to participate in the immune function regulation and mediate the chemotaxis of other immune cells[33,41,42]. Neutrophils promote the progression of HCC by interacting with macrophages and regulatory T cells (Tregs). Large numbers of neutrophils predict poor tumor status[43,44]. Conversely, adaptive immune cells include B cells, plasma cells, and effector T cells. A normal liver provides a tolerant microenvironment that inhibits innate and adaptive immunity in homeostasis and prevents inflammation or tissue damage in the liver[45,46].

The immune system of the liver plays a vital role in controlling the occurrence and development of HCC. The interaction between innate and adaptive immunity can lead to effective antitumor immunosurveillance[47]. Tumor cells, Tregs, inhibitory B lymphocytes, and other inhibitory cells mediate the tumor microenvironment by regulating negative costimulatory molecules to achieve immune escape [48]. In addition, myeloid suppressor cells (MDSCs) or M2-polarized tumor-associated macrophages generate an inflammatory microenvironment, which can also serve as a medium for tumor initiation, angiogenesis, and metastasis[49]. Transforming growth factor beta (TGF-β) is the primary mediator for this activity[50] and plays a central role in inflammation, fibrogenesis, and immunomodulation in the HCC microenvironment[51,52]. Therefore, controlling the synthesis and activation of TGF-β during tumor progression is important.

PRINCIPLES OF HEPATOCELLULAR IMMUNOTHERAPY AND IMMUNE CHECKPOINT INHIBITOR

Tumor cells inhibit immune checkpoint overactivation and express corresponding ligands to achieve an immune escape[53]. We previously studied various immunosuppressive receptors, including programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), lymphocyte-activation gene 3, T cell immunoglobulin and mucin domain containing-3, and B- and T-lymphocyte attenuator[54,55]. For example, the inhibitory receptor on T cells, PD-1, can be expressed in various immune cell types and binds to programmed death ligand-1 (PD-L1) of the corresponding target cells to inhibit the effects of T cells. CTLA-4 is expressed on the surface of activated T cells by competing with cluster of differentiation 28 (CD28) and binding to CD80 and CD86 to reduce the co-inhibitory signal of CD28 and induce T cell apoptosis. Meanwhile, CTLA-4, an essential gene in Treg differentiation, development, and maintenance of cell functions is highly expressed in Tregs[56]. The concept of blocking inhibitory immune receptors and activating the antitumor function of reinvigorated immune cells has been experimentally demonstrated and translated into the clinical treatment of many types of tumors[57]. Inhibitors of PD-1, PD-L1, and CTLA-4, known as immune checkpoint inhibitors (ICIs), are an essential part of immunotherapy for many tumors including melanoma, non-small cell lung cancer, and colorectal cancer[58]. ICIs, which can block the influence of negative immune costimulatory molecules, can exhibit antitumor activity and kill tumor cells by promoting and upregulating the activation of T cells, thereby restoring normal physiological functions of the human body[59]. ICIs have shown that effective immune response can exterminate tumor cells. Current approaches of immunotherapy were shown in Figure 1. Some ICIs and their related targets are summarized in Figure 2.

SINGLE IMMUNE AGENT THERAPY

Nivolumab

Nivolumab was approved in 2017 for patients with recurrent HCC who showed no respond to sorafenib treatment[60]. Nivolumab showed noble safety and tolerability in the phase of escalation (0.1-10 mg/kg) in the CheckMate 040 study. Only 12 of 48 patients (25%) experienced grade 3 or 4 AEs, and no deaths linked to nivolumab treatment were confirmed. In the phase of dose expansion (3 mg/kg), ORR, disease control rate (DCR), and mPFS were 20%, 40%, and 4 mo, respectively. Compared with the phase of escalation, the indices of the dose-expansion phase were significantly improved[61]. In the CheckMate

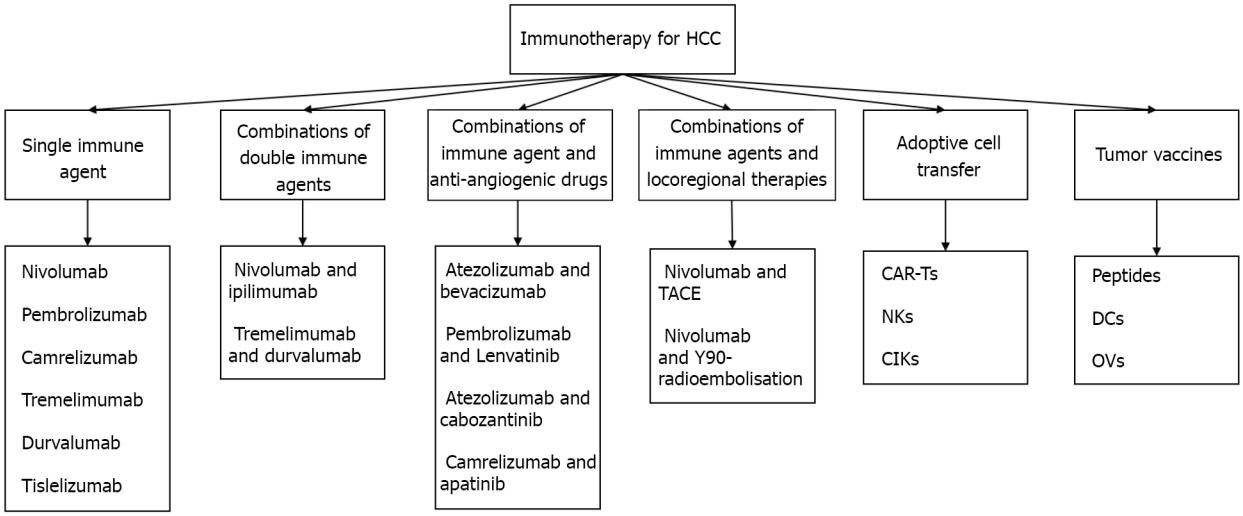

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Figure 1 Current approaches of immunotherapy. CAR-Ts: Chimeric antigen receptor expressing T cells; CIKs: Cytokine-induced killer cells; DCs: Dendritic cells; HCC: Hepatocellular carcinoma; NKs: Natural killer cells; OVs: Oncolytic virus; TACE: Transcatheter arterial chemoembolization.

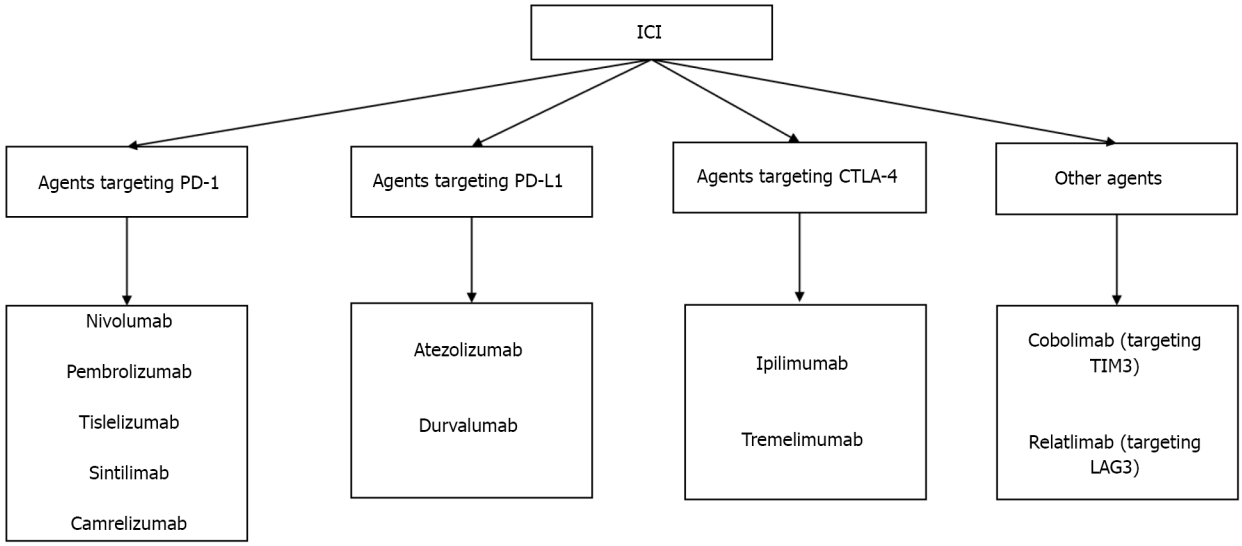

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Figure 2 Immune targets and immune checkpoint inhibitors. CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; ICI: Immune checkpoint inhibitor; LAG3: Lymphocyte-activation gene 3; PD-1: Programmed cell death protein 1; PD-L1: Programmed death ligand 1; TIM3: T cell immunoglobulin and mucin domain containing-3.

040 study (NCT01658878) (Table 1), a single nivolumab showed an enduring response, controlled safety, and satisfactory survival in patients with advanced HCC. As the CheckMate 040 study lacked a randomized control, the CheckMate 459 randomized trial (NCT02576509) (Table 1) was conducted to evaluate the efficacy of nivolumab *vs* sorafenib in a first-line setting. Although nivolumab did not significantly improve mOS (16.4 mo *vs* 14.7 mo, hazard ratio [HR]: 0.85; P = 0.075) compared with sorafenib, a lower proportion of grade 3 or 4 treatment-related adverse events (AEs), persistent response frequency, and clinical activity make nivolumab a broader treatment prospect[62].

Pembrolizumab

Pembrolizumab, an anti-PD-1 monoclonal antibody (mAb), has demonstrated promising antineoplastic effects and safety in a variety of malignant tumors[63]. KEYNOTE-224 study (NCT02702414) (Table 1) was conducted to evaluate the efficacy and safety of pembrolizumab in patients with recurrent HCC with no response to sorafenib. The results included ORR of 17%, DCR of 62%, mPFS of 4.9 mo, mOS of 12.9 mo, and grade 3 or 4 AEs that occurred in 25% of the clinical trial participants. Therefore, the Food and Drug Administration (FDA) approved pembrolizumab for treating unresectable intermediate and advanced HCC in November 2018[64]. Pembrolizumab showed good efficacy and a controllable safety

Table 1 Activity of single immune checkpoint inhibitor from the clinical trials

Drugs (dose)	Other treatment	Targets	Trial identifier	Patient group	*n*	mOS in mo	ORR, %	DCR, %	mPFS in mo	Phase	Setting
Nivolumab (3 mg/kg every 2 wk)	No	PD-1	NCT01658878	Advanced HCC	214	NR	20.0	64.0	4.00	I-II	1L
Nivolumab (240 mg every 2 wk)	*vs* Sorafenib	PD-1	NCT02576509	Advanced HCC	371	16.40	15.0	55.0	3.70	III	1L
Pembrolizumab (200 mg every 3 wk)	No	PD-1	NCT02702414	Advanced HCC	104	12.90	17.0	62.0	4.90	II	2L
Pembrolizumab (200 mg every 3 wk)	*vs* Placebo	PD-1	NCT02702401	Advanced HCC	278	13.90	18.3	62.2	3.00	III	2L
Pembrolizumab (200 mg every 3 wk)	No	PD-1	NCT02658019	Advanced HCC	29	11.00	32.0	46.0	4.50	II	2L
Camrelizumab (200 mg every 2 wk)	*vs* Camrelizumab (200 mg q3w)	PD-1	NCT02989922	Advanced HCC	109	14.20	11.9	47.7	2.30	II	2L
Camrelizumab (200 mg every 3 wk)	*vs* Camrelizumab (200 mg q2w)	PD-1	NCT02989922	Advanced HCC	108	13.20	17.6	44.0	2.00	II	2L
Durvalumab (1500 mg every 4 wk)	*vs* T300+D and tremelimumab	PD-L1	NCT02519348	Unresectable HCC	104	13.60	10.6	37.5	2.07	II	Mix
Durvalumab (1500 mg every 4 wk)	*vs* T300+D and sorafenib	PD-L1	NCT03298451	Unresectable HCC	389	16.56	17.0	54.8	3.65	III	1L
Tremelimumab (750 mg every 4 wk)	*vs* T300+D and durvalumab	CTLA-4	NCT02519348	Unresectable HCC	69	15.10	7.2	49.3	2.69	II	Mix
Tislelizumab (5 mg/kg every 3 wk)	No	PD-1	NCT02407990	Advanced HCC	50		12.2	51.0		Ib	2L

1L: First-line therapy; 2L: Second-line therapy; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; DCR: Disease control rate; HCC: Hepatocellular carcinoma; mOS: Median overall survival; mPFS: Median progression free survival; NR: Not reached; ORR: Overall response rate; PD-1: Programmed cell death protein 1; PD-L1: Programmed death ligand 1; T300+D: Dose of tremelimumab (300 mg IV, cycle 1) combined with durvalumab (1500 mg IV once every 4 wk).

profile in patients with advanced HCC who had previously received sorafenib; therefore a worldwide phase 3 study of pembrolizumab (KEYNOTE-240) (NCT02702401) (Table 1) was conducted. In the second-line treatment of advanced HCC, mOS of pembrolizumab and placebo were 13.9 mo *vs* 10.6 mo (HR: 0.77), mPFS was 3.3 mo *vs* 2.8 mo (HR: 0.70), and OS and PFS did not meet the specified criteria for statistical significance. Improvements in ORR, DCR, PFS, and OS with pembrolizumab treatment were consistent with the results of the single-cohort KEYNOTE-224 study (Table 1). The difference in ORR (18.4% *vs* 4.4%) favored pembrolizumab[65]. Accelerated FDA approval was acquired for pembrolizumab use for treating advanced HCC in patients who failed to respond to prior sorafenib therapy.

Camrelizumab

Camrelizumab, an immunoglobulin G4 (IgG4) anti-PD-1 mAb, is used to treat several cancers including lymphoma, lung cancer, esophageal cancer, and HCC[66,67]. Camrelizumab showed significant antitumor efficacy and tolerance in patients with advanced solid tumors in phase 1 trials[68-70]. To continue evaluating the activity and safety of camrelizumab as a second-line or higher treatment for advanced or recurrent HCC, a randomized phase 2 trial (NCT02989922) (Table 1) was conducted. A total of 217 patients with advanced HCC were randomly assigned in a 1:1 ratio to two groups, including 2 wk of camrelizumab (3 mg/kg) (n = 109) treatment and 3 wk of camrelizumab (3 mg/kg) (n = 108) treatment. At the end of data cutoff, survival metrics from the 2- or 3-wk group, including mOS (14.2 mo *vs* 13.2 mo), mPFS (2.3 mo *vs* 2 mo), DCR (47.7% *vs* 44 %) and ORR (11.9% *vs* 17.6%) showed good antitumor activity. In terms of safety, grade 3 or 4 AEs occurred in 47 patients (22%)[71].

Compared with other PD-1 inhibitors, camrelizumab experienced a significantly lower DCR (44.2% *vs* 55% with nivolumab[62] in sorafenib-patients and 47.7% *vs* 62% with pembrolizumab[64] in the second-line setting after sorafenib use) and shorter mPFS (2.1 mo *vs* 4.9 mo with pembrolizumab and 3.7 mo

with nivolumab). Overall, camrelizumab demonstrated potential antitumor efficacy and safety. However, the efficacy of single camrelizumab was limited; hence, a combination with targeted agents and other ICIs are needed to improve the efficacy.

In March 2020, camrelizumab was approved by the Chinese Food and Drug Administration for treating patients with advanced HCC who had received sorafenib or chemotherapy with oxaliplatin. Camrelizumab is also the first PD-1 inhibitor with HCC indications approved in China, which is a breakthrough in immunotherapy in China.

Tremelimumab

Tremelimumab is a human IgG2 mAb that blocks the binding of CTLA-4[72]. ORR was 17.6% with a DCR of 76.4% in a clinical trial of tremelimumab in patients with HCC and chronic HCV. Surprisingly, tremelimumab showed satisfactory antitumor activity, antiviral activity, and safety in patients with advanced HCC developed from HCV-induced liver cirrhosis. However, the first trial of tremelimumab for HCC included only 20 patients and therefore could not account for chance results caused by multiple clinical covariates[73]. In a phase 2 clinical trial of tremelimumab in combination with durvalumab for HCC (NCT02519348) (Tables 1 and 2), 326 patients were assigned to four cohorts, namely the tremelimumab monotherapy arm (750 mg once every 4 wk [seven doses] and then once every 12 wk), durvalumab monotherapy arm and T300+D arm (tremelimumab 300 mg plus durvalumab 1500 mg [one dose each during the first cycle] followed by durvalumab 1500 mg once every 4 wk), and T75+D arm (750 mg once every 4 wk [seven doses] and then once every 12 wk). The tremelimumab monotherapy arm represented the first large cohort of HCC patients receiving anti-CTLA-4 monotherapy. The ORR was 7.2%, DCR was 49.3%, mOS was 15.1 mo, and mPFS was 2.69 mo. Although the ORR of this cohort was the lowest (7.2%), the mOS was the second longest, and the median duration of response (mDOR) was prolonged (23.95 mo). However, the grade 1-4 AEs of T300+D were highest (82.4%), whereas that of grade ≥ 3 AEs of tremelimumab monotherapy was the highest (43.5%). Among the four arms, the tremelimumab monotherapy received the highest dose of tremelimumab; therefore, serious AEs were considered to be dose-related to tremelimumab. Compared with tremelimumab monotherapy, the combination of T300+D significantly enhanced antitumor efficacy[74].

Durvalumab

In the phase 2 clinical trial of tremelimumab in combination with durvalumab for HCC (NCT02519348) (Tables 1 and 2) mentioned before, 104 patients with HCC who had progressed on, were intolerant to, or refused sorafenib were randomly assigned to receive durvalumab monotherapy; ORR was 10.6%, DCR was 37.5%, mOS was 13.6 mo, and mPFS was 2.07 mo[74]. Meanwhile, in a phase 3 trial (NCT03298451) (Table 1) of tremelimumab in combination with durvalumab for HCC, the durvalumab monotherapy arm was non-inferior to sorafenib in ORR (17% *vs* 5.1%) and mOS (16.56 mo *vs* 13.77 mo). Compared with durvalumab monotherapy in the phase 2 study, durvalumab in this phase 3 study had significantly increased activity with an ORR of 17%, DCR of 54.8%, mPFS of 3.65 mo, and mOS of 16.56 mo[75].

Tislelizumab

Tislelizumab (BGB-A317) is a humanized IgG4 mAb with high affinity and binding specificity for PD-1. Unlike nivolumab and pembrolizumab, tislelizumab evades the efficacy mediated by Fc gamma R1 (FcγR1) and minimizes the binding of macrophages to FcγR; this may mitigate potential adverse interactions with other immune cells, including macrophages and MDSCs[76-78]. Tislelizumab has demonstrated satisfactory tolerability and significant antitumor activity in patients with advanced HCC. Fifty advanced HCC patients who had previously received other antitumor therapies were reported in the HCC cohort, with an ORR of 12.2% (95% confidence interval [CI]: 4.6-24.8), a DCR of 51% (95%CI: 36.3-65.6), and an average DOR of 15.7 mo. Preliminary safety and antitumor activity support the continued exploration and development of tislelizumab in patients with advanced HCC[79,80]. Therefore, the phase 2 open-label clinical trial of tislelizumab (NCT03419897) further explored the efficacy and safety of Tislelizumab in the second-line treatment of advanced HCC and a phase 3 randomized controlled trial (NCT03412773) is currently evaluating the efficacy and safety of tislelizumab and sorafenib as a first-line treatment for unresectable HCC. These results will provide more options for treating advanced and recurrent HCC.

COMBINATION OF DOUBLE IMMUNE AGENTS THERAPIES

Nivolumab and pembrolizumab have demonstrated antitumor properties in treating advanced HCC. PD-1/PD-L1 inhibitors and CTLA-4 inhibitors influence T cell response through a complementary mechanism to enhance antitumor efficacy[81]. These positive results inspired the study of the combination of PD-1/PD-L1 inhibitors with CTLA-4 inhibitors with an aim of longer survival and higher response rates. Several combinations of ICIs have been tested to prove their efficacy (Table 2), whereas some remain in the experimental research and development stage (Table 3). According to the

Table 2 Activity of combinations of immune checkpoint inhibitors from the clinical trials

Drugs	Targets	Other treatment	Trial identifier	Patient group	*n*	mOS in mo	ORR, %	DCR, %	mPFS in mo	Phase	Setting
Nivolumab + ipilimumab	PD-1; CTLA-4	No	NCT01658878	Advanced HCC	50	22.80	32.0	54.0		I/II	1L
Durvalumab + tremelimumab	PD-L1; CTLA4	*vs* Durvalumab and tremelimumab	NCT02519348	Unresectable HCC	75	18.70	24.0	45.3	2.17	I/II	2L
Durvalumab + tremelimumab	PD-L1; CTLA4	*vs* Durvalumab and sorafenib	NCT03298451	Unresectable HCC	393	16.40	20.1	60.1	3.78	III	1L

1L: First-line therapy; 2L: Second-line therapy; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; DCR: Disease control rate; HCC: Hepatocellular carcinoma; mOS: Median overall survival; mPFS: Median progression free survival; ORR: Overall response rate; PD-1: Programmed cell death protein 1; PD-L1: Programmed death ligand 1.

Table 3 Activity of combinations of an immune checkpoint inhibitor and a vascular endothelial growth factor inhibitor from clinical trials

Drugs	Other treatment	Targets	Trial identifier	Patient group	*n*	mOS in mo	ORR, %	DCR, %	mPFS in mo	Phase	Setting
Atezolizumab + bevacizumab	*vs* Sorafenib	PD-L1; VEGF	NCT03434379	Unresectable HCC	326	19.20	27.3	74.0	6.90	III	1L
Pembrolizumab + lenvatinib	No	PD-1; VEGFR	NCT03006926	Unresectable HCC	104	22.00	36.0	88.0	8.60	Ib	1L
Sintilimab + IBI305	*vs* Sorafenib	PD-1; VEGF	NCT03794440	Unresectable HCC	380	NR	21.0	72.0	4.60	III	1L
Atezolizumab + cabozantinib	*vs* Sorafenib	PD-L1; VEGFR	NCT03755791	Advanced HCC	432	15.40	11.0	78.0	6.10	III	1L
Camrelizumab + apatinib	No	PD-1; VEGFR	NCT03463876	Advanced HCC	70	NR	34.3	77.1	5.70	II	1L
Camrelizumab + apatinib	No	PD-1; VEGFR	NCT03463876	Advanced HCC	120	NR	22.5	75.8	5.50	II	2L

1L: First-line therapy; 2L: Second-line therapy; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; DCR: Disease control rate; HCC: Hepatocellular carcinoma; mOS: Median overall survival; mPFS: Median progression free survival; NR: Not reached; ORR: Overall response rate; PD-1: Programmed cell death protein 1; PD-L1: Programmed death ligand 1; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor.

preliminary results of the nivolumab and ipilimumab combination compared with nivolumab monotherapy, the ORR (34%) and mOS (22.8 mo) significantly increased with the combination of PD-1 and CTLA-4[82] (NCT01658878) (Table 2). The rate of AEs was significantly higher with the combination of nivolumab and ipilimumab than with nivolumab monotherapy. More than 50% of patients in the Checkmate 040 study required corticosteroids, and the discontinuation rate was 22%, due to tolerably high immunotoxicity[61]. Similar results that showed the antitumor activity of dual immunoblockers being superior to that of single drug were also observed in tremelimumab and durvalumab for patients with unresectable HCC (NCT02519348). Compared with tremelimumab or durvalumab monotherapy, T300+D showed the most encouraging benefit-risk profile[74], which promotes T300+D to enter into phase 3 clinical trial (NCT03298451). For the 393 patients, the ORR was 20.1%, DCR was 60.1%, mOS was 16.4 mo, and mPFS was 3.7 mo (Table 2). Durvalumab was not inferior to tremelimumab; however, the combination of T300+D showed superior efficacy and a favorable benefit-risk profile compared with durvalumab and tremelimumab monotherapy. Compared with the combination of nivolumab and ipilimumab, the incidence of immunotoxicity requiring systemic corticosteroids in the T300+D regimen was 24.3%. The discontinuation rate was only 10.8% due to AEs[75]. Overall, the results of these two studies demonstrated that PD-1/PD-L1 and CTLA-4 had different and complementary antitumor mechanisms.

COMBINATION OF IMMUNE AGENTS AND ANTI-ANGIOGENIC DRUG THERAPIES

Combination of atezolizumab and bevacizumab

The overexpression of vascular endothelial growth factor (VEGF) is important in the occurrence and development of HCC. Anti-angiogenic drugs, including sorafenib, lenvatinib, and bevacizumab, are capable of targeting platelet-derived growth factor receptor, VEGF receptor (VEGFR), fibroblast growth factor receptor, hepatocyte factor receptor (c-KIT), and other proteins to inhibit tumor angiogenesis. Anti-VEGFR drugs (sorafenib and lenvatinib) effectively reduce VEGFR-mediated immune suppression and promote T cell activity in the tumor environment[83,84]. Sorafenib was the first anti-VEGFR drug used to treat advanced HCC in the past decade. Since then, until the emergence of atezolizumab in combination with bevacizumab, no treatment has surpassed the first-line efficacy of sorafenib[18,85]. In a phase 1b randomized cohort trial comprising 119 patients, atezolizumab in combination with bevacizumab resulted in significantly higher mPFS (7 mo) and ORR (36%) than atezolizumab monotherapy[86]. In the IMbrave150 clinical trial (NCT03434379) (Table 4), compared with sorafenib, the combination of atezolizumab (PD-L1) and bevacizumab (a vascular epidermal growth factor inhibitor) reduced the risk of death by 42% and extended mPFS and mOS by 2.5 and 5.8 mo (median follow-up 15.6 mo), respectively. The results showed an ORR of 27.3%, DCR of 74%, mPFS of 6.9 mo, and mOS of 19.2 mo. Notably, the ORR of this combination even reached more than twice that of sorafenib[85]. With long-term follow-up, to the best of our knowledge, this combination had the longest mOS observed in a phase 3 trial for HCC until now. In terms of safety, the grade ≥ 3 AEs of the combination occurred in 160 patients (49%), which were consistent with the known AEs of each drug [87]. The combination of atezolizumab and bevacizumab was approved by the FDA for treating patients with advanced or recurrent HCC who had not previously received systemic treatment[88].

Combination of pembrolizumab and lenvatinib

Lenvatinib was not statistically inferior to sorafenib in a phase 3 trial comparing lenvatinib with sorafenib as the first-line treatment for unresectable HCC. Compared with sorafenib, lenvatinib showed significant and clinically significant improvements in ORR, PFS, and TTP[21]. However, pembrolizumab also exhibited substantial antitumor activity and safety. Lenvatinib, in combination with pembrolizumab, has received accelerated approval for the treatment of advanced tumors that do not have high microsatellite instability or mismatch repair defects[89]. The encouraging preliminary trial data has led to a phase 1b study for the combinations of lenvatinib and pembrolizumab to treat unresectable HCC (NCT03006926) (Table 4). Surprisingly, the combination achieved an ORR of 46.0% (95%CI: 36.0%–56.3%, mRECIST standard) and a DCR of > 85% (regardless of the RECIST category). mPFS and mOS were 9.3 and 22 mo, respectively. The combination of lenvatinib and pembrolizumab showed no new AEs[90]. Based on the interim data from this study, the FDA granted lenvatinib in combination with pembrolizumab as first-line therapy for advanced HCC. The combination is being studied in a randomized phase 3 trial (NCT03713593) and compared with the first-line treatment of unresectable or metastatic HCC using lenvatinib (Table 3).

Other combination of ICIs and antiangiogenic drugs therapy

ICIs combined with anti-angiogenic agents open a new avenue for treating HCC. In contrast, FDA-approved first-line combination therapies for HCC are only available in a few regions worldwide. Therefore, alternative therapies need to be developed and approved. Currently, PD-1/PD-L1 checkpoint inhibitors, CTLA-4 checkpoint inhibitors, TKI, along with other antitumor agents are undergoing randomized phase 1–3 trials as monotherapy or combination therapy (Table 3). Cabozantinib, approved in 2019 by the FDA as a second-line treatment of sorafenib, has shown promising antitumor activity. COSMIC312 (NCT03755791) evaluated the combination of cabozantinib and atezolizumab *vs* sorafenib as first-line systemic therapy for HCC. Compared with sorafenib, the combination arm significantly improved PFS (HR: 0.63; 99%CI: 0.44–0.91; $P = 0.0012$; mPFS 6.8 mo *vs* 4.2 mo). However, OS was not improved[91] (HR: 0.90; 96%CI: 0.69-1.18; $P = 0.438$). At the end of 2020, the ORIENT-32 trial, which enrolled 571 HCC patients without systemic therapy, reported that combination of sintilizumab (PD-1) and bevacizumab biosimilar (IBI305) was significantly superior to sorafenib in terms of OS and PFS, as shown in Table 4. After a median follow-up of 10 mo, the mOS was not achieved in the combination line (sintilizumab and IBI305), while it was 10.4 mo in the sorafenib group (HR: 0.57; 95%CI: 0.43–0.75; $P < 0.0001$); mPFS (4.6 mo, 95%CI: 4.1–5.7) was significantly prolonged (HR: 0.56, 95%CI: 0.46-0.70; $P < 0.0001$)[92]. In early 2021, camrelizumab (PD-1) in combination with apatinib (a selective VEGFR-2 tyrosine kinase inhibitor) was assessed in phase 2 (NCT03463876) as the first- and second-line treatment for advanced HCC. Significant antitumor activity was achieved in ORR, DOR, and OS for both first- and second-line treatments[93] (Table 4). Encouraging antitumor properties continue to emerge in the new combination therapies with ICIs and TKIs, which will provide options for recurrent HCC treatment.

Table 4 Ongoing phase I-III trials testing immune checkpoint inhibitors in advanced hepatocellular carcinoma

Drugs	Other treatment	Targets	Trial identifier	Patient group	Status	*n*	Estimated completion date	Phase	Setting
Single ICI									
Pembrolizumab	Placebo	PD-1	NCT03062358	Advanced HCC	Active, not recruiting	454	June 30, 2023	III	2L
Tislelizumab	*vs* Sorafenib	PD-1	NCT03412773	Advanced HCC	Active, not recruiting	674	May 1, 2022	III	1L
Durvalumab	No	PD-L1	NCT04294498	Advanced HCC	Recruiting	43	December 31, 2023	II	2L
Tislelizumab	*vs* Sorafenib	PD-1	NCT03419897	Unresectable HCC	Active, not recruiting	249	June 30, 2022	II	2L
Combination of ICIs									
Nivolumab + ipilimumab	*vs* Sorafenib and lenvatinib	CTLA-4, PD-1	NCT04039607	Advanced HCC	Recruiting	728	September 30, 2019	III	1L
Sintilimab + IBI310	*vs* Sorafenib	PD-1, CTLA-4	NCT04720716	Advanced HCC	Recruiting	490	February 7, 2021	III	1L
Combination of ICIs and antiangiogenic drugs									
Nivolumab + regorafenib	No	PD-1, VEGFR	NCT04310709	Unresectable HCC	Recruiting	42	May 30, 2023	II	1L
Pembrolizumab + lenvatinib	Placebo and lenvatinib	PD-1, VEGFR	NCT03713593	Advanced HCC	Recruiting	750	December 31, 2023	III	1L
Pembrolizumab + futibatinib	No	PD-1, FGFR	NCT04828486	Advanced HCC	Recruiting	25	May 6, 2024	II	2L
Pembrolizumab + regorafenib	No	PD-1, VEGFR	NCT03347292	HCC	Active, not recruiting	57	September 26, 2022	I	1L
Pembrolizumab + sorafenib	No	PD-1, VEGFR	NCT03211416	Advanced or metastatic HCC	Recruiting	41	December 7, 2022	I/II	1L
Pembrolizumab + cabozantinib	No	PD-1 VEGFR	NCT04442581	Advanced HCC	Recruiting	29	September 13, 2024	II	1L
Camrelizumab + apatinib	No	PD-1, VEGFR	NCT04826406	HCC	Recruiting	40	August 30, 2023	II	1L
Camrelizumab + lenvatinib	No	PD-1, VEGFR	NCT04443309	Advanced HCC	Recruiting	53	August 1, 2023	I/II	1L
Camrelizumab + apatinib	*vs* Sorafenib	PD-1 VEGFR	NCT03764293	Advanced HCC	Active, not recruiting	543	June 1, 2022	III	1L
Toripalimab + lenvatinib	No	PD-1, VEGFR	NCT04368078	Advanced HCC	Recruiting	76	April 1, 2023	II	2L
Tislelizumab + regorafenib	No	PD-1, VEGFR	NCT04183088	Advanced HCC	Recruiting	125	March 1, 2025	II	1L
Tislelizumab + lenvatinib	No	PD-1, VEGFR	NCT04401800	Locally advanced or Unresectable HCC	Recruiting	66	December 1, 2022	II	1L
Sintilimab + lenvatinib	No	PD-1, VEGFR	NCT04042805	Advanced HCC	Recruiting	36	August 30, 2024	II	1L
Sintilimab + anlotinib	No	PD-1, VEGFR	NCT04052152	Advanced HCC	Recruiting	20	December 30, 2021	II	1L
Sintilimab + IBI305	*vs* Sorafenib	PD-1, VEGFR	NCT03794440	Advanced HCC	Active, not recruiting	595	December 1, 2022	II/III	1L
Sintilimab + regorafenib	*vs* Regorafenib	PD-1, VEGFR	NCT04718909	Unresectable HCC	Recruiting	180	December 31, 2022	II	1L
Sintilimab + donafenib	No	PD-1, VEGFR	NCT05162352	Advanced HCC	Recruiting	30	May 1, 2023	II	1L

Atezolizumab + lenvatinib or sorafenib	*vs* Sorafenib or lenvatinib	PD-L1, VEGFR	NCT04770896	Unresectable HCC	Recruiting	554	October 8, 2024	III	2L
Atezolizumab + bevacizumab	No	PD-L1, VEGFR	NCT04829383	Unresectable HCC	Recruiting	50	July 1, 2024	II	1L
Atezolizumab + bevacizumab	No	PD-L1, VEGFR	NCT04732286	Unresectable HCC	Active, not recruiting	100	September 25, 2023	III	1L
Atezolizumab + bevacizumab	No	PD-L1, VEGFR	NCT04487067	Unresectable HCC	Active, not recruiting	152	July 31, 2023	IIIb	1L
Durvalumab + tivozanib	No	PD-L1, VEGFR	NCT03970616	Advanced HCC	Recruiting	42	August 1, 2022	I/II	Mix
Durvalumab + lenvatinib	No	PD-L1, VEGFR	NCT05312216	Unresectable HCC	Not yet recruiting	25	April 1, 2022	II	1L
Durvalumab + bevacizumab	Placebo	PD-L1, VEGFR	NCT03847428	High risk of recurrence HCC	Active, not recruiting	877	May 31, 2024	III	1L

1L: First-line therapy; 2L: Second-line therapy; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; FGFR: Fibroblast growth factor receptor; HCC: Hepatocellular carcinoma; ICI: Immune checkpoint inhibitor; NR: Not reached; PD-1: Programmed cell death protein 1; PD-L1: Programmed death ligand 1; VEGFR: Vascular endothelial growth factor receptor.

COMBINATION OF IMMUNE AGENTS AND LOCOREGIONAL THERAPIES

Some locoregional therapies for HCC, including radiotherapy, RFA, TACE and HAIC, can release or produce altering substances from cancer cells to stimulate the aggregation of DCs into tumor tissues. This can upregulate the expression and antigenicity of tumor-associated antigens (TAAs) and trigger injury-related molecular patterns to induce "immunogenic cell death"[94-96]. Locoregional therapies can induce the release of proinflammatory cytokines to activate and expand innate and adaptive immune cells (NK and cytotoxic T cells) and reduce the activity of immunosuppressive cells (Tregs and MDSCs)[97-100]. Meanwhile, immunotherapy can not only improve the hypoxic microenvironment in tumors and enhance the effect of radiotherapy by inducing vascular normalization through a T cell-dependent pathway but also enhance the immune induction effect of radiotherapy to slow the growth of distant tumors (abscopal effect). Radiotherapy and immunotherapy synergize to exert more potent local effects in the irradiated tumors[101,102]. The IMMUTACE trial initially evaluated the efficacy of nivolumab plus TACE in 49 patients with mid-stage HCC; the ORR was 71.4% (95%CI: 56.8%-83.4%), including 16.3% complete responses (CRs) and 55.1% partial responses. Despite the small number of patients in each group, subgroup analyses did not reveal differences in treatment responses[103]. In the CA 209-678 study (NCT03033446) of Y90-radioembolisation followed by nivolumab in 36 patients with advanced HCC, the ORR of 30.6% compared favorably with an ORR of approximately 20% noted with Y90-radioembolisation. Notably, 81% of patients showed regression of radiation-field target lesions. This combination is safe and tolerable with grade 3–4 treatment-related AEs or serious AEs noted in 14% of patients[104]. Many clinical trials of locoregional therapies combined with ICIs are being conducted successively (Table 5). This combination is expected to become the mainstream treatment for HCC in the future.

ADOPTIVE CELL TRANSFER

Adoptive cell transfer is a form of passive therapy in which immune cells are activated and expanded *in vitro* and then reinfused into the patient. These immune cells commonly used include NKs, tumor-infiltrating lymphocytes, lymphokine-activated killer cells, cytokine-induced killer cells (CIKs), and CAR-T cells.

NK cells

NKs can recognize tumor cells based on the expression of ligands for inhibitory and stimulant NK receptors[40]. Encouraging clinical trials results in which autologous lymphocytes containing NK cells were transfused into HCC patients after ablation or resection had shown that extended NKs have significant cytotoxic effects on HCC cells[105]. Furthermore, extended NKs significantly enhanced the anti-HCC cytotoxicity of sorafenib[106]. Multiple phase 2 trials are being conducted to evaluate the use of NKs in patients after hepatectomy (NCT02008929) or TACE (NCT02854839). However, how to cultivate high purity NKs is still a problem to be solved.

Table 5 Ongoing clinical trials that combine locoregional therapies with immune checkpoint inhibitors

Main intervention methods	Comparison arms	Trial identifier	Status	Estimated or actual enrollment	Patient group	Phase
Pembrolizumab + RAF/MWA/brachytherapy/TACE	*vs* Pembrolizumab + RAF/MWA/brachytherapy/TACE	NCT03753659	Active, not recruiting	30	Early-stage HCC	II
Nivolumab + TACE	No	NCT03572582	Active, not recruiting	49	Intermediate-stage HCC	II
Pembrolizumab + TACE	No	NCT03397654	Active, not recruiting	26	HCC	I/II
Durvalumab + tremelimumab + TACE/RAF/cryoablation	*vs* Durvalumab + tremelimumab	NCT02821754	Active, not recruiting	54	Advanced HCC	II
Durvalumab + tremelimumab + TACE	No	NCT03638141	Recruiting	30	Intermediate-stage HCC	II
Durvalumab + tremelimumab + bevacizumab + TACE	No	NCT03937830	Recruiting	22	Advanced HCC	II
Durvalumab + bevacizumab + TACE	*vs* Durvalumab + TACE *vs* TACE	NCT03778957	Active, not recruiting	724	Intermediate-stage HCC	III
Apatinib + camrelizumab + HAIC	No	NCT04191889	Recruiting	84	Advanced HCC	II
Pembrolizumab + SBRT	No	NCT03316872	Recruiting	30	Advanced HCC	II
Durvalumab + tremelimumab + SBRT	No	NCT03482102	Recruiting	70	Advanced HCC	II
Nivolumab + curative resection/RAF	*vs* Curative resection/RAF	NCT03383458	Active, not recruiting	545	Resected HCC	III
Durvalumab + bevacizumab + curative resection/RAF	*vs* Durvalumab + curative resection/RAF *vs* Curative resection/RAF	NCT03847428	Active, not recruiting	877	Resected HCC	III
Ipilimumab + nivolumab + TACE	*vs* Nivolumab + TACE + placebo *vs* TACE + placebo + placebo	NCT04340193	Active, not recruiting	26	Intermediate-stage HCC	III
Lenvatinib + pembrolizumab + TACE	*vs* Placebo + placebo + TACE	NCT04246177	Active, not recruiting	950	Incurable/non-metastatic HCC	III
Nivolumab + DEB TACE	*vs* DEB TACE	NCT04268888	Recruiting	522	Intermediate-stage HCC	II/III

HAIC: Hepatic artery infusion chemotherapy; HCC: Hepatocellular carcinoma; MWA: Microwave ablation; RAF: Radiofrequency ablation; SBRT: Stereotactic body radiation therapy; TACE: Transcatheter arterial chemoembolization.

CIK cells

CIKs are heterogeneous cells with non-major histocompatibility complex-restricted tumor killing activity. After being cultured *in vitro*, CIKs can secrete a variety of cytokines to improve the internal microenvironment of tissues and organs and enhance the killing activity of immune cells[107]. A phase 3 clinical trial (NCT00699816) including 230 patients showed that adjuvant CIK immunotherapy improved PFS and OS in patients with HCC after curable surgical resection, RFA, or percutaneous ethanol injection[105]. CIKs may have a significant impact on adoptive immunotherapy regimens in patients with primary HCC.

CAR-T cell therapy

CAR-T therapy is a developing immunotherapy approach for treating malignant tumors. Due to the great success of CAR-T therapy in the treatment of CD19-positive hematological malignancies, such as a CR rate of up to 90% with anti-CD19 CAR-T cells in B-cell acute lymphoblastic leukemia[108-111], two CAR-T cell therapies, Kymriah® and Yescarta®, were approved by the FDA for lymphoma studies in 2018 and 2017, respectively. Because of this lymphoma breakthrough, CAR-T's application in treating solid tumors, such as HCC, has also been explored. Glypican-3 (GPC-3), a member of the GPC family, is a 70 kDa heparan sulfate proteoglycan overexpressed in HCC and associated with poor diagnosis and prognosis[112-115]. Several clinical trials have evaluated the safety and efficacy of GPC-3 CAR-T cells. Shanghai Renji Hospital combined lymphodepleting chemotherapy with GPC-3 CAR-T cells in 13 patients with GPC3-positive HCC and confirmed the antitumor efficacy and safety of GPC3 CAR-T cells (NCT02395250)[116]. GPC-3 CAR-T cells combined with sorafenib may be a promising option for treating of HCC[117].Chongqing Xinqiao Hospital has attempted to combine TACE with CAR-T to treat

GPC3-positive advanced HCC (NCT03084380). Other clinical trials are recruiting patients to improve the efficacy of intratumoral or intravenous administration of GPC3-CART cells (NCT03130712, NCT02715362, NCT04951141, NCT03198546, and NCT05155189). In conclusion, GPC-3 is a promising target for future therapeutic strategies in HCC. Mucin 1 glycoprotein (MUC-1)[118,119] and epithelial cell adhesion molecule (EpCAM)[120] are two transmembrane glycoproteins that can be overexpressed during the occurrence and development of HCC and can be used as biomarkers and therapeutic targets for HCC. One clinical trial of MUC-1 CAR-T cells (NCT02587689) and two clinical trials of EpCAM CAR-T cells (NCT03013712 and NCT02729493) are ongoing. Alpha-fetoprotein (AFP), which is overexpressed in HCC, is another potential therapeutic target being explored. However, AFP is a glycoprotein of the cellular endocrine system and expression and is therefore considered inappropriate for the CAR. Some researchers have designed a highly specific antibody (Ab) of the (AFP)-MHC complex to be expressed as the CAR and found that CAR-T cells of this Ab had an apparent inhibitory effect on HCC; this provided a promising new approach for HCC immunotherapy[121].

TUMOR VACCINE

Tumor vaccines are active immunotherapies that require the injection of tumor antigens, including viruses, DNA, peptides, and tumor cell-expressed genes, into patients to trigger TAA-specific immune responses and mediate powerful antitumor effects[122]. Therapeutic tumor vaccines include peptides, DCs, whole-cell vaccines, oncolytic viruses, and DNA reagents.

Peptides

Several peptide-based cancer vaccines have been assessed for HCC treatment. As a biomarker of HCC, AFP was constructed as a peptide vaccine, used in 2 patients with AFP-expressing tumors and showed high levels of AFP-specific CD8+T cell expression and apparent safety (NCT00093548). GPC-3 is highly expressed in most malignant tumors and is rarely in normal tissues; therefore, GPC-3 is considered an ideal TAA for developing cancer vaccines[114,123]. The GPC-3 vaccine is well-tolerated and safe[124, 125]. Similarly, multidrug resistance-associated protein 3 (MRP3), a vector-type transporter, highly expressed and associated with various cancers[126], is a great potential candidate for tumor vaccine development. In a phase 1 trial, the MRP3-derived peptides (MRP3765) showed promising safety and antitumor properties in 12 HLA-A24-positive HCC patients. MRP3-specific T-cell responses were induced in 8 patients (72.7%) and the mOS was 14 mo (95%CI: 9.6-18.5)[127]. Other TAAs, including synovial sarcoma X breakpoint 2, NY-ESO-1, human telomerase reverse transcriptase and melanoma-associated antigens family A, can also be valuable targets for HCC immunotherapy, but no clinical trials have verified the clinical response to these antigens in HCC[128]. Although peptide vaccines have achieved some success in terms of safety, tolerability, and mOS improvement, they have fewer clinical benefits and more stringent screening conditions than ICIs.

DC vaccines

DCs as APCs can stimulate T cells and increase the antitumor effect[129]. Peripheral monocytes were isolated *in vitro* and the DC population was expanded by adding cofactors (granulocyte-macrophage colony-stimulating factor or IL-4). Mature DCs are activated with autologous tumor lysates (TLs) or specific TAAs. Finally, these cells are reinfused into the patient to stimulate the adaptive cells to mount an antitumor immune response[36,130,131]. Currently, several clinical trials have confirmed the immunogenicity and safety of DCs. In a phase 1 trial of 17 patients with HCC treated with immunoprimers (ilixadencel), 73% had an increased frequency of tumor-specific CD8+-T cells in their peripheral blood[132]. Meanwhile, a phase 1 clinical trial in Japan injected DCs pulsed with TLs into 10 patients with unresectable HCC. All patients had an excellent immune tolerance; 1 patient experienced significant tumor shrinkage, while two experienced considerable tumor marker decrease[133]. In another phase 2 study, the intravenous administration of mature DCs pulsed with tumor lysate (HepG2) showed promising antitumor properties and safety in 35 patients with HCC[134]. When DCs were combined with TACE, tumor-specific immune responses were enhanced more effectively than when TACE was used alone[135]. Multiple clinical trials on DCs are in progress (NCT01821482, NCT02638857, NCT02882659, NCT03674073, and NCT03203005). A growing body of evidence suggests that DC vaccines have general safety and antitumor properties as primary therapy and adjunct to other established therapies. DC vaccines are promising mainstream immunotherapy for HCC.

Oncolytic viruses

An oncolytic virus (OV) is a specially modified intracellular pathogen that can achieve an antitumor response by massive replication in tumor cells, leading to direct lysis of tumor cells to produce soluble TAAs[136,137]. OVs have been shown to improve ORR and mOS in advanced melanoma (NCT00769704)[138]. Currently, adenovirus and vesicular stomatitis virus are the main oncolytic viruses used to treat HCC, which can preferentially infect HCC tumor cells, followed by the herpes simplex virus and vaccinia virus[139]. In a recent randomized phase 2 trial (NCT00554372), JX-549 (Pexa-Vec)

was injected into the tumors of 30 HCC patients, and mOS was significantly longer in the high-dose group than in the low-dose group (14.1 mo *vs* 6.7 mo) (HR: 0.39; P = 0.020)[140]. Unfortunately, the phase 2b trial (NCT01387555), which compared Pexa-Vec to placebo as second-line therapy in patients with advanced HCC with no response to sorafenib therapy, did not achieve its OS[141]. A phase 3 trial (NCT02562755) is currently underway, which compares the safety and efficacy of sorafenib with Pexa-Vec against sorafenib alone in HCC. Currently, two clinical trials are underway to evaluate the efficacy of the combination of OVs and ICIs in HCC (NCT03647163 and NCT03071094)[142].

CONCLUSION

The rapid development of immunotherapy has changed the traditional treatment modalities for recurrent HCC. Immunotherapy can play a unique role in the comprehensive treatment of HCC, including prolonging and improving quality of life and even curing HCC. Several clinical trials have attempted to evaluate the antitumor properties and safety of ICIs and their combinations in recurrent HCC, and have reported encouraging results. Although ICIs are the leading immunotherapy for recurrent HCC, other immunotherapy modalities including CAR-T cells, DC vaccines, and OVs are rapidly evolving. Among the multiple treatment options for recurrent HCC, achieving satisfactory results with single immunotherapy has become challenging. The development of synergistic immunotherapy may be a promising direction for HCC treatment in the future. In addition, immunosuppression of HCC remains a significant obstacle for immunotherapy drugs in which they must exert their antitumor properties. Another priority is to actively exploring the mechanisms of immunotherapy resistance or overcoming immune drug resistance through multiple antitumor drugs. Immunotherapy can lead to future breakthroughs and progress in treating recurrent HCC.

FOOTNOTES

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REVIEW

Molecular methods for colorectal cancer screening: Progress with next-generation sequencing evolution

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Abstract

Currently, colorectal cancer (CRC) represents the third most common malignancy and the second most deadly cancer worldwide, with a higher incidence in developed countries. Like other solid tumors, CRC is a heterogeneous genomic disease in which various alterations, such as point mutations, genomic rearrangements, gene fusions or chromosomal copy number alterations, can contribute to the disease development. However, because of its orderly natural history, easily accessible onset location and high lifetime incidence, CRC is ideally suited for preventive intervention, but the many screening efforts of the last decades have been compromised by performance limitations and low penetrance of the standard screening tools. The advent of next-generation sequencing (NGS) has both facilitated the identification of previously unrecognized CRC features such as its relationship with gut microbial pathogens and revolutionized the speed and throughput of cataloguing CRC-related genomic alterations. Hence, in this review, we summarized the several diagnostic tools used for CRC screening in the past and the present, focusing on recent NGS approaches and their revolutionary role in the identification of novel genomic CRC characteristics, the advancement of understanding the CRC carcinogenesis and the screening of clinically actionable targets for personalized medicine.

Key Words: Colorectal cancer; Gut microbiota; Colorectal cancer screening; Next-generation sequencing

Core Tip: Due to the multitude of host and microbial genetic factors, the optimization of colorectal cancer (CRC) biomarkers remains difficult. The advent of next-generation sequencing (NGS) methods has facilitated the identification of previously unrecognized CRC-related genomic alterations and the CRC relationship with gut microbial composition. Hence, we have summarized the diagnostic tools used for CRC screening in the past and the present, focusing on the revolutionary role of NGS approaches in the identification of novel genomic CRC characteristics, the advancement of understanding the CRC carcinogenesis and the screening of clinically actionable targets for personalized medicine.

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INTRODUCTION

Currently, colorectal cancer (CRC) represents the third leading cause of cancer-related deaths in men and women worldwide, and the American Cancer Society estimates that the number of new colon and rectum cancer cases in the United States in 2022 will be around 106180 and 44850, respectively[1]. Despite the great progress of modern medicine, such as the development of novel therapeutic methods and the advent of new high throughput sequencing technologies, the mortality of CRC patients remains relatively high due to the lack of specific biomarkers and therapies.

Nowadays, CRC incidence largely varies across the world, and it appears to be positively correlated with the Human Development Index. For instance, in 2020 Norway, the Netherlands and Denmark reported the highest age-standardized incidence rates (41.9, 41.0 and 40.9 cases per 100000 persons, respectively) while Guinea, Gambia and Burkina Faso showed the lowest age-standardized incidence rates (3.3, 3.7 and 3.8 cases per 100000 persons, respectively)[2]. Usually, these variations reflect differences in the availability of screening services and other factors such as geographic location, environmental factors (*e.g.*, polluted surface water sources), economic status and dietary and lifestyle habits[3].

At present, considering the difficulties in implementing significant lifestyle changes or common primary prevention strategies, screening and early detection represent the most powerful public health tool to reduce CRC mortality[4]. In general, an acceptable screening marker can only be considered by the health community if it respects specific parameters such as simplicity, safety and accuracy and has a known and defined suitable cutoff level[5]. Colonoscopy is considered the gold standard test for detecting CRC and promoting effectiveness in reducing its incidence and mortality. However, its high cost, invasiveness and reduced availability of necessary equipment hinder the establishment of organized screening settings, especially in poor countries[6].

In recent years, massive efforts have focused on next-generation sequencing (NGS) approaches to identify genes and microorganisms that are significantly associated with the malignancy due to the emerging evidence that intestinal microbial dysbiosis constitutes a crucial environmental factor in CRC onset and development[7]. Moreover, metagenomics approaches, considered a real revolution in the screening and diagnosis of different cancers, are also useful for the identification of novel potential markers for CRC diagnosis[8]. Hence, in this review, we summarized the diagnostic tools used for CRC screening in the past and the present, focusing on recent NGS approaches.

FECAL OCCULT BLOOD TEST

Since the 1970s, stool-based CRC screening was considered a successful non-invasive method with proven effectiveness given by the detection of high-risk polyps and early-stage malignancies that dramatically reduced CRC incidence and death[9] (Figure 1). The fecal occult blood test (FOBT) currently represents the early analysis for CRC screening that is recommended by the National Screening Committee[10]. This method is based on the detection of occult blood by measuring the non-protein portion of hemoglobin, the heme group, present in the stool. In particular, the heme present in a stool sample reacts with hydrogen peroxide-based developer to oxidize guaiac-infused paper, resulting

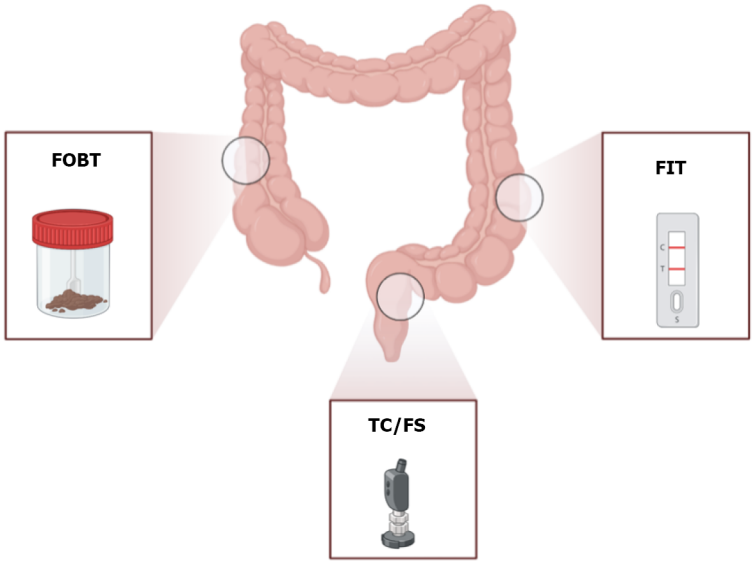

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Figure 1 Stool-based and visual colorectal cancer screening methods. FIT: Fecal immunochemical test; FOBT: Fecal occult blood test; FS: Flexible sigmoidoscopy; TC: Total colonoscopy.

in a blue color[4]. In general, FOBT has been shown to reduce both the incidence and the risk of CRC death with the advantages of ease of use and cheaper than other alternative screening approaches[11] (Table 1). Despite this, the FOBT method presents some limitations, such as the low sensitivity for colorectal adenomas that may not bleed or the specificity of the method that can be influenced by diet or drugs[12]; hence, Young *et al*[13] affirmed that FOBT is only suitable for limited colonoscopy resources with a need to constrain the test positivity rate[13].

FECAL IMMUNOCHEMICAL TEST

Different immunoassay methods have been used to measure the development of antibody-globin complexes, including immunochromatography, immunoturbidimetry and enzyme-linked immunosorbent assay (Table 1)[14]. For instance, the fecal immunochemical test (FIT) is used for the detection of microscopic amounts of blood present in the stool during defecation *via* the utilization of antibodies targeted to globin molecules (Figure 1). The antibodies preferably target lower gastrointestinal bleeding, making FIT easy to use, sensitive to low concentrations of globin and sufficiently flexible to adjust the cutoff concentration for positivity (the cutoff is usually selected with a risk threshold that would produce a specificity of 96.9% in the study group, matching the specificity of FIT at a cutoff of 20 μg Hb/g feces)[13,15]. Imperiale *et al*[16] tested individuals at average risk for CRC having an age comprised between 50 years and 84 years and documented that FIT detected 48 out of 65 colon cancers, showing a sensitivity of 73.8% and specificity of 96.0%[16]. The same specificity was observed among participants with negative results on colonoscopy, suggesting that FIT had fewer false positive results compared to stool DNA testing[17]. On the other hand, weaknesses of FIT tests are the low clinical sensitivity for both cancers (73%, 80%, 82% and 79% for CRC stages I, II, III and IV, respectively) and advanced adenomas (16%-34%) when used at a low cutoff and the limited detection of upper gastrointestinal bleeds because the hemoglobin undergoes degradation by digestive enzymes with a consequent reduction of the binding to FIT antibodies[18].

FLEXIBLE SIGMOIDOSCOPY AND TOTAL COLONOSCOPY

Randomized controlled trials showed that the visual inspection of colic mucosa through flexible sigmoidoscopy (FS) decreased CRC mortality and incidence by 22%-31% and 18%-23%, respectively (Figure 1)[19]. Overall, FS represents a safe test, but its use is limited to the distal colon and a combined strategy using FS and FOBT/FIT only increases the endoscopic workload and reduces patient participation without solving the problem. Instead, total colonoscopy allows direct visualization and polyp removal over the whole colon (Figure 1), has a very high sensitivity and specificity for CRC and is usually used as confirmatory for all other screening strategies (Table 1)[20]. Although total colonoscopy can lead to a decrease in CRC incidence (66%-90%) and mortality (31%-65%), many features (*e.g.*,

Table 1 Summary of the main diagnostic approaches for colorectal cancer screening

Technology	Approach	Sample types	Targeted and colorectal marker	Sensitivity/specificity for CRC	Advantages	Disadvantages
Chemical and immuno-chromatographic test	FOBT	Stool	Heme of hemoglobin	4%-25%/95%	(1) Non-invasive; (2) Reduction of mortality (asymptomatic patients); (3) Colorimetric indicator; (4) Rapid and easy-to-carry out (self-testing); and (5) Commercially available test	(1) Low sensitivity for non-bleeding adenoma and advanced adenoma; (2) Specificity influenced by diet or drugs; (3) Must be done annually; (4) Risk of false positive results; (5) Three consecutive samples needed; (6) Only detects the blood present in the external layer of the stool; and (7) Confusing interpretation of the test results
	FIT	Stool	Globin molecules	62.0%-100%/94.9%	(1) Easy to use; (2) Flexible cutoff concentration; (3) Sensitive to low concentrations of globin; (4) Single sample needed; (5) Combined with FOBT inferred mortality; and (6) No dietary restriction	(1) Insensitive to digested hemoglobin; (2) Poor sensitivity for advanced adenoma; (3) Sensitivity based on threshold value of hemoglobin; and (4) Detect more distal neoplasms
Visual inspection	FS	Distal colon	Polyps	100%/100%	(1) Reduce colorectal cancer mortality and incidence; and (2) High susceptibility to detect adenomas	(1) Invasive process; (2) Not suitable for diabetic or psychotropic patients; (3) Expensive; (4) Serious harms for colonoscopy that increase with age; (5) Sigmoidoscopy was not effective for female screening (high risk for proximal colorectal cancer); and (6) Moderate-to-severe pain was reported for patients (bleeding, anxiety, *etc*)
	TC	Entire colon				
Sanger sequencing methodology	Single gene sequencing	Tissue; liquid biopsy	A specific gene in human tumor DNA cells	High sensitivity (input of DNA mutated quantity < 1%)	(1) Non-invasive (blood/liquid biopsy); (2) Some mutations were prominent in colorectal cancer; (3) Bioinformatic analysis not required; (4) Simple and less time consuming; and (5) No specialized instrument in laboratory	(1) Requires high-quality DNA; (2) Heterogenous mutations genes; (3) Risk of contamination with normal tissue; and (4) Low coverage sequencing
	ddPCR	Liquid biopsy. Tissue	Short amplicon sizes (< 100 bp) of human DNA	Very high sensitivity (input of mutated DNA quantity < 0.1% even with degraded DNA)	(1) Monitoring tumor burden in response to treatment and indicator of disease progression; (2) Precise measurement of copy number of mutated DNA and lower probability error (without standard samples); (3) Minimally invasive process; (4) Detects specific mutations; (5) Independent prognostic factor; and (6) Large target mutation	(1) No ability to detect benign lesions from plasma due to insufficient tumor burden; (2) Need an expensive instrument; (3) Limited prime-probe sets for each single nucleotide change; (4) No information in tumor-associated protein profiling; (5) Possibility of contamination with normal tissue; (6) Not strictly tumor specific; and (7) Necessity of cell search system
	MT-sDNA	Stool	Specific genes in human tumor DNA cells	66%-94%/90%-96%	(1) Non-invasive test; (2) Acceptable cost; (3) Potential credibility; (4) No dietary restrictions (including food and medications); and (5) Widespread accessibility and multiple commercialized prototypes	(1) Lack of standardization or optimization of fecal DNA panels for high sensitivity and specificity; (2) Risk of contamination by microbial DNA; (3) No defined optimal interval for screening individuals; (4) Poor sensitivity for advanced adenoma; and (5) Must be repeated every 3 years
	Idylla system	Tissue; liquid biopsy	Specific genes in human tumor DNA cells	High sensitivity (input of DNA mutated quantity < 1%)	(1) Fully automated; (2) Real-time based-PCR molecular diagnosis system; (3) Without pre-analytical DNA extraction; (4) Lower cost and time requested for results; (5) Easily	(1) No detection of complex genomic variants; (2) Unknown mutations were not detected; (3) Cannot detect rare and complex genomic variants not included in the reference range; and (4) Less suitable when new

					implemented in routine laboratory workflow; (6) Wide range of CRC-related mutations; and (7) Very sensitive to detect the most common CRC mutation	gene mutations appear
	Custom panel sequencing	Tissue; liquid biopsy	Specific genes in human tumor DNA cells	95%-100%/99%-100%	(1) Decreased sequence cost; (2) Greater sequencing depth; (3) Simple and less time consuming; (4) Robust and tissue efficient; (5) Massive parallel multigene sequencing; and (6) Provide additional information (TMB levels/relevant mutated genes/heredity cancer genes)	(1) Low coverage sequencing; (2) There is no standardized procedure; and (3) Relatively long turnaround time of 3 d
Next generation sequencing	WGS/WES	Tissue; liquid biopsy	All exome and all genome in human tumor DNA cells	95%-100%/99%-100%	(1) Detection of large-scale mutations; (2) High coverage sequencing; (3) Complete definition of the genomic landscape for WGS; and (4) Complete mutation analysis panel without the repeated testing cost and reuse of material	(1) Require bioinformatics specialists; (2) Expensive; (3) Require good quality DNA; and (4) Relatively long turnaround time of 3 d
	Third generation sequencing	Tissue/stool/liquid biopsy	(1) Specific genes/WES/WGS in human tumor DNA cells; and (2) Microbes in stool	95%-100%/99%-100%	(1) Identification of large-scale rearrangement; (2) Sequencing errors do not release rearrangement; (3) High coverage sequencing; and (4) Fast and real time molecular diagnosis system	(1) High percentage of somatic errors; (2) Require bioinformatic specialists for assembling and analysis in laboratory; (3) Need specialized equipment in laboratory; and (4) Cannot detect some somatic mutations
	Metagenomic analysis	Stool/tissue	Microbial DNA in stool by Shotgun (all the DNA) or metabarcoding DNA (16S, ITS1, ITS2, 18S, *etc*)		(1) Microbial flora was more abundant than human cells in stool; (2) Benign lesions do not release human cells in stool; (3) Noninvasive diagnostic test; (4) Microbiota seems to play a role major in initiation and progression of CRC; (5) Test can be potentially used on all pathogen groups; and (6) Microbiota dysbiosis induces methylation of host genes	(1) Complex bioinformatics analysis; (2) Expensive; (3) Microbiota composition depends on sample preparation, conservation, extraction protocol and many other factors; (4) Need a healthy control group; (5) Many microorganisms (virus, bacteria, fungi) have not been identified and sequenced; (6) Metabarcoding analysis provides only taxonomic affiliation based in small region; and (7) Analysis results depends on reference database

CRC: Colorectal cancer; ddPCR: Droplet digital polymerase chain reaction; FIT: Fecal immunochemical test; FOBT: Fecal occult blood test; FS: Flexible sigmoidoscopy; MT-sDNA: Multi-target stool DNA; TC: Total colonoscopy; TMB: Tumor mutational burden; WES: Whole exome sequencing; WGS: Whole genome sequencing.

invasive, expensive and painful) dramatically reduce its acceptability as a first-line screening test; moreover, proper training programs for endoscopists as well as continuous quality assurance are necessary[21].

METHODS-BASED ON SANGER DNA SEQUENCING

It is currently well established that CRC development relies upon a stepwise acquisition of several chromosome mutations. The model of the adenoma-carcinoma progression, based on the accumulation of multiple mutations and epigenetic alterations, has been widely accepted[22]. Overall, there are two types of mutational events in sporadic CRC. The first concerns about 85% of all patients and consists of

frequent mutations in *APC*[23], *TP53*[24], *KRAS*[25], *BRAF*, *TTN*, *PIK3CA*[26], *FBXW7*[27] and *SMAD4* genes[28]; the second concerns 15% of CRC-sporadic patients and is characterized by a high level of hypermethylation of the *MLH1* gene, responsible for DNA mismatch repair[29]. Additionally, a different complement of mutations in somatic genes has also been described[30].

Single gene sequencing

Considering their role in resistance to multiple treatment strategies, genotyping of gene mutations currently represents an important diagnostic and therapeutic tool (Figure 2). For instance, a mutation in *APC*, a tumor suppressor gene highly mutated in 57% of CRC cases and involved in DNA replication and repair processes, has been documented to strongly influence the chemotherapy response[31]. Also, *SMAD4* gene mutations were observed in 2%-20% of CRC cases and were usually associated with poor response to cetuximab treatment[32]. In addition, several *RAF* mutations have been implicated in the induction of genomic instability, driving the proliferation of cancer cells[33], while heterogeneous *KRAS* mutations have been identified in almost 40% of CRC patients[34] (with a substitution in the G12C position as the most common detected), having a consequent association with anti-epidermal growth factor receptor treatment resistance[35].

To better represent the cancer heterogeneity using NGS technology, Ye *et al*[36] proposed a protocol for conducting rigorous systematic reviews and meta-analyses on the accuracy of *KRAS* mutation detection in CRC using non-invasive liquid biopsy samples[36]. Generally, liquid biopsies represent the collection of tumor-derived biomarkers in the blood or other body fluids, such as urine, saliva, stool or cerebrospinal fluid. Circulating tumor DNA (ctDNA), circulating tumor cells and exosomes are the most common tumor-related biomarkers assessed on liquid biopsy so far[37]. Moreover, the Food and Drug Administration recently approved a liquid biopsy test to analyze the frequency of *KRAS*, *NRAS* and *BRAF* hotspot mutations in ctDNA that could represent good CRC prognostic factors[38].

Multi-target stool DNA test

The multi-target stool DNA (MT-sDNA) test allows the identification of specific gene mutations in human tumor DNA cells separately from the more abundant microbial DNA in the stool (Figure 2). During the last few years, several key technological advances have led to increasingly accurate approaches to stool DNA testing including: (1) The use of a DNA preservative swab for stool collection; (2) The improvement of the target capture and amplification methods; and (3) The identification of new informative marker panels[39]. Zou *et al*[40] produced a methyl-binding domain protein bound to a column of nickel-agarose resin to increase the assay sensitivity for detecting methylated DNA markers in stool[40]. Subsequently, multiple prototypes of MT-sDNA test were commercialized, but only two were approved in August 2014 by the Food and Drug Administration for screening people at average risk for CRC aged over 50 years[29]. To date, both the American Cancer Society and the United Services Preventive Services Task Force affirmed that the MT-sDNA test can be repeated every 3 years to provide a decrease in CRC incidence and mortality with an acceptable cost and have approved this test for screening people of ages 45 years to 49 years[41,42].

Moreover, Heigh *et al*[43] performed a targeted single assay test with aberrant methylation of *BMP3* alone and detected sessile serrated polyps with a sensitivity of 66% and a specificity of 91%[43]. Although additional biomarkers can be used by including multiple targets that reach the 21-target MT-sDNA test, no increase in the sensitivity or specificity was observed[44]. In general, most studies agree that MT-sDNA is effective to detect CRC with only a few exceptions. In fact, Imperiale *et al*[16] detected 60 out of 65 colon cancers by MT-sDNA test with an estimated sensitivity of 92.3% and a specificity of 90%, confirming that the MT-sDNA test is more sensitive than FIT, especially for the detection of lesions with high-grade dysplasia or sessile serrated polyps (≥ 1 cm). Overall, the method sensitivity varied from 62% to 91% for cancer and from 27% to 82% for advanced adenomas, with a specificity of 93% to 96% in people with normal findings on colonoscopy[45].

The advancement of the genetic knowledge in CRC and their related mutational events would improve the efficiency and the sensitivity of MT-sDNA tests by increasing the target DNA genes. Nowadays, MT-sDNA tests include quantitative molecular assays for *KRAS* mutations, *NDRG4* and *BMP3* methylation and β-actin and include eleven different DNA sequences commonly seen in colon polyps/cancers[46]. Therefore, as confirmed by a retrospective study conducted by Weiser *et al*[47] on 368494 subjects, the MT-sDNA test represents the most recommended CRC screening tool because of its widespread accessibility and higher sensitivity compared with other previously described methods such as FIT and FOBT (Table 1)[47].

Droplet digital polymerase chain reaction

Droplet digital polymerase chain reaction (ddPCR) is recognized as an established and trustworthy approach for clinical cancer research due to its high sensitivity (almost 74% for CRC) in comparison to traditional standard procedures, even in degraded samples[48] (Figure 2). This method consists of an enrichment strategy that allows the detection of low-level mutations by amplification of single DNA molecules without the need for standard reference curves. It is considered much easier, faster and less error-prone than real-time quantitative PCR[49]. Nowadays, ddPCR is commonly used for detecting

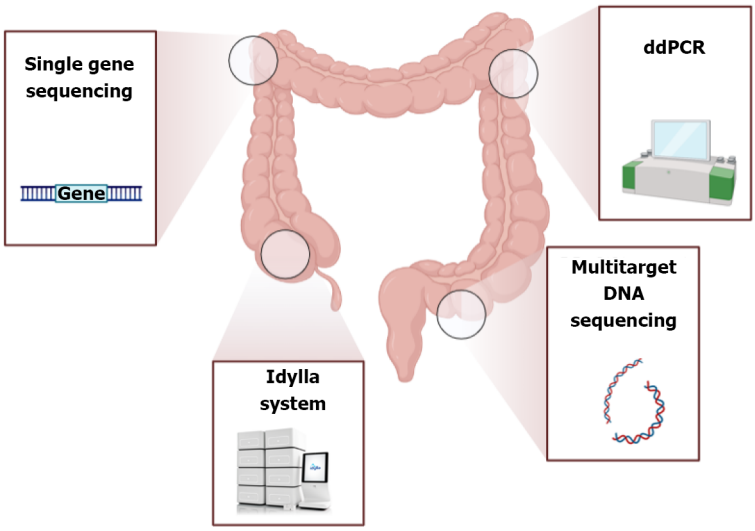

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Figure 2 Colorectal cancer screening based on Sanger DNA sequencing. ddPCR: Droplet digital polymerase chain reaction.

rare alleles as molecular markers in plasma samples of pre- and postoperative CRC patients not only because of its high sensitivity for detecting tumor DNA (even with a very small fraction or degraded DNA) but also to monitor disease progression and the emergence of drug resistance[50]. Through this method, Taly *et al*[51] documented seven common mutations in codons 12 and 13 of the *KRAS* oncogene from plasma samples of CRC patients, demonstrating the clinical utility of multiplex ddPCR to screen multiple mutations with a sensitivity sufficient to detect mutations in circulating DNA obtained by non-invasive blood collection[51].

In the same context, ddPCR platforms using OncoBEAM technology demonstrated high sensitivity for plasma detection of *KRAS* mutations[52], and overall ddPCR has been largely applied to the detection and quantification of mutated genes including *KRAS*[53], *BAT26*[54], *ITGA6* and *ITGA6A*[55] and hypermethylated *GRIA4*, *VIPR2*[56] and *VIM*[57] from both ctDNA or fecal DNA of CRC patients. Recently, Garrigou *et al*[58] proposed the screening of modifications in methylated ctDNA as a biomarker to monitor tumor evolution of CRC patients at different stages and concluded that it could be a universal approach to follow tumor burden of CRC patients as compared with mutated ctDNA, which requires previous tumor mutation identification[58]. To summarize, although there are many advantages of ddPCR including the high sensitivity and the large range of target mutations, its major limitation is represented by the lower availability of primer/probe sets (Table 1)[59].

The Idylla approach

The Idylla system (Biocartis, Mechelen, Belgium) consists of a cartridge-based fully automated medical device able to perform an innovative technology that consists of a conventional TaqMan reporter system and novel chemistry known as PlexPCR (amplicons containing a small region with a sequence different from that of target DNA) simultaneously with a PlexZyme (specific amplicon sequence-matched reporter probe) that allows multiplexing of numerous gene targets in one assay[60] (Figure 2). Hence, due to its ability to easily detect a wide range of CRC-related mutations, the Idylla approach can be easily implemented in pathology laboratories to reduce turnaround time[61]. It currently represents a feasible and validated test for *KRAS*, *NRAS* and epidermal growth factor receptor mutations in formalin-fixed paraffin-embedded tissues[62] and for *BRAF* hotspot mutations in plasma samples[63].

In addition, the Idylla system can be used to confirm uncertain outcomes of doubtful NGS results and/or in case of scarce tissue material within a few hours. For instance, Zwaenepoel *et al*[64] evaluated the clinical performance of the Idylla method in 330 CRC samples and demonstrated that this technology was able to give results in less than 2.5 h with only two invalid results. Many authors tested the full panel of CRC gene targets (*BRAF*, *KRAS* and *NRAS*) and found that the concordance between Idylla and NGS was 100% for *BRAF* and *KRAS* mutations and 94% for *NRAS*[65]. Therefore, this methodology is highly accurate for detecting frequent mutations and minimizing the contamination risk, in addition to reducing cost per test when compared with NGS or some conventional PCR assays. However, rare and/or complex genomic variants, which are not included in the reference ranges, cannot be detected by the Idylla system, and continuous improvement of its biomarker panel is necessary to guarantee efficient diagnosis[66].

METHODS BASED ON NGS TECHNOLOGIES

Since the 2000s, and in coincidence with the emergence and development of new high-throughput sequencing technologies, many analyses have been undertaken to examine genetic susceptibility to diseases through genome-wide association studies. Zanke *et al*[67], using a multistage genetic association approach comprising 7480 CRC patients and 7779 controls, recognized a wide association of markers in chromosomal region 8q24, the same site where the *SMAD7* gene is located[67]. In addition, a genome-wide association study performed by Broderick *et al*[68], consisting of the genotyping of 550163 tag single nucleotide polymorphisms in 940 individuals with familial CRC and 965 controls, identified three single nucleotide polymorphisms in the *SMAD7* gene[68]. Subsequently, Tomlinson *et al*[69] confirmed these results and elucidated other markers in chromosomal regions of 8q23.3 and 10p14 at which common variants can influence the risk of CRC development[69].

NGS-based diagnostic assays are increasingly adopted especially with decreasing sequencing costs. In the early stage, sequencing technologies were used to target driver genes known to contribute to CRC, but recently larger chromosomal regions have been targeted exploiting the potential of these technologies in multigene sequencing by using a very low amount of biological material from liquid or tissue biopsy samples. In this step, many efforts have been made to standardize sequencing procedures and data analyses and to generate databases that store the sequencing information. Clinicians and research communities can use this information to provide better quality care[70].

Early in 2010, The Cancer Genome Atlas project conducted a genome-scale analysis of samples obtained from 276 CRC patients, analyzed exome sequences, DNA copy number, promoter methylation and messenger RNA and microRNA expression and concluded that 16% of CRC samples were found to be hypermutated, 77% of patients displayed one or both breakpoints leading to translocation in an intergenic region and 7% of patients reported a translocation involving the *TTC28* gene (an inhibitor of tumor cell growth) located on chromosome 22[71]. Furthermore, the Pan-Cancer Analysis of Whole Genomes, the International Cancer Genome Consortium and The Cancer Genome Atlas projects recently described 2658 whole genomes of tumor samples and their matching normal tissues, not only of CRC but of 38 different cancer types, providing insights into the nature and timing of the many mutational processes that shape large and small-scale somatic variation in the cancer genome[65].

According to the improvement of NGS approaches, different sequencing platforms have been developed (Illumina, Ion Torrent, SOLiD, PacBio and Nanopore) that are classified in terms of maximum output, reads per run, accuracy, run time, amount of nucleic acids necessary for analysis and reads length. In particular, they can generate short (*e.g.*, SOLiD, Ion Torrent, Illumina) or long reads (*e.g.*, PacBio, Nanopore). While short reads sequencing does not exceed 300 base pairs and is more suitable for CRC diagnosis, long reads sequencing determines a better coverage of the genome and is more adaptable for large deletion/insertion determination or chromosomal rearrangement[72]. Considering that both short-read and long-read sequencing have their benefits and flaws depending on the experimental aim, it is important to remark that when somatic alterations in oncogenes and tumor suppressor genes are stable throughout the tumor clonal evolution, chromosomal alterations and copy number variation (CNV) could be lost during cancer progression[73].

In addition, CRC represents one of the most interesting fields of NGS application because of its great quantity of activating mutations; in fact, next-gen techniques enable the identification of novel mutations/altered genes or genomic rearrangements allowing the discovery of new possible treatments [74]. In general, there are three more common NGS-based methods used for CRC studies: Custom panel; whole genome sequencing (WGS); or whole exome (WES) sequencing and third-generation sequencing approaches. In general, large-scale mutations were identified by WGS of tumor DNA, while point mutations were identified by targeted sequencing (Table 1).

Custom panel sequencing

During the last decade, several pipelines based on NGS approaches have been developed, and additional somatic mutations and chromosomal aberrations were detected in CRC samples (Figure 3). To simplify routine adoption of NGS tools, Zheng *et al*[75] considered a custom-designed panel of genes of only 2.2 Mb (exons and partial introns of cancer driver of more than 600 genes) and deduced a 9-loci model for detecting microsatellite instability (MSI) with 100% sensitivity and specificity compared with MSI and 84.3% overall concordance with immunohistochemistry staining[75]. Many authors have undertaken the simultaneous sequencing of many driver genes including low allele frequencies using NGS technologies and have emphasized the importance of the fine classification of mutational status as some cancers were associated with poor prognosis treatment[76]. In this regard, the comprehension of the wide heterogeneity of CRC lesions seems to be an extremely important point for tracing the therapeutic approach of the patient and developing effective strategies for early CRC detection and prevention.

Liquid biopsy samples have been investigated more than tumor tissue samples because of their non-invasiveness and their better representation of cancer heterogeneity[77]. In this context, Myint *et al*[78] developed a multiregional NGS approach from circulating cell-free DNA using a customized targeted CRC panel consisting of all coding exons of 116 genes, 22 genes recurrently amplified/deleted, 51 copy number regions, 121 MSI regions and 2 gene fusions (*RSPO2* and *RSPO3*) and confirmed the

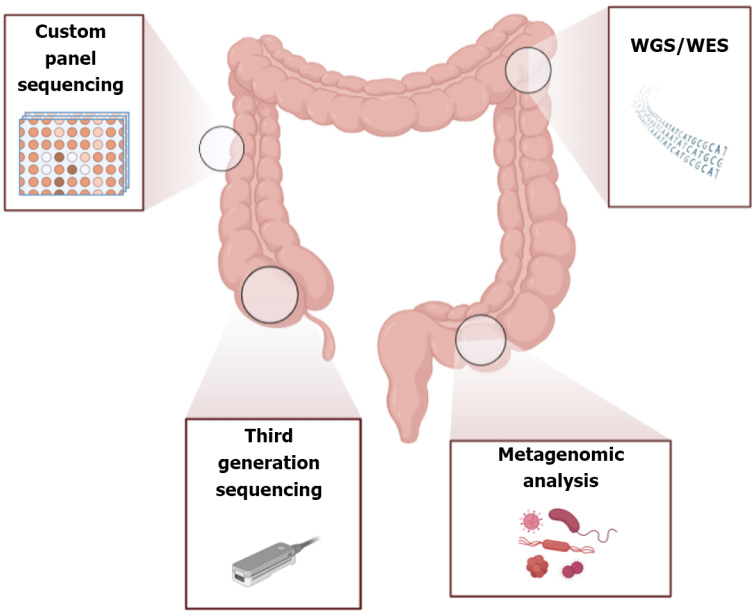

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Figure 3 Next-generation sequencing-based colorectal cancer screening. WES: Whole exome sequencing; WGS: Whole genome sequencing.

widespread genetic heterogeneity in six adenoma samples, which affected the driver genes *MMR*, *APC*, *PIK3CA*, *TP53* and *SMAD4*[78]. Additionally, based on an NGS analysis of a panel of 324 CRC-associated genes, Stahler *et al*[79] documented frequent single nucleotide variations in the *TP53*, *APC*, *KRAS*, *PIK3CA*, *BRAF*, *SMAD4* and *FBXW7* genes, and copy number alterations in the *MYC* and *FLT3* genes[79].

Furthermore, Leary *et al*[80] developed a "personalized analysis of rearranged ends" approach, which can identify translocations and copy number alterations in CRC and other solid tumors. In addition, personalized analysis of rearranged ends can detect 57 regions containing putative somatic rearrangements, with an average of 14 rearrangements per sample[80]. Moreover, targeted sequencing strategies based on short reads and CNV determination could represent a good strategy for CRC studies. In fact, Gould *et al*[81] confirmed that an NGS approach using short fragments presented a sensitivity > 96% and a specificity > 99% for detecting samples with CNVs in the terminal five exons of *PMS2*[81].

Additionally, Corti *et al*[82] developed multiple DNA NGS approaches coupled with the computational and bioinformatics algorithm "IDEA" to target a WES of about 30 Mb, a custom panel of genes of about 603 Kb (frequently mutated genes) and another of 918 Kb (intron-exon junction to precisely identify the genomic breakpoint)[82]. Currently, IDEA represents a flexible and comprehensive pipeline for the management of CRC patients and is suitable for identifying several genetic alterations from a non-invasive sample (ctDNA) such as single nucleotide variants, insertions and deletions, gene copy-number alterations and chromosomal rearrangements in the *KRAS*, *BRAF*, *PIK3CA* and *ERBB2* genes (usually involved in drug resistance). In general, sequencing of smaller target regions provides greater sequencing depth which allows for better recognition of low gene frequency variation. Hence a customized gene approach is more suitable for clinical oncology laboratories for many advantages such as the simplicity, low cost and fast of the method and the non-need for bioinformatics specialists in the laboratories (Table 1).

WES and WGS

The contribution of MSI to the tumor mutational burden (TMB) due to a defective mismatch repair system is considered important in about 15% of CRC patients. According to the phenotype, MSI tumors can be divided into two distinct MSI phenotypes: MSI-high and MSI-low[83]. Recently, considering that the defective mismatch repair phenotype is crucial to define the efficacy of immune checkpoint inhibitor treatment, Xiao *et al*[84] used WES to evaluate the immune microenvironment and 2539 microsatellite loci in a group of 98 CRC patients. They concluded that the microenvironment of TMB-high was significantly more immune-responsive than TMB-low[84]. On the other hand, Gurjao *et al*[85] demonstrated the presence of a novel alkylating mutational signature, identified through the WES of 900 CRC patients and predicted that *KRAS* p.G12D, *KRAS* p.G13D and *PIK3CA* p.E545K driver mutations were mainly targeted by the alkylating signature in non-hypermutated patients[85].

Moreover, Chang *et al*[86] performed the WES of DNA obtained from tumor tissues of 32 surgical CRC patients and identified the well-known recurrent mutations in the *APC*, *TP53*, *KRAS* and *FBXW7*

genes and unreported mutations in additional 14 genes[86]. Furthermore, many authors confirmed that WGS largely contributed to determining the significant role of non-coding regions such as enhancers, transcription factor binding sites, promoters and 3'untranslated regions in CRC carcinogenesis[87]. In addition, WGS was used to demonstrate that metastatic lesions were enriched in gene mutations affecting PI3K-AKt signaling, cell adhesion and extracellular matrix processes[88]. Finally, Dashti *et al* [89] conceived a new technique based on a novel concept called 'gene-motif,' which identified seven CRC subtypes that can be effectively used to develop a personalized treatment[89].

In comparison to WES, the WGS approach has the advantage of increasing the overall variant accuracy and poor coverage but is more expensive and requires fresh-frozen tumor material to perform analysis of the highest quality (Figure 3 and Table 1).

Third-generation sequencing

Third-generation sequencing of long reads has been developed and represents the most suitable approach for the identification of deletion/duplication breakpoints and complex structural variants and CNV-neutral rearrangements such as inversions and large intronic insertions[90] (Figure 3). Indeed, many studies affirmed that long-read sequencing technologies have potential advantages over existing alternatives especially when pathogenic variants are in complex genomic regions, such as the recurrent *PMS2* insertion-deletion variant. Using a locus-specific amplicon template, Watson *et al*[91] undertook Nanopore long-read sequencing to assess the CRC diagnostic accuracy of this platform. Pairwise comparison between sequencing results derived from short-read NGS and unidirectional Sanger sequencing and the consensus Nanopore dataset revealed 100% sequence identity[91]. Furthermore, reads produced by Nanopore oxford technology were able to identify both the 5' and 3' junctions and revealed detailed insertion sequence information[92].

METAGENOMICS ANALYSIS OF GUT DYSBIOSIS IN CRC PATIENTS

Genetic factors that concern somatic mutations in *KRAS*, *APC*, *p53*, mismatch repair genes and other chromosomal aberrations explain less than 35% of all diagnosed CRCs, and many environmental exposures seem to modulate the cancer risk[93]. For instance, metagenomics studies based on 16S rRNA sequencing that has been recently conducted have documented the presence of more than a thousand microbial species in the human gastrointestinal tract carrying more than 100 times as many genes as the human genome[94] (Figure 3).

Therefore, considering the high microbial diversity in humans and their contribution to host health and pathological or malignant conditions, it was suggested that about 20% of the global cancer burden can be linked to microbial agents[95]. However, in addition to the several factors that can considerably modify the gut microbiota (GM) composition (*e.g.*, age, sex, nationality, dietary and lifestyle habits, drugs or alcohol abuse)[96], multiple experimental challenges can influence the results of GM studies such as sampling methods and consistency[97], storage sample conditions[98], DNA extraction methods [99], type of primers used and pipelines adopted for data analyses[100]. For all these reasons, it is very hard to define a baseline microbial community for healthy people, especially due to the impossibility of obtaining biopsy samples from healthy controls. Therefore, tumor-adjacent tissue has been regarded as the healthy control, but many efforts have recently been expended to standardize the experimental and analytical methods[101]. The two most common metagenomics approaches for GM characterization are shotgun sequencing and metabarcoding.

These NGS-based approaches both contain three basic steps: Library preparation; sequencing; and data analysis. Sequencing libraries are typically created by fragmenting DNA and adding specialized adapters to both ends to allow the DNA fragments to bind to the sequencer flow cell. Due to unique barcodes added to each library that are used to distinguish between the libraries during data analysis, multiple libraries can be pooled together and sequenced in the same run (a process known as multiplexing). During the next sequencing step of the NGS workflow, the sequencer amplifies the DNA fragments, resulting in millions of copies of single-stranded DNA. In detail, chemically modified nucleotides bind to the DNA template strand through natural complementarity, and each nucleotide contains a fluorescent tag and a reversible terminator that blocks the incorporation of the next base. The fluorescent signal indicates which nucleotide has been added, and the terminator is cleaved so the next base can bind. After reading the forward DNA strand, the reads are washed away, and the process repeats for the reverse strand. After sequencing, the instrument software identifies nucleotides (a process called base calling) and the predicted accuracy of those base calls. Finally, data analysis can be performed with standard tools or with customized techniques[102].

While shotgun sequencing results in a very complicated data output (*i.e.* a huge amount of information that can be up to 1.5 terabases per run) because it simultaneously provides functional and taxonomic information about bacteria, fungi, viruses and a variety of other microorganisms, metabarcoding has a less complex data output and provides only taxonomic information about the bacterial (16S region sequencing) or fungal (ITS sequencing) composition of the sample (Table 1)[103]. Thus, metagenome-wide association studies have identified a correlation between many microbial species/

gene markers and CRC, promoting the development of an affordable diagnostic test using both stool and tissue samples[104,105].

CRC-associated bacteria

Much evidence has documented GM involvement in different diseases, including CRC. In particular, recent reports have demonstrated a bacterial driver-passenger model for CRC initiation and progression and showed that the first epithelial transformations can be supported by certain intestinal bacteria[106]. In 2012, Tjalsma *et al*[107] proposed a bacterial driver-passenger model for CRC in which pathogenic driver bacteria interact transiently with host cells to initiate CRC development and are then replaced by other passenger bacteria species that were unable to colonize healthy colon tissue but benefitted from altered metabolism of tumors cells[107]. To date, Wang *et al*[108] have identified *Bacillus spp.*, *Bradyrhizobium spp.*, *Methylobacterium spp.* and *Streptomyces spp.* as potential driver bacteria and *Fusobacterium spp.* and *Campylobacter spp.* as certain and abundant passenger bacteria[108].

Moreover, Luan *et al*[109] characterized the mucosa-adherent fungal microbiota of paired biopsy samples of adenomas and adjacent healthy tissue from 27 subjects using barcoded high-throughput sequencing that targeted the ITS region and reported a different fungal composition in patients with different adenoma stages and identified the phylum Glomeromycota as a possible powerful CRC marker[109]. Consistently, recent findings obtained through the WGS approach demonstrated that the Ascomycota/Basidiomycota ratio could represent a potential novel marker for early CRC detection [110]. Furthermore, Coker *et al*[111] used a shotgun metagenomics approach to evaluate the role of the archeome in colorectal carcinogenesis and found distinct archaea clusters in fecal samples from CRC patients, patients with adenomas and healthy subjects, with the CRC patients showing significant enrichment of halophilic archaea and depletion of methanogenic archaea[111].

Several metagenomic analyses of CRC patients have documented an over-representation of *Fusobacterium nucleatum* (*F. nucleatum*) in both tissue or stool samples in comparison to healthy controls[112]. Interestingly, in a large cohort of 616 participants, Yachida *et al*[113] demonstrated that the shift in the GM composition between CRC patients and healthy controls occurred in the very early stages of CRC development. In particular, the relative abundance of *F. nucleatum* was significantly elevated continuously from intramucosal carcinoma to more advanced stages, while *Atopobium parvulum* and *Actinomyces odontolyticus* were significantly increased only in multiple polypoid adenomas and/or intramucosal carcinomas[113]. Recently, in addition to *F. nucleatum*, several bacteria, such as *Bacteroides fragilis*, *Escherichia coli*, *Streptococcus bovis*, *Enterococcus faecalis*, *Peptostreptococcus anaerobius* and *Lachnoclostridium spp.*, have been reported to be enriched in stool or tissue samples of CRC patients compared to healthy ones[114-118].

Moreover, an association between specific bacterial species and antitumor responses have been reported; for instance, a positive correlation between the abundance of *Bifidobacterium longum* or Ruminococcaceae members and the efficiency of CRC immunotherapy has been documented[119]. *Eubacterium limosum, Ruthenibacterium lactatiformans*, *Fusobacterium ulcerans*, *Bacteroides uniformis*, *Paraprevotella xylaniphila* and *Alistipes senegalensis* improved the effectiveness of immune checkpoint inhibitors[120].

Because the GM composition can be modified by probiotic and prebiotic supplementation, which can help maintain intestinal microbial homeostasis and mitigate dysbiosis, many reports have evaluated their effect on colorectal carcinogenesis. Overall, recent systematic reviews suggested that prebiotics may have a protective effect on the progress of CRC, while the administration of certain probiotics in patients with CRC reduced the side effects of chemotherapy, improved the outcomes of surgery, shortened hospital stays and decreased the risk of death[121,122]. However, the findings are still conflicting, and none determined changes in bacterial richness and diversity that are usually reduced in CRC patients. Thus, further studies are needed to better understand the prebiotic and probiotic effects in CRC patients.

CRC-associated bacterial metabolites

Accumulating evidence has suggested that GM modulates the CRC progression, and its metabolites can play a crucial role in this scenario. The rapid development of technologies such as mass spectrometry and nuclear magnetic resonance have documented different profiles of microbial metabolites between CRC patients and healthy subjects. For instance, lower bile acid hydrolase and β-galactosidase abundances and higher levels of leucine, tyrosine, valine, choline, colibactin, gallocin, formyl methionyl leucyl phenylalanine, *Bacteroides fragilis* toxin and trimethylamine-N-oxide have been associated with CRC development[123-125]. Furthermore, the total amount of short chain fatty acids, the main metabolites produced by the bacterial anerobic fermentation of indigestible polysaccharides that exert various and fundamental functions for the host, was significantly lower in fecal and plasma samples of CRC patients compared to both patients with adenomatous polyps and healthy controls. Therefore, these metabolites could represent novel potential non-invasive diagnostic biomarkers for CRC[126,127]. In addition, recent investigations have reported that the GM plays a critical role in the effectiveness of anti-CRC treatments, including chemotherapy as well as immunosuppressive agents. For instance, it has been reported that the effectiveness of CRC treatment with 5-fluorouracil is enhanced by certain microbial metabolites[128]. The supplementation with probiotics or prebiotics could increase chances of

therapeutic success[129].

CONCLUSION

Since the advent of NGS approaches, many molecular techniques for the diagnosis of CRC from invasive or non-invasive sampling have emerged and have significantly increased the number of known genes and mutations linked to CRC. However, due to the multitude of host and microbial genetic factors and the complexity of the tumor environment, the optimization of a CRC biomarker remains difficult, especially in stool samples, in which the complexity of the lesion environment seems to play a key role[50]. Thus, the development of a biological method to find stable, sensitive and specific markers in non-invasive samples such as feces or plasma remains an arduous challenge to be carried out. Furthermore, despite the great progress in metagenomics methods and bioinformatics tools, WES and WGS are still feasible only in expert centers. Only limited pieces of genomic information are currently clinically relevant for the care of CRC patients, and the list of predictive actionable genomic biomarkers is quite short[86]. Apart from the identification of novel microbial biomarkers, new CRC-associated molecules are under evaluation for CRC screening, such as circular RNA and Piwi-interacting RNA. These advances in the identification of microbial markers and the improvement of non-invasive diagnostic capabilities and their applications in guiding precision cancer therapies are poised to change the diagnosis of CRC and select and monitor the treatments in the near future due to the increasingly adopted precision medicine for the care of CRC patients.

FOOTNOTES

Author contributions: Abbes S and Baldi B contributed to the conceptualization and revision of the literature; Abbes S contributed to the original draft preparation; Sellami H, Amedei A and Keskes L contributed to critical revision and supervised the manuscript; Abbes S and Baldi S wrote the review and edited; Amedei A and Keskes L contributed to the funding acquisition; All authors have read and agreed to the published version of the manuscript.

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MINIREVIEWS

Genetic heterogeneity of colorectal cancer and the microbiome

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Abstract

In 2020, the International Agency for Research on Cancer and the World Health Organization's GLOBOCAN database ranked colorectal cancer (CRC) as the third most common cancer in the world. Most cases of CRC (> 95%) are sporadic and develop from colorectal polyps that can progress to intramucosal carcinoma and CRC. Increasing evidence is accumulating that the gut microbiota can play a key role in the initiation and progression of CRC, as well as in the treatment of CRC, acting as an important metabolic and immunological regulator. Factors that may determine the microbiota role in CRC carcinogenesis include inflammation, changes in intestinal stem cell function, impact of bacterial metabolites on gut mucosa, accumulation of genetic mutations and other factors. In this review, I discuss the major mechanisms of the development of sporadic CRC, provide detailed characteristics of the bacteria that are most often associated with CRC, and analyze the role of the microbiome and microbial metabolites in inflammation initiation, activation of proliferative activity in intestinal epithelial and stem cells, and the development of genetic and epigenetic changes in CRC. I consider long-term studies in this direction to be very important, as they open up new opportunities for the treatment and prevention of CRC.

Key Words: Gut microbiota; Bacterial metabolites; Colorectal cancer; Colorectal polyp; Stem cells; Epigenetic changes

Core Tip: Most cases of colorectal cancer (CRC) develop from colorectal polyps that can progress to intramucosal carcinoma and CRC. A large number of studies have indicated that the gut microbiota may play a key role in the initiation and progression of CRC. The mini-review discusses current ideas about the role of the microbiome and microbial metabolites in inflammation initiation, activation of proliferative activity in intestinal epithelial and stem cells and development of genetic and epigenetic changes in CRC. Further research in this direction is of great interest since these studies may contribute to the development of new CRC prevention and treatment methods.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer type worldwide and the second most common cause of cancer death[1]. Risk factors for developing CRC include age, male sex[2-4], family history of CRC, and genetic predisposition (for example, polyposis and nonpolyposis CRC, including Lynch syndrome), which are detected in 5% of patients with CRC[2,5,6]; benign tumors (adenomatous polyps and sessile serrated polyps) associated with sporadic CRC[3]; inflammatory bowel diseases[7,8]; obesity and type 2 diabetes[9-11]; smoking[3,4,12]; and alcohol consumption[13]. Currently, increasing evidence is accumulating that the intestinal microbiome plays a key role in the initiation and progression of CRC and affects the effectiveness of this pathology treatment. Moreover, intestinal dysbiosis, which is characterized by a decrease in the microbiota diversity and an overgrowth of pathogenic and conditionally pathogenic flora, is associated with many risk factors for CRC, such as inflammatory bowel disease, obesity, type 2 diabetes, and a diet characterized by a high intake of fat, red meat and processed meat [14-16]. To discuss the role of the gut microbiota in the development and progression of CRC, as well as in the development of genetic and epigenetic changes in CRC, a search was made in the PubMed database (https://pubmed.ncbi.nlm.nih.gov/) and Reference Citation Analysis (https://www.referencecitationanalysis.com/) for studies published up to December 31, 2022, using a combination of text keywords "gut microbiota", "bacterial metabolites", "CRC", "colorectal polyp", "stem cells", and "epigenetic changes". A total of 2042 unique results were identified, which were screened individually by title and abstract, and 222 references were included in this review based on the role of the microbiome and microbial metabolites in inflammation initiation, activation of proliferative activity in intestinal epithelial and stem cells, in the development and progression of CRC, and development of genetic and epigenetic changes in CRC.

CHANGES IN THE GUT MICROBIOTA IN CRC

Accumulating evidence indicates that the gut microbiota may play a key role in the initiation and progression of CRC, acting as an important metabolic and immunological regulator[7,17-20] and influencing both the efficacy and toxicity of cancer treatment[21]. A number of studies have shown that patients with CRC and polyps had a significant increase in *Bacteroides massiliensis*, *Bacteroides ovatus*, *Bacteroides vulgatus* and *Escherichia coli (E. coli)*[22], *Atopobium parvulum* and *Actinomyces odontolyticus* [23], *Fusobacterium nucleatum (F. nucleatum)*[24,25] and *Bacteroides fragilis (B. fragilis)*[25,26], as well as *Porphyromonas asaccharolytica*, *Parvimonas micra (P. micra)*, *Prevotella intermedia*, *Alistipes Finegoldii* and *Thermanaerovibrio acidaminovorans*[25]. Other authors have established an association of CRC and adenomas with *Parvimonas*, *Peptostreptococcus*, *Prevotella*, *Butyricimonas*, *Alistipes* and *Odoribacter*[27-29]. Coker *et al*[30], in addition, noted dysbiosis of the intestinal fungal microflora and archaea in patients with CRC, namely, a decrease in methanogenic archaea, Saccharomycetes, and Pneumocystidomycetes but enrichment of halophilic archaea and Malasseziomycetes in fecal samples[30].

A recent study by Liu W in 2020 found a significant reduction in the richness and diversity of the intestinal flora in patients with CRC and polyps compared with healthy individuals[31]. In healthy individuals, *Bacteroidetes* (52.14%) and *Firmicutes* (35.88%) predominated. When analyzing the microbiome in healthy individuals, patients with polyps, adenomas and CRC, a decrease in *Bacteroidetes* (from 52.14% - 53.92% - 52.46% to 47.06%) and *Firmicutes* (from 35.88% - 29.73% - 24.27% - up to 25.36%) and an increase in the share of *Proteobacteria* (from 9.33% - 12.31% - 16.51% - to 22.37%) were revealed. In addition, in patients with CRC, there was a decrease in the number of *Clostridium butyricum*, producing butyrate, *Streptococcus thermophiles*, producing lactic acid[32], and other intestinal symbiotic bacteria, such as *Bifidobacterium animalis* and *Streptococcus thermophiles*[33]. In African Americans with a high risk

of developing CRC, the main representatives of the intestinal microbiome were *Bacteroides*, with a high level of microbial gene expression, which encode the production of secondary bile acids (BAs). At the same time, in the intestinal microbiome of indigenous inhabitants of rural Africa with a low risk of developing CRC, the main representative was *Prevotella*, with a high level of expression of genes encoding methanogenesis and production of hydrogen sulfide[34].

It has been established that bacteria with proven pro-carcinogenic activity are capable of forming biofilms, which are communities of microorganisms embedded in the matrix of extracellular polymeric substances that are produced by the biofilm bacteria themselves and include proteins, polysaccharides and nucleotides in different proportions. Bacterial biofilms reduce the penetration of antibiotics, increase the permeability of the epithelium due to the loss of E-cadherin, and promote the contact of bacteria with epithelial cells and bacterial translocation[35,36]. Dejea *et al*[37] demonstrated that invasive polymicrobial bacterial biofilms are detected in 89% of cases in right-sided CRC[37]. They were more often determined in areas of normal mucosa remote from the tumor, and their composition was identical to the microbial composition in the tumor tissue. The presence of biofilms has been associated with increased levels of interleukin 6 (IL-6) and activation of signal transducer and activator of transcription 3 (STAT3), a decrease in epithelial cell E-cadherin, and increased proliferation of crypt epithelial cells in normal colonic mucosa[37]. Drewes *et al*[38] showed that these biofilms are enriched in *B. fragilis*, *F. nucleatum*, *P. micra*, and *Peptostreptococcus stomatis*[38].

Of particular interest are the causes of dysbiosis in patients with CRC. It is known that the qualitative and quantitative composition of the microbiome can be influenced by various factors, such as age, infection, diet, stress, inflammation, and drugs[39,40]. Genetic changes (congenital or acquired) with age can contribute to a change in the microenvironment, which leads to a change in the microbiome[39]. The gut microbiota can change under the influence of chemotherapy and immunotherapy for CRC, which, in the end, can affect its effectiveness[41]. In addition, inflammatory changes associated with colorectal carcinogenesis can also alter the gut microbiome[40]. The use of resveratrol, an anti-inflammatory stilbenoid, in a mouse model of CRC not only reduced inflammation and CRC development but also altered the gut microbiota, leading to an increase in colonic butyrate, which has antitumor effects[42]. It is possible that the anti-inflammatory effect underlies the reduction in the risk of CRC with regular use of aspirin[43].

It is believed that the specific tumor microenvironment can also influence the microbiome. This is indirectly evidenced by the fact that the populations of intestinal bacteria “on the tumor” and “outside the tumor”, as well as in the intestinal lumen, associated with the intestinal mucosa and associated with the tumor tissue, differ within the tumor host[39,44]. It is believed that pro-oncogenic "bacteria drivers" associated with colorectal carcinogenesis induce epithelial DNA damage, which leads to a change in the microenvironment of bacteria. In the future, a change in the microenvironment can lead to a decrease or disappearance of “bacteria drivers” and the population of the formed niche by “bacteria passengers”[45-48]. In a number of experimental studies, it was noted that genetic disorders of gastric mucosal cells, for example, in mice with gene knockouts (*e.g.,* Tlr5, Il10, T-bet and Rag2), led to changes in the microbiome in the colon[49]. In patients with CRC with deficient DNA mismatch repair (dMMR) and proficient MMR (pMMR), there were significant differences in the composition of the intestinal microbiome. The dMMR group was dominated by *Fusobacteria, Firmicutes, Verrucomicrobia*, and *Actinobacteria* at the phylum level and *Fusobacterium, Akkermansia, Bifidobacterium, Faecalibacterium, Streptococcus*, and *Prevotella bacteria* at the genus level, while the pMMR group was dominated by *Proteobacteria* at the phylum level and *Serratia, Cupriavidus* and *Sphingobium* at the genus level[50]. Table 1 shows the characteristics of a number of bacteria associated with the development of CRC.

Notably, CRC rarely develops in the small intestine. To some extent, this may be due to the peculiarities of their epithelial and mucosal barrier[51], as well as differences in the quantitative and qualitative gut microbiota composition[52-54]. In particular, the small intestine has (1) A higher density of natural killer T cells, intraepithelial lymphocytes, eosinophils and dendritic cells[51]; (2) A higher oxygen concentration[52]; and (3) A higher concentration of antibacterial substances[55]. At the same time, in the small intestine, the expression of multiple Toll-like receptors (TLRs)[56,57] and microbial cell concentrations are lower[52,53] than those in the large intestine. In addition, a number of authors have noted significant differences in the immune system of the large and small intestines[58,59]. It is believed that tolerance to food and bacterial antigens is formed in the proximal small intestine, which has a thinner layer of mucus, providing closer contact with epithelial and immune cells, and the immune system is aimed at stopping local and systemic inflammation[59]. The duodenum and upper jejunum contain a class of CD103+CD11b+ dendritic cells unique to the gut that are involved in the formation of regulatory T cells[60]. It has been established that in response to food and bacterial antigens, lymph nodes draining the small intestine predominantly induce tolerogenic responses, while lymph nodes draining the large intestine activate proinflammatory T-cell responses[59,61]. The tolerogenic effect of oral administration of antigens was comparable to the systemic anti-inflammatory effect of dexamethasone[59]. These data suggest that the low incidence of CRC in the small intestine may be associated with a low level of inflammatory reactions in response to the intestinal microbiota, given that inflammation is one of the key players in colorectal carcinogenesis.

Table 1 Features of some bacteria associated with colorectal cancer

Virulence factors, clinical and experimental data	CRC pathways
B. fragilis	
- Contains B. fragilis toxin (BFT) that is zinc-dependent metalloprotease toxin[185,186]; -Associated with colitis[186], low-grade dysplasia, tubular adenomas, and serrated polyps[163], Lynch syndrome and familial adenomatous polyposis[187]; - More often associated with left-sided CRC [163]; - Characterized by biofilms formation[38]; - Associated with unfavorable CRC prognosis[123]; - Potentiates oncogenesis in the distal colon in mice[188]	-Induction of stepwise cleavage of E-cadherin and stimulates cell proliferation[185]; - Synthesis of cytokines, incl. IL-17↑[26,123,163,188]; - Activation of the WNT/β-catenin pathway → c-Myc transcription and translation↑ → cell proliferation↑[189]; - STAT3 activation in the mucosal immune cells → mucosal permeability↑[163,186,188]; - NF-κB activation →Th17c↑[163]; - STAT3 and NF-κB activation→ COX-2 and MMP-9 expression↑[123,163]; - BRAF and KRAS mutations, expression of MLH1↓[123]; - APC mutations [187]
E. coli	
- Contains Colibactin and Cytotoxic necrotizing factor 1 (CNF1)[190]; - Can carry the pathogenicity island pks (pks + E. coli), which encodes a set of enzymes that synthesize Colibactin[191]; - Associated with inflammatory bowel disease and CRC[192]; - More frequently found in CRC biopsies than in healthy mucosa[191]; - More common in advanced stages of CRC [193,194]; - Potentiates induction of invasive cancer in mice[192]; – Differences in the frequency of pks + E. coli in patients with CRC, adenomas and in healthy people were not detected[195]	- Induction of DNA double-strand breaks[196]; - DNA alkylation[190]; - Decrease of tumor-infiltrating T lymphocytes (CD3+ and CD8 T cells) and increases colonic inflammation[197]; - Activation of angiogenesis[198] and epithelial-mesenchymal transition[199]; - Modulates activity Rho GTPases (signaling G-proteins of Ras subfamily), thereby affecting the actin cytoskeleton, that contributes to the disruption of cell adhesion (due to the reorganization of the expression of E-cadherin, β-catenin, zonula occludens-1 (ZO-1) and caveolin- 1), the reduction of phagocytosis and improve epithelial cell motility[200,201]; - Induction of cell proliferation[191]; - Depletion of host mismatch repair proteins *via* bacterially secreted EspF effector protein[142]
F. nucleatum	
- Contains adhesion protein FadA, Fap2 и RadD[96]; - Synthesis of toxic metabolites: Secondary bile acids, trimethylamine N-oxide, hydrogen sulfide, heme, nitrosamines, heterocyclic amines and polyaromatic hydrocarbons[96]; - Characterized by biofilms formation[38]; - Often detected in adenomas[38,202], tumor samples with high grade dysplasia [203], carcinoma tissue[44,202,204], distant CRC metastases[125]; - More frequently found in CRC biopsies than in healthy mucosa[205]; - Associated with proximal tumor localization[161,204,206,207], higher depth of invasion[206], higher clinical stage[206,207], low tumor differentiation[206,207], lymph node metastases and low survival rate[204]; - Promotes CRC induction in a traditional experimental model in mice[202]	- Associated with MLH1 methylation[206,207]; MSI-H[161,206-208]; CIMP-H [161,207]; BRAF mutation[206,207]; - FadA-dependent activation of the E-cadherin/β-catenin pathway → cell proliferation↑ and expression of E-cadherin↑[96]; - Activation of p38 MAPK and NF-κB signaling pathways → synthesis IL-6, IL-8 and IL-18↑[204]; - Inhibition of NK cell cytotoxicity by the Fap2 protein and an increase in the number of myeloid suppressor cells[96, 204,208]; - Regulates miR21 expression *via* the TLR4/MYD88/NFκB[96,202, 208,209]
Streptococcus gallolyticus (Sg)	
- More frequently found in CRC biopsies than in healthy mucosa[210,211]; - Circulation of Sg in the blood in patients with CRC is most likely associated with dysfunction of the epithelial barrier[212]; - In a mouse xenograft model of CRC, Sg promotes tumor growth[211]	- Induction of cell proliferation through WNT/β-catenin pathway[211] and modulation of extracellular matrix[213]; - Increase in expression of c-Myc and cyclin D1 proteins[211]; - Stimulation of proliferation of the intestinal epithelium by some Sg strains which is associated with their ability to adhere to the intestinal epithelium and the genetic characteristics of the host cells [211,214]
Enterococcus faecalis (Ef)	
- Contains superoxide[215]- Promotes CRC induction in Il10 $^{-/-}$ mice experimental model[215]	- Promotes chromosome instability[215]; - *Ef* polarize colon macrophages to produce endogenous mutagens → initiation CIN → expression of progenitor and tumor stem cell markers[216]; - Induces gene mutation and endogenous transformation through microbiome-induced bystander effects[217]; - Activation of the WNT/β-catenin pathway[217]; - Activation of transcription factors c-Myc, Klf4, Oct4 and Sox2[217]

APC: Adenomatous polyposis coli; BFT: *B. fragilis* toxin; BRAF: B-Raf proto-oncogene; CIMP: CpG island methylator phenotype; CIN: Initiate chromosomal instability; CNF: Cytotoxic necrotizing factor 1; COX2: Cyclooxygenase 2; FadA: Fatty acid desaturase A; KRAS: Kirsten ras; MAPK: Mitogen-activated protein kinase; MLH1: MutL homolog 1; MPP-9: Metalloproteinase-9; MSI: Microsatellite instability; MYD88: Myeloid differentiation primary response 88; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NK: Natural killer; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; SMAD4: Small mothers against decapentaplegic 4; TLR: Toll-like receptor; TP53: Tumor protein 53; ZO-1: Zonula occludens-1.

Interestingly, the gut microbiota in right-sided and left-sided CRC also differ significantly. *Prevotella*, *Selenomonas*, and *Peptostreptococcus* are the most abundant phyla in right-sided CRC, while *Clostridium perfringens*, *F. nucleatum*, *Escherichia/Shigella*, and *Leptotrichia* are found at a higher abundance in left-sided CRC[62,63]. A recent study by Keskin *et al*[64] reported an association of CRC with patient height [64]. Unfortunately, the authors did not compare the risk of cancer development in the right and left halves of the colon depending on the patient height, but it can be assumed that an increased risk of CRC may be associated with elongation of the intestine, which leads to a decrease in the concentration of antimicrobial peptides and an increase in bacterial density in the distal part of the intestine.

It is important to note that the study of gene markers of the intestinal microbiota in colorectal polyps and CRC has made it possible to develop effective predictive models that not only identify individuals at high risk of developing adenomas and CRC[65] but also predict the survival of patients with CRC

[66]. These studies open up new possibilities for screening for CRC but also for the prevention and treatment of this pathology.

GUT MICROBIOTA AND MECHANISMS OF COLON CANCER INITIATION

According to modern concepts, CRC is a progressive process involving a sequence of cellular mutations during the transition from adenoma to carcinoma[67,68]. Most cases of CRC (> 95%) are sporadic and develop from polypoid adenomas that can progress to intramucosal carcinoma (stage 0) and CRC[69]. The risk of developing CRC is associated with (1) The genetic predisposition to CRC, which determines the proliferation rate of colon epithelial cells and their susceptibility to cell death, as well as the features of their metabolism that affect the formation of a protective mucous layer; (2) The state of the colon mucosa local immunity; and (3) The intestinal microbiome, which, in turn, can influence the stability of the cell genome, metabolism and immune response[70]. At the same time, it should be taken into account that the influence of the microbiota on the development of CRC may be indirect. Thus, in a study by Wang *et al*[71], it was noted that in patients with H. pylori-associated atrophic gastritis or intestinal metaplasia, the risk of developing colorectal polyps and CRC was 2.19 and 3.05 times higher, respectively, than in patients without H. pylori infection. Multiple and adenomatous polyps were significantly more common in the group of infected patients than in the uninfected group[71].

Factors that determine the role of the microbiota in CRC carcinogenesis include: (1) Inflammation; (2) The impact of bacterial metabolites on the activation of signaling pathways associated with proliferation, apoptosis, epithelial-mesenchymal transformation and their genotoxicity; (3) Changes in intestinal stem cell function; and (4) Disorders of local and systemic immunity and others[72,73]. Let us consider them in more detail.

Gut microbiota and inflammation

The interaction of bacteria and other microorganisms with the intestinal epithelium is carried out through TLRs and nucleotide-binding oligomerization domain receptors. Enterocytes express TLR2, TLR3, TLR4, TLR5 and TLR9. Most TLRs are localized on the basolateral membrane, while TLR2, TLR3, and TLR9 are also expressed on the apical surface[74,75]. Basolateral TLR stimulation results in a signaling cascade, activation of nuclear factor-kappaB (NF-κB), and increased secretion of cytokines and chemokines, including tumor necrosis factor-α (TNF-α), IL-6, IL-12, IL-18, C-X-C motif chemokine ligand 8 (CXCL8), and C-C motif chemokine ligand 20 (CCL20). Cytokines activate immunocompetent cells, thereby promoting inflammation and proliferation of the intestinal epithelium[76]. It is believed that with an increase in the number of intestinal bacteria, hypersecretion of cytokines contributes to bacterial invasion into the dense layer of mucus. Bacterial invasion into the mucus layer and interaction with the epithelium may cause early stages of cellular transformation with a deficiency in DNA MMR as a consequence of increased epithelial proliferation[77].

Importantly, the composition and quantity of the intestinal microbiota are regulated by antimicrobial peptides produced by Paneth cells and IgA present in intestinal mucus[78]. On the one hand, mucus determines the distribution and organization of the microbiota in the intestine and protects epithelial cells and crypts against bacterial colonization. However, on the other hand, the presence of microbiota is absolutely necessary for the correct location of intestinal mucus[79]. Mice treated with antibiotics had a thinner layer of mucus, and it was permeable to bacteria. Moreover, epithelial stem cell replication was impaired, and antibiotic-treated mice were more susceptible to colitis induced by physical or chemical exposure[80,81].

Gut microbiota metabolites and proliferative activity of the intestinal epithelium

Bacterial metabolites play a key role in maintaining intestinal homeostasis. As noted above, the synthesis of intestinal mucins is regulated by the gut microbiota. However, intestinal mucus not only protects epithelial cells from bacterial invasion but also serves as a bacterial breeding ground. These bacteria include *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Bifidobacterium bifidum*, *B. fragilis*, *Ruminococcus gnavus* and *Ruminococcus torques*. These species generate short-chain fatty acids (SCFAs) during fermentation using glycans as an energy source[80,82]. SCFAs are carboxylic acids resulting from bacterial anaerobic fermentation predominantly of dietary fibers in the intestine, where acetate and propionate are produced by *Bacteroidetes* (gram-negative bacteria) and butyrate is produced by *Firmicutes* (gram-positive bacteria) in human intestines[83]. It is assumed that the effect of butyrate on intestinal epithelial cells (IECs) and on tumor cells is different[84]. Butyrate, the main source of energy in normal IECs, maintains low levels of Wnt signaling and normal proliferative activity[84,85]. In germ-free mice and in specific pathogen-free (SPF) mice treated with antibiotics selective for gram-positive bacteria, a decrease in proliferative activity in IECs was noted. Oral administration of chloroform-treated SPF mouse feces to sterile mice or oral administration of SCFAs restored the proliferative activity of IECs[86].

At the same time, in intestinal stem cells (ISCs) and tumor cells, Wnt hyperactivation by butyrate leads to increased transcription of proteins involved in apoptosis of colon cancer cells and a decrease in cell proliferative activity[87]. It is believed that hyperactivation of Wnt signaling in tumor cells raises Wnt activity levels to the range leading to apoptosis, while stimulation of normal colon cells with BAs likely results in moderate levels of Wnt activity that promote proliferation[85,88].

The following antitumor effects of butyrate have been described: Butyrate suppresses miR-92a expression *via* c-Myc, which reduces colon cancer cell proliferation and stimulates apoptosis[89]; butyrate reduces the phosphorylation of AKT serine/threonine kinase 1 (Akt1) and extracellular signal–regulated kinases ½ by blocking histone deacetylase 3 (HDAC3) activity with subsequent cell motility inhibition and inhibiting any subsequent cell movement, which impedes CRC cell metastasis and invasion[90]; and butyrate activates miR-203, which inhibits CRC cell proliferation, colony formation and invasion and promotes apoptosis of CRC cells[91].

An interesting hypothesis has been proposed to explain the antitumor effect of butyrate by the fact that colon cancer cells prefer glucose to butyrate as an energy source owing to the Warburg effect pathway. As a result, colon cancer cells accumulate large amounts of butyrate, which acts as an HDAC inhibitor, disrupting gene transcription[92]. Butyrate can enter the nucleus directly, inhibit HDAC1 and cause a decrease in the levels of short chain acyl-CoA dehydrogenase (SCAD), which is the primary process in the catalysis of mitochondrial butyrate oxidation[93]. This reduces the autoxidation of butyrate in CRC cells and allows butyrate to accumulate in carcinoma cells, thereby inhibiting the development of CRC by inhibiting CRC cell proliferation and activating apoptosis.

At the same time, a number of studies have shown the pro-oncogenic effect of butyrate. In an experimental model of Lynch syndrome, its ability to induce the formation of reactive oxygen species (ROS) and promote the accumulation of 8-oxo-7,8-dihydro-2'-deoxyguanosine damage in MMR-deficient cells was noted. In an experiment on mice, DNA damage, polyp formation, and tumorigenesis were reduced by treatment with the antioxidant Vit C or N-acetylcysteine[94,95]. These data may be important, as 5% of patients with Lynch syndrome and approximately 15% of patients with sporadic CRC carry mutations or epigenetically silenced MMR genes[94].

Gut microbiota metabolites and genotoxicity

It should be noted that a number of bacterial metabolites have direct procarcinogenic effects. These include secondary BAs, trimethylamine N-oxide, hydrogen sulfide, polyamines and others[96-99]. It is known that high concentrations of secondary BAs, caused, in particular, by the Western diet, promote inflammation, activation of Wnt and NF-κB signaling pathways, oxidative DNA damage, and disruption of mitotic activity, which ultimately leads to hyperproliferation and invasiveness of colon cells[68,85,100]. Secondary BAs can cause destruction of cell membranes and local disorders of the intestinal epithelium that stimulate the repair mechanisms, which is involved in the excessive proliferation of undifferentiated cells. Hydrophobic BAs can also contribute to increased ROS and reactive nitrogen species that cause oxidative stress, DNA and protein damage and disruption of base excision repair pathways[93,100,101]. In addition, secondary BAs promote epidermal growth factor receptor (EGFR) activation through phospholipid acid aggregation, which induces EGFR dimerization/oligomerization and stimulates EGFR-mitogen-activated protein kinase signaling and cell proliferation [93,102].

The genotoxicity of microbial metabolites may be associated with the biotransformation of a number of xenobiotics, such as heterocyclic amines (HCAs). HCAs are formed as a result of the thermal processing of foods, including oils, grains and vegetables, and processed meats[103]. The intestinal microbiota can metabolize them into molecules with increased mutagenic activity, which have pronounced genotoxic and mutagenic properties. HCAs can promote the development of malignant neoplasms of the intestine, liver, lung, breast, and other tumors[104]. Their carcinogenicity is associated with mutations in proto-oncogenes and tumor suppressor genes, including KRAS, HARAS, adenomatous polyposis coli (APC), β-catenin, and tumor protein 53 (TP53)[105]. However, the intestinal microbiota can metabolize food-derived HCAs, facilitating their excretion with feces or conversion into less toxic compounds[103]. These processes involve bacterial enzymes produced by some lactic acid bacteria and probiotics. A decrease in the number of taxa with their activity was noted in patients with CRC[106].

Gut microbiota and Paneth cells

Paneth cells are the source of secreted proteins, such as Wnt3, EGF, and Notch ligand Delta-like (Dll) 4 and Dll1, which are crucial for stem cell support[107-110]. They are in direct contact with crypt base columnar cells expressing leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), known as a stem cell marker for colon cancer and gastric cancer. Due to their proximity to LGR5+, Paneth cells affect the function of ISCs by activating the canonical Wnt/β-catenin signaling pathway and producing Wnt3, which binds to Frizzled receptors to improve the function of Lgr5 stem cells[109,111]. There is an assumption that the development of adenomas and CRC is preceded by the appearance of metaplastic Paneth cells as a protective antibacterial and inflammatory reaction caused by altered microbial activity [78,110]. In epithelial injury followed by inflammation, when rapid barrier repair is needed, Paneth cells are able to acquire stem cell characteristics through dedifferentiation upon Notch and stem cell factor/c-

kit signaling, thereby promoting epithelial repair[109,112,113]. In addition to Paneth cells, other differentiated IECs can also acquire stem cell properties under certain conditions and participate in the repair of epithelial damage[113]. When studying tissue samples of polyps, normal tissues and CRC, it was found that some polyps and CRC cells demonstrate a stem-like phenotype. Advanced polyps contain an increased number of stem-like cells, regulatory T cells and a subtype of precancerous fibroblasts[114].

Gut microbiota and immunity

The intestine is an important immune organ that not only protects against external pathogens but also participates in the formation of immune tolerance to food substrates and the normal intestinal microbiome. At the same time, the intestinal microbiome is directly involved in the formation of local and systemic immunity by activating the synthesis of IgA and interferons and activating immune cells [115]. Thus, germ-free animals are more susceptible to viral and bacterial infections and have reduced digestive enzyme activity, cytokine production and serum immunoglobulin levels, smaller Peyer's patches and fewer intraepithelial lymphocytes[116].

A number of experimental studies have demonstrated an association between the gut immune system, gut microbiota, and CRC risk[117-119]. Hurtado *et al*[117] showed that the induction of experimental CRC is associated with increased synthesis of the proinflammatory cytokine IL17A by immune cells, for activation of which specific microbes are needed, such as *B. fragilis*[117]. Activation of IL17A receptors leads to activation of the NF-κB transcriptional pathway and induction of IL6 production by epithelial cells, which, in turn, activates STAT3 in epithelial cells, promoting their survival and proliferation[118]. At the same time, it should be noted that the role of Th17, IL17 and regulatory T cells (Tregs) in CRC is ambiguous and can be associated with both oncogenic and antitumor activity, as evidenced by the data of experimental and clinical studies[117,119]. The interaction between the intestinal immune system, microbiota, and CRC is complex and multipolar and depends on the stage of the tumor process, the tumor microenvironment, the ratio of bacteria in the intestine with oncogenic and antitumor properties, diet, and other factors[119-121]. This issue still requires detailed study, especially given that modulation of the immune system by the gut microbiome can directly influence the outcome of CRC treatment[122].

GUT MICROBIOTA AND PROGRESSION OF CRC

A growing body of research suggests that the gut microbiome can influence not only the initiation but also the progression of CRC[72]. It has been established that high infection of the tumor tissue with "pro-oncogenic" bacteria, for example, *P. micra*, *F. nucleatum* and *B. fragilis*, is associated with more advanced stages of CRC and with a decrease in patient survival[44,123,124]. Some authors have noted positive correlations between the number of *F. nucleatum* in the tumor and the presence of metastases in regional lymph nodes[44]. Furthermore, pro-oncogenic bacteria, such as *F. nucleatum*, have been found not only in primary colorectal tumors but also in metastatic lesions of the liver[125] and lungs[126].

The influence of the intestinal microbiome on the progression of CRC can be carried out by various mechanisms, for example, by modulating the tumor microenvironment and initiating the processes of inflammation, angiogenesis, epithelial-mesenchymal transition (EMT) and immunological tolerance that promote cancer metastasis[33,127,128]. For example, it has been found that a high abundance of *F. nucleatum* in tumor tissue is associated with increased expression of TNF-α, β-catenin and NF-κB, while high B. fragilis level is positively related with COX-2, metalloproteinase-9, NF-κB, and the presence of Kirsten ras (KRAS) and B-Raf proto-oncogene (BRAF) mutations[123]. In an experiment, *F. nucleatum* infection promoted increased tumor cell migration and translation of factors associated with EMT, which promoted CRC metastases to the lungs and liver[126,129]. *F. nucleatum*-infected cells have been shown to secrete specific exosomes that increase the ability of tumor cells to migrate *in vitro* and promote tumor metastasis *in vivo*[130]. *F. nucleatum* may also promote immune evasion by binding the Fap2 protein to the immunosuppressive T-cell immunoglobulin and ITIM domain receptor, which inhibits T-cell activation and induces the death of human natural killer cells[131,132]. In addition, the gut microbiome may influence macrophage polarization and the formation of neutrophil extracellular traps, which may promote tumor metastasis through local invasion, increase vascular permeability, and facilitate immune escape and colonization[133,134].

A number of authors drew attention to the similarity of the mechanisms of tumor and bacterial metastasis (dissemination), which, in their opinion, indicates a possible role of bacterial translocation in the progression of CRC[135,136]. Translocation is the process of penetration of bacteria into the intestinal epithelium and beyond into the lymphatic vessels and nodes, blood vessels and distant organs. Translocation mechanisms may be associated with a violation of the composition and amount of mucus, remodeling of tight junctions, invasive bacterial properties, local and systemic inflammation, alcohol, infection, dysbacteriosis, drug treatment, and other factors[137]. In particular, it was found that *E. coli*, depending on the virulence regulator VirF, destroys the intestinal vascular barrier that controls the spread of bacteria along the gut-liver axis. When it is disturbed, bacteria disseminate to the liver, stimulate the formation of a premetastatic niche, and promote the recruitment of metastatic cells.

Elevated levels of vesicle-associated protein-1 (PV-1), a marker of disruption of the intestinal vascular barrier, are associated with dissemination of hepatic bacteria and metachronous distant metastases[136].

It should be noted that the assessment of the microbiome role in the progression of CRC is important, as it determines the feasibility of using antibiotic therapy in patients with CRC. A number of experimental studies have shown that the use of antibiotic therapy against F. nucleatum contributed to a decrease in tumor cell proliferation and overall tumor growth[125]. Depletion of intratumoral bacteria in experimental breast cancer significantly reduced lung metastasis without affecting primary tumor growth[127]. Moreover, some researchers have noted the positive effect of antibiotic therapy in the treatment of malignant neoplasms, including CRC[138].

GUT MICROBIOTA AND GENETIC HETEROGENEITY IN CRC

Microbiome and genetic and epigenetic changes in CRC

In 1990, Fearon and Vogelstein offered a multistage genetic model for the formation of CRC[69]. According to this model, the formation of CRC occurs due to the accumulation of several genetic and epigenetic changes in key tumor suppressor genes and the activation of oncogenes[69,139]. The first mutations in CRC most often affect the APC gene. These mutations confer a selective growth advantage on normal IECs and result in slow-growing intestinal adenomas. The appearance of the 2nd mutation over time, for example, in the KRAS gene, increases the growth rate of adenomas. Accelerated proliferation of epithelial cells contributes to the accumulation of new mutations in genes such as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), small mothers against decapentaplegic 4 (SMAD4) and TP53, which ultimately leads to the emergence of a malignant tumor with invasive and metastatic properties. These mutations give epithelial cells certain advantages over other cells and are called "driver" mutations[140].

The authors proposed two main pathways of CRC formation. The first pathway accounts for 85% of all CRCs and involves inhibition of Testis-Specific GTPase expression and APC. The second pathway is associated with mutational inactivation of proteins involved in MMR, namely, the mutS homolog 2 (MSH2), mutL homolog 1 (MLH1), and post meiotic segregation 2 (PMS2) genes. This pathway is seen in 15% of all sporadic cases of CRC[69,139,141].

Currently, CRC is considered a heterogeneous group of tumors with different mechanisms of carcinogenesis and macro- and microscopic characteristics, as well as with different 5-year survival prognoses. A growing body of evidence points to an important role of the gut microbiome in the development of genetic and epigenetic changes in CRC[142-145]. It is believed that there are two main mechanisms by which the microbiome influences the genome or epigenome of the intestinal epithelium in CRC: the ability of intestinal microbes to induce a pro-carcinogenic inflammatory response and the production of secondary metabolites by intestinal microbes[146-149]. In addition, a number of microbial toxins, such as cytolethal distending toxin (CDT), colibactin and *B. fragilis* toxin, can directly cause DNA damage and trigger mutations[150].

Of particular interest is the role of the gut microbiota in hereditary syndromes. A number of studies have noted an increase in the number of *Bacteroides*, *Parabacteroides distasonis*, *Faecalibacterium prausnitzii*, *Ruminococcus bromii*, the *Pseudomonadaceae family*, *E. coli*, *Klebsiella species*, *Porphyromonas* and *Methanobrevibacter* in Lynch syndrome[151] and *B. fragilis* and *E. coli* in familial adenomatosis[35]. It is known that Lynch syndrome, which is characterized by a high risk of developing CRC and other forms of malignant tumors, is associated with a mutation of the MSH2 gene, which encodes a protein of the same name and plays an important role in DNA repair. The MSH2 protein is involved in the elimination of errors that occur during DNA replication in the process of preparing a cell for division. In Msh2-Lynch mice, there was an increase in the expression of genes associated with immunity, activation of K-Ras and genes responsible for the epithelial-mesenchymal transition and an increase in the number of Lyz1 and Wnt3 transcripts, which indicates increased proliferation of stem cells due to Wnt production by Paneth cells and activation of inflammatory pathways. In experimental Lynch syndrome, in MSH2-Lynch mice with MSH2-deficient intestinal epithelial crypts, the use of the methylating drug temozolomide resulted in a fivefold increase in the number of MSH2-deficient crypts and caused tumors in all experimental animals. Transfer of Msh2-Lynch mice from a normal room to a specific SPF room resulted in an almost complete loss of the intestinal tumor phenotype and increased survival of the experimental animals. SPF mice showed a decrease or absence of *Lactobacillus* and *Epsilonproteobacteria taxa* in the feces[77]. In addition, transplantation of cryopreserved conventional feces into SPF mice did not restore the tumor phenotype but increased the rate of epithelial renewal and accelerated the development of microsatellite instability (MSI). A number of authors have also demonstrated that depletion of the gut microbiota by antibiotics reduced the development of colitis and cancer formation in experimental animals[152].

Main ways of developing sporadic CRC

Analysis of genetic and epigenetic changes in CRC made it possible to identify the main mechanisms of development of sporadic CRC and offer molecular genetic classifications of CRC[153-156]. Thus, at present, it is customary to consider three main ways of developing sporadic CRC (Table 2). Of particular

Table 2 The main pathways for the development of sporadic CRC and their relationship to gut dysbiosis

Pathway	Clinical features	Genetic and epigenetic disorders
The chromosomal instability (CIN) – 38%-85%[157,158]	- Associated with traditional adenomas[158]; - Associated with *Fusobacterium, Escherichia/Shigella u Leptotrichia*[63]; - CIN-H tumors are more frequently located on the left colon (87%), are associated with lymphocytic infiltration (82%), are more common in older patients (73%) and are associated with worse prognosis[157]	- Alteration in chromosome number or structure, loss of heterozygosity and aneuploidy[158]; - Mutations in the APC gene, a negative regulator of β-catenin-dependent Wnt signaling pathway (in 70%-80% of cases)[158]; - Mutations in TP53, KRAS, and PIK3CA genes and tumor suppressor genes SMAD2, SMAD4, and DCC[157,158]; - 20%-60% of tumors are CIN-H[158]
The CpG island methylator phenotype (CIMP) – 15%-30%Three groups based on the degree of gene methylation: High CIMP (CIMP -H); low CIMP (CIMP -L) and negative (CIMP-)[157,158]	- Associated with sessile serrated polyps[158,161,218]; - Associated with *F. nucleatum*[161,208], and *E. faecalis*[219]; - Data on the clinical features of CIMP-H tumors are controversial:- CIMP-H tumors are more frequently located in the left colon (67%) and are more common in men (68%) and in older patients (73%)[157,158]; - CIMP-H tumors are more common in females (68%) and in older patients (73%) and are associated with smoking, alcohol consumption, overweight, Western diet, right colon cancer, tumor lymphocytic infiltration, and poor differentiation[159,220,221]; - CIMP-H tumors are associated with worse prognosis[157,158,198,221,222];- CIMP-H tumors with MSI-H and BRAF mutations are more common in the proximal colon, whereas CIMP-L tumors with KRAS mutations are more common in the distal colon[158,159]	- Data on the presence of mutations in BRAF, PIK3CA, KRAS and TP53 genes are contradictory:- KRAS mutation in 27% tumors, PI3KCA mutation in 27%, and BRAF mutation in 7% tumors[157]; - Mutations in BRAF and PIK3CA genes and absence of mutations in KRAS and TP53 genes[154,159,161]; - Methylation of the MINT1, MINT2, MINT31, p14, p16 and MLH1 genes[154,159,161]; - 25% to 60% of tumors are MSI-H[157-159]
The microsatellite instability (MSI) – 15%-20%Three groups based on the number of microsatellites associated: High MSI (MSI-H); low MSI (MSI-L), and microsatellite stable (MSS)[158]	- 20% of cases are hereditary[158]; - Associated with *F. nucleatum*[124,161,208], *E. faecalis*[219] and *P. micra*[124]; - MSI-H are more frequently located on the right colon (86,7%), are more common in older patients(80%), and have a good prognosis at an early stage[157,158]	- High frequency of replication errors in MLH1 and MSH3 mismatch repair (MMR) genes[158]; - Hereditary CRC is associated with germline mutations in MMR genes: MLH1, MSH2, MSH6, and PMS2[158]; - 20%- 70% of tumors are CIMP-H[157,158]; - Often BRAF mutation (40%)[158]; - Mutations in the ACVR2A, TGFBR, MSH3, and MSH6 genes, as well as in the RNF43, RNF213, and ZNRF3 Wnt regulatory pathways[158]; - APC, TP53 and KRAS mutations are rare[158]

APC: Adenomatous polyposis coli; CIMP: CpG island methylator phenotype; CIN: Chromosomal instability; KRAS: Kirsten ras; MMR: Mismatch repair; MSI: Microsatellite instability; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; SMAD4: Small mothers against decapentaplegic 4; TP53: Tumor protein 53.

interest is their association with the gut microbiota[153].

Wielandt *et al*[157] defined a fourth pathway for the development of sporadic CRC: tumors with low or negative MSI-, chromosomal instability (CIN)- and CpG island methylator phenotype (CIMP)- status (30%) that are left-sided, 39% have KRAS mutations and are associated with better survival[157]. At the same time, attention was drawn to significant differences in the assessment of the clinical characteristics of MSI, CIN and CIMP subtypes of CRC, the frequency of BRAF, KRAS, PIK3CA, TP53 and other gene mutations within these subtypes, and the frequency of genetic subtype combinations among themselves. For example, Parmar *et al*[158] showed that CIMP-H tumors are more frequently located on the left colon and are more common in men, while Advani *et al*[159] determined that these tumors are more common in women and are associated with right colon cancer. We believe that these contradictions are associated with a large number of possible genetic subvariants of CRC, and in this connection within one subtype, variants with opposite characteristics are possible.

Notably, the listed pathways are also characteristic of CRC predecessors. In particular, it has been established that tumors derived from sessile serrated polyps are more often located on the right side of the colon and are associated with BRAF mutation, MSI-H and CIMP-H. They have been associated with human intestinal spirochetosis[160] and an increase in the relative abundance of *F. nucleatum*[161].

Molecular pathological classifications of CRC

Currently, two main molecular pathological classifications of CRC have been proposed[155,156]. The first classification based on complex molecular analysis (array and sequencing technologies) distinguishes two groups of CRCs. The first group includes hypermutated tumors (approximately 16%)[155]. They are characterized by microsatellite instability (MSI) due to defective MMR (approximately 13%) or exonuclease domain mutations (approximately 3%). The second group consists of nonhypermutated tumors (approximately 84%) and microsatellite-stable (MSS) cancers with Wnt pathway dysregulation and frequent gene mutations, including APC, KRAS, PIK3CA, SMAD4, and TP53[155]. The second classification proposed by Guinney *et al*[156] includes 4 consensus molecular subtypes (CMSs)[156].

CMS1 (17% of cases) is associated with a high frequency of CIMP-H and MSI-H; mutations in BRAF (48%), KRAS (29%), and APC (42%); activation of the JAK/STAT pathway; impaired MMR system; serrated polyps; diffuse immune infiltration; and diagnosis of CRC on stage I-III with good prognosis

and worse survival after relapse.

CMS2 (47% cases) is associated with CIMP- and MSS; mutations in BRAF (62%), KRAS (34%), APC (80%); Wnt & Myc activation; epithelial signature; tubular adenoma; diagnosis of CRC on Stage II (39%) and good prognosis and good survival after relapse.

CMS3 (13% of cases) is associated with CIMP- and MSS; mutations in BRAF (30%), KRAS (83%), and APC (91%); epithelial signature; metabolic dysregulation; tubulovillous adenoma with serrated features; and intermediate prediction.

CMS4 (23% cases) is associated with CIMP- and MSS; mutations in BRAF (60%), KRAS (37%), APC (77%); stromal signature; EMT; TGF-b activation angiogenesis; serrated polyps; diagnosis of CRC on Stage III-IY with poor prognosis and worse survival after relapse.

Approximately 80%-90% of CRCs fall into one of the four major transcriptional subgroups, and the remaining CRCs are heterogeneous cases exhibiting "mixed or indeterminate" gene expression patterns with varying features of these subtypes[153,156,158,162].

At present, a link between the microbiota and CMS has only been established for the CMS1 subtype, which was associated with an increase in the relative abundance of Fusobacteria and Bacteroidetes and a decrease in *Firmicutes* and *Proteobacteria*[163]. In particular, increases in the abundance of *F. nucleatum*, *Porphyromonas gingivalis*, *Tannerrella forsythia*, *Treponema denticola*, *P. micra* and *Peptostreptococcus stomatis* were noted[163,164]. For other molecular subtypes, this question remains open.

GUT MICROBIOTA AND CRC TREATMENT

Numerous studies suggest that the gut microbiome may influence the efficacy of CRC drug therapy, namely, the efficacy of hematopoietic cell transplantation, chemotherapy, and immunotherapy[122,165-167]. The influence of the intestinal microbiota on the immunotherapy of solid tumors has been studied the most. It has been established that the intestinal microbiota is able to influence the expression of immune checkpoints, both reducing and increasing the effectiveness of immunotherapy[166,167]. Thus, patients with increased levels of *Firmicutes* and *Verrucomicrobia* almost always had a better response to immune checkpoint inhibitor therapy, while in patients with increased levels of *Proteobacteria*, the treatment was ineffective[167]. Moreover, the targeted use of prebiotics and probiotics has contributed to improving the effectiveness of immunotherapy due to the immunomodulatory effect, in particular, by increasing the production of butyrate[168], inhibiting inflammation and reducing oxidative stress[169], and activating CD8+ T- cells[170].

At the same time, the intestinal microbiome can influence the development of resistance to immunotherapy, which is observed in 60%-70% of cases. It has been established that resistance to immunotherapy may be associated with low antigenicity of tumor cells, impaired priming of antigen-specific naive lymphocytes, including as a result of pretreatment with chemotherapy or radiation therapy, functional depletion of tumor-infiltrating lymphocytes, and other factors[122]. Routy *et al*[122] showed that the clinical response to immune checkpoint inhibitor therapy is positively correlated with the relative abundance of the mucin-degrading bacterium *Akkermansia muciniphila* (*A. muciniphila*)[122]. Subsequent studies have shown that *A. muciniphila* improves metabolic functions and immune responses in the host and has the potential to treat inflammatory bowel disease, obesity, and type 2 diabetes[171], diseases associated with the risk of CRC. The important role of *A. muciniphila* in reducing intestinal barrier permeability and the synthesis of intestinal peptide hormones, increasing the level of Tregs in the general population of CD4 T cells and reducing the levels of IL-6 and IL-1β expression was noted[172]. In an experiment, administration of A. muciniphila to mice for 4 wk accelerated the proliferation of LGR5+ ISCs, promoted the differentiation of Paneth and goblet cells in the small intestine and colon, and reduced intestinal damage caused by radiation and methotrexate[173]. The authors suggest that *A. muciniphila* may be one of the key components that maintains a healthy bacterial community by increasing the availability of mucin sugars[174].

The gut microbiota can also influence the efficacy of chemotherapy by inducing CRC chemoresistance [175]. The mechanisms of development of chemoresistance under the influence of microbiota may be associated with a violation of autophagy mechanisms[166] or a change in the metabolism of chemotherapeutic drugs, for example, gemcitabine[176].

Discussions continue in the scientific world about the effect of antibiotic therapy on the effectiveness of therapy for malignant neoplasms, including CRC. A number of meta-analyses have shown that antibiotic therapy can reduce the survival of patients with malignant neoplasms[177], while other authors have shown that antibiotics can inhibit tumor growth or metastases and improve treatment outcomes[125,127,138,178-180]. Thus, the depletion of intratumoral bacteria in experimental breast cancer significantly reduced lung metastasis without affecting the growth of the primary tumor[127]. At the same time, Routy *et al*[122] demonstrated that dysbiosis associated with malignant disease or antibiotic use can influence primary resistance to programmed cell death protein 1 (PD-1) blockade in mice with tumors and cancer patients. In mice with experimental MCA-205 sarcoma and RET melanoma, treatment with broad-spectrum antibiotics significantly compromised the antitumor effects and survival of mice treated with PD-1 mAb alone or in combination with cytotoxic T lymphocyte-

associated antigen-4 mAb. Moreover, the authors demonstrate that in patients with non-small cell lung cancer, renal cell carcinoma, or urothelial carcinoma, the use of antibiotics before or 1 mo after the first mAb administration resulted in a significant reduction in progression-free survival and overall survival. The authors believe that the deterioration of the immunotherapy effect is due to a decrease in the number and species diversity of the intestinal microflora. Oral administration of *A. muciniphila* to experimental animals resulted in increased expression of CD4+ T cells expressing the small intestine–associated chemokine receptor CCR9 and/or the Th1-associated chemokine receptor CXCR3 in mesenteric lymph nodes and tumor-draining lymph nodes[122].

Despite the impressive results obtained in this study, some heterogeneity of patient groups in terms of nosology, the prevalence of the tumor process, the number of A. muciniphila, and antibacterial therapy regimens is embarrassing. In particular, in the group of patients with renal cell carcinoma treated with antibacterial therapy, there were significantly more patients with liver metastases than in the group of patients who did not receive antibacterial therapy. Moreover, regardless of whether patients received antibacterial therapy or not, *A. muciniphila* and *E. hirae* were the commensals most significantly associated with a favorable clinical outcome. This raises a reasonable question: Are the best survival rates associated with the absence of antibacterial therapy or are they due to the noted differences?

We believe that further study of the feasibility of using targeted antibacterial therapy and probiotics in patients with CRC is relevant. It seems logical that if certain types of bacteria can be associated not only with the initiation but also with the progression of cancer, then the elimination of pathogenic bacteria can improve long-term treatment outcomes. A number of researchers have demonstrated the effect of antibacterial therapy in the treatment of malignant neoplasms[179-182].

In connection with the above, it can be stated that the effect of antibacterial therapy on the effect of immunotherapy and the survival of patients with malignant neoplasms is insufficiently unexplored. Contradictions in the results obtained require a detailed study with stratification of patients depending on the type and stage of cancer, the type of antibiotics used and the characteristics of the patient microbiome, as well as the nature of changes in the microflora as a result of treatment. Considering the literature data, macrolides (azithromycin, clarithromycin), doxycycline and salinomycin seem to be the most promising groups of antibacterial drugs[138,179-182]. At the same time, the sensitivity of "pro-oncogenic" strains to antibacterial drugs requires a detailed study, since under modern conditions, polyresistance to antibacterial drugs is not so rare[183,184].

CONCLUSION

Thus, the results of numerous studies confirm the important role of the gut microbiome in the development of intestinal polyps and CRC. The microbiota and its metabolites affect the severity of inflammatory changes in the intestine and activation of signaling pathways associated with proliferation, apoptosis, and epithelial-mesenchymal transformation and may contribute to the accumulation of genetic mutations. In addition, intestinal bacteria can affect the effectiveness of immunotherapy and chemotherapy and the survival of CRC patients, as they are associated with the formation of immunological tolerance, priming of tumor antigens, and the qualitative and quantitative characteristics of immune cells infiltrating the tumor. Changing the microbiome aimed at eliminating pro-oncogenic bacteria and microorganisms and saturating it with a population of microorganisms responsible for correcting immune defense may be one of the promising directions not only in the prevention but also in the treatment of sporadic CRC. In this regard, further study is required to determine the role of specific bacteria in the progression of CRC and their influence on the effectiveness of antitumor therapy. Considering that CRC is a heterogeneous disease with different mechanisms of carcinogenesis, macro- and microscopic characteristics, as well as an unclear prognosis, further study of the connection between the intestinal microbiota and molecular genetic subtypes of CRC is of great interest. The results of these studies may contribute to the development of new approaches to the diagnosis, treatment and prevention of CRC.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Xiaojianzhong decoction prevents gastric precancerous lesions in rats by inhibiting autophagy and glycolysis in gastric mucosal cells

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Abstract

BACKGROUND

Gastric precancerous lesions (GPL) precede the development of gastric cancer (GC). They are characterized by gastric mucosal intestinal metaplasia and dysplasia caused by various factors such as inflammation, bacterial infection, and injury. Abnormalities in autophagy and glycolysis affect GPL progression, and their effective regulation can aid in GPL treatment and GC prevention. Xiaojianzhong decoction (XJZ) is a classic compound for the treatment of digestive system diseases in ancient China which can inhibit the progression of GPL. However, its specific mechanism of action is still unclear.

AIM

To investigate the therapeutic effects of XJZ decoction on a rat GPL model and the mechanisms underlying its effects on autophagy and glycolysis regulation in GPLs.

METHODS

Wistar rats were randomly divided into six groups of five rats each and all groups except the control group were subjected to GPL model construction for 18 wk. The rats' body weight was monitored every 2 wk starting from the beginning of modeling. Gastric histopathology was examined using hematoxylin-eosin staining and Alcian blue-periodic acid-Schiff staining. Autophagy was observed using transmission electron microscopy. The expressions of autophagy, hypoxia, and glycolysis related proteins in gastric mucosa were detected using immunohistochemistry and immunofluorescence. The expressions of the following proteins in gastric tissues: B cell lymphoma/Leukemia-2 and adenovirus E1B19000 interacting protein 3 (Bnip-3), microtubule associated protein 1 light chain 3 (LC-3), moesin-like BCL2-interacting protein 1 (Beclin-1), phosphatidylinositol 3-kimase (PI3K), protein kinase B (AKT), mammalian target of rapamycin (mTOR), p53, AMP-activated protein kinase (AMPK), and Unc-51 like kinase 1 (ULK1) were detected using western blot. The relative expressions of autophagy, hypoxia, and glycolysis related mRNA in gastric tissues was detected using reverse transcription-polymerase chain reaction.

RESULTS

Treatment with XJZ increased the rats' body weight and improved GPL-related histopathological manifestations. It also decreased autophagosome and autolysosome formation in gastric tissues and reduced Bnip-3, Beclin-1, and LC-3II expressions, resulting in inhibition of autophagy. Moreover, XJZ down-regulated glycolysis-related monocarboxylate transporter (MCT1), MCT4, and CD147 expressions. XJZ prevented the increase of autophagy level by decreasing gastric mucosal hypoxia, activating the PI3K/AKT/mTOR pathway, inhibiting the p53/AMPK pathway activation and ULK1 Ser-317 and Ser-555 phosphorylation. In addition, XJZ improved abnormal gastric mucosal glucose metabolism by ameliorating gastric mucosal hypoxia and inhibiting ULK1 expression.

CONCLUSION

This study demonstrates that XJZ may inhibit autophagy and glycolysis in GPL gastric mucosal cells by improving gastric mucosal hypoxia and regulating PI3K/AKT/mTOR and p53/AMPK/ULK1 signaling pathways, providing a feasible strategy for the GPL treatment.

Key Words: Xiaojianzhong decoction; Gastric precancerous lesions; Autophagy; Glycolysis; Gastric mucosal cells; Herb

Core Tip: Xiaojianzhong Decoction is a classic compound for the treatment of digestive system diseases in ancient China, and it also shows good curative effect in the treatment of gastric precancerous lesions (GPL). Many studies have shown that the levels of autophagy and glycolysis influence the progression of GPL. Our study found that Xiaojianzhong Decoction can improve the pathological manifestations of gastric mucosa in GPL rats, and inhibits the expression of autophagy, glycolysis-related proteins and mRNA. Xiaojianzhong decoction (XJZ) prevented the increase of autophagy levels by decreasing gastric mucosal hypoxia, activating the phosphatidylinositol 3-kimase/protein kinase B/mammalian target of rapamycin pathway, inhibiting the p53/AMP-activated protein kinase pathway activation and Unc-51 Like kinase 1 (ULK1) Ser-317 and Ser-555 phosphorylation. In addition, XJZ improved abnormal gastric mucosal glucose metabolism by ameliorating gastric mucosal hypoxia and inhibiting ULK1 expression.

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INTRODUCTION

Gastric precancerous lesions (GPLs) are histopathological changes of the gastric mucosa that are prone to cancer, mainly including intestinal metaplasia (IM) and dysplasia (Dys), accompanied by local mucosal inflammation and atrophy[1]. As an early pathological process before the occurrence of gastric cancer (GC), GPL is reversible, and preventing the progression of GPL can reduce the morbidity and

mortality of GC, so it is significant to carry out early prevention and treatment in the GPL stage. Epidemiological surveys show that the incidence of GPL in patients undergoing endoscopy in East Asia is as high as 23.2%; among them, 32.0% have IM and 10.6% have Dys, and the incidence of GC in these patients is 23.8% and 7.3%, respectively. Thus, the prevention and treatment of GPLs are crucial and urgent[2].

GPL occurs in the epithelial cells and glands of the gastric mucosa. The continuous action of multiple factors such as *Helicobacter pylori* infection and inflammatory injury causes gastric mucosal cells to atrophy or decrease in number, and the abnormal repair of the gastric mucosa causes the replacement of lamina propria glands by goblet cells and the gradual development of IM and Dys. Autophagy is a process in which cells autonomously generate autophagosomes to phagocytose intracellular functionally damaged or senescent cell structures and then combine with lysosomes for digestion, energy production, and reuse by the body. As an important factor affecting the fate of IM and Dys cells, autophagy is present throughout the course of GPL[3]. An appropriate level of autophagy maintains intracellular homeostasis and avoids the accumulation of gene damage or even canceration. The complex local microenvironment comprising hypoxia, ischemia, and dysregulation of the signaling molecule activation in GPLs leads to increased autophagy. This increase in autophagy not only helps the GPL dysplastic cells to repair damaged organelles, maintain the stability of the internal environment, and ensure survival but also increases the damage and death of normal gastric mucosal cells[4,5]. Thus, inhibition of high levels of autophagy in gastric mucosal cells may be effective in preventing GPL progression. However, there is currently a lack of drugs that can inhibit autophagy in gastric mucosal cells and effectively treat GPL. Moreover, the complex local microenvironment of the GPL gastric mucosa is a favorable condition for activating glycolysis. Many studies have shown that cells in the GPL stage have high levels of glycolysis. Glycolysis accelerates adenosine triphosphate (ATP) production and promotes the proliferation of dysplastic cells in the gastric mucosa and the accumulation of lactate. The end product of this process promotes the formation of an acidic microenvironment in the gastric mucosa which facilitates tumor occurrence and development[6,7]. In summary, the activation of glycolysis and high levels of autophagy may together contribute to GPL deterioration.

Accumulating evidence indicates that natural medicines and their formulations play a unique role in the treatment of digestive diseases. In recent years, the discovery of natural medicines has occupied an important position in the field of new drug development. It is very promising to find safer and more effective natural medicines and foods against GPL. Xiaojianzhong decoction (XJZ), the formula of which comes from the ancient Chinese medical practitioner Zhang Zhongjing's *Treatise on Febrile and Miscellaneous Diseases*, is a classic traditional Chinese medicine compound for the treatment of gastrointestinal diseases and contains six edible herbal medicines: Maltose, cassia twig, paeonia lactiflora, ginger, licorice, and jujube. Studies have found that the main components of these drugs have certain therapeutic effects on the diseased gastric mucosa. For example, cinnamaldehyde and 6-shogaol can inhibit autophagy of gastric mucosal cells, inhibit Hp reproduction, and have anti-inflammatory and analgesic effects[8,9]; paeoniflorin can regulate immune function, act as antioxidants, and inhibits glycolysis[10].

Therefore, in this study, we investigated the therapeutic effects of XJZ on GPL and its underlying mechanisms by constructing a rat GPL model with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), with the aim of assessing the potential of XJZ as a therapeutic drug for GPL.

MATERIALS AND METHODS

Chemicals and reagents

The ingredients-maltose, cassia twig, paeonia lactiflora, ginger, licorice, and jujube-of XJZ were purchased from Xingshengde Pharmaceutical Co., Ltd. (Xi'an, China). MNNG and Tretinoin were purchased from Solarbio Technology Co., Ltd. (Beijing, China). Superoxide dismutase (SOD) and malondialdehyde (MDA) biochemical detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China). Alanine aminotransferase (ALT) and creatinine (Cr) biochemical detection kits were purchased from Changchun Huili Biotechnology Co., Ltd. (Changchun, Jilin Province, China). Caudal-type homeobox protein 2 (CDX-2), Ki-67, p62, Sirtuin 6 (SIRT6), microtubule associated protein 1 Light chain 3 (LC-3), and moesin-like BCL2-interacting protein 1 (Beclin-1) antibodies were purchased from Proteintech Group, Inc (Wuhan, Hubei Province, China). Phosphatidylinositol 3-kimase (PI3K)/p-PI3K, protein kinase B (AKT)/p-AKT, mammalian target of rapamycin (mTOR)/p-mTOR, Unc-51 Like kinase 1 (ULK1)/p-ULK1 Ser-317/p-ULK1 Ser-555, p53/p-p53, and AMP-activated protein kinase (AMPK)/p-AMPK antibodies were purchased from ABCAM (United States). Hypoxia-inducible factor-1α (HIF-1α), B cell lymphoma/leukemia-2 and adenovirus E1B19000 interacting protein 3 (Bnip-3), and CD147 antibodies were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, Hubei Province, China). Trizol and Western blot chemiluminescence reagents were purchased from Ambion Thermo Fisher Scientific CN (Shanghai, China).

Animal model construction and sampling

The male Wistar rats used in this study, which were provided by Beijing Spelford Laboratory Animal Co., Ltd, were housed in specific pathogen free-class animal rooms. All the animal experiments were approved by the Experimental Animal Ethics Committee of Shaanxi University of Chinese Medicine. Every effort was made to reduce the number of animals used and alleviate their suffering. As presented in Figure 1A, routinely fed with water and food. They were acclimatized for 1 wk and then divided into six groups (five rats per group) according to random number method: Control, model (MNNG, 180 mg/L, free to be drunk in the dark; hot ranitidine-salt solution, 2.25 g/L, 40 °C; and 20% ethanol, 2.50 mL/d), XJZ-L (low dose of XJZ, 1.875 g/kg; MNNG, 180 mg/L; hot ranitidine-salt solution, 2.25 g/L, 40 °C; and 20% ethanol 2.50 mL/d), XJZ-M (middle dose of XJZ, 3.75 g/kg; MNNG, 180 mg/L; hot ranitidine-salt solution, 2.25 g/L, 40 °C; 20% ethanol 2.50 mL/d), XJZ-H (high dose of XJZ, 7.50 g/kg; MNNG, 180 mg/L, hot ranitidine-salt solution, 2.25 g/L, 40 °C; and 20% ethanol 2.50 mL/d), and tretinoin (tretinoin, 0.04 g/kg; MNNG, 180 mg/L; hot ranitidine-salt solution, 2.25 g/L, 40 °C; and 20% ethanol, 2.50 mL/d). The model was constructed by the MNNG compound modeling method (MNNG was free to be drunk in the dark, and hot ranitidine-salt solution and 20% ethanol were alternately gavaged) combined with "starvation-satiation alternation". After 18 wk, the control and model groups were gavaged with saline, and the remaining four groups were gavaged with the corresponding concentrations of drugs. After 4 wk of intervention, pentobarbital anesthesia was administered. Blood was collected from the abdominal aorta and centrifuged; next, the serum was extracted and frozen in an ultra-low temperature refrigerator. The whole stomach was cut along the greater curvature, and the tissue at the junction between the gastric body and the gastric antrum was taken. Two pieces of flat gastric tissue were cut. One was fixed in 2.5% glutaraldehyde, and stored at 4 °C for transmission electron microscopy; the other was fixed in 4.0% paraformaldehyde for immunofluorescence, immunohistochemistry, and pathological staining. The remaining tissues were placed in cryovials and frozen with liquid nitrogen for protein and mRNA detection.

High-performance liquid chromatography

First, we collected 9 g of paeonia lactiflora, 9 g of cassia twig, 6 g of licorice, 9 g of ginger, six jujubes (24 g), and 30 g of maltose and added 11.5 times of water. The mixture was soaked for 30 min, decocted for 1.5 h, and then filtered. Next, eight times of water was added to the filter residue, and the mixture was decocted for 1 h and filtered. The filtrates were combined. Maltose (30 g) was added to melt, and the mixture was concentrated to 1. 05 mg/mL of raw drug and then centrifuged. Subsequently, 1 mL of the centrifuge was taken, and methanol was added until the volume was 10 mL. After coarse filtration, the filtrate was passed through a 0.22 μm microporous membrane to obtain the injection solution. Chromatographic column: Acuity-I-Class UPLC-BEH C18 column (2.1 mm × 100.0 mm, 1.7 μm); mobile phase: Acetonitrile (A)-0.1% phosphoric acid water; volume flow rate: 0.2 mL/min; column temperature: 30 °C; and injection volume: 2 μL.

Body weight monitoring

Each rat was weighed every 16 d. At each measurement, the rats were weighed several times until stable weight values were obtained continuously to ensure accurate monitoring results.

Histopathological analysis

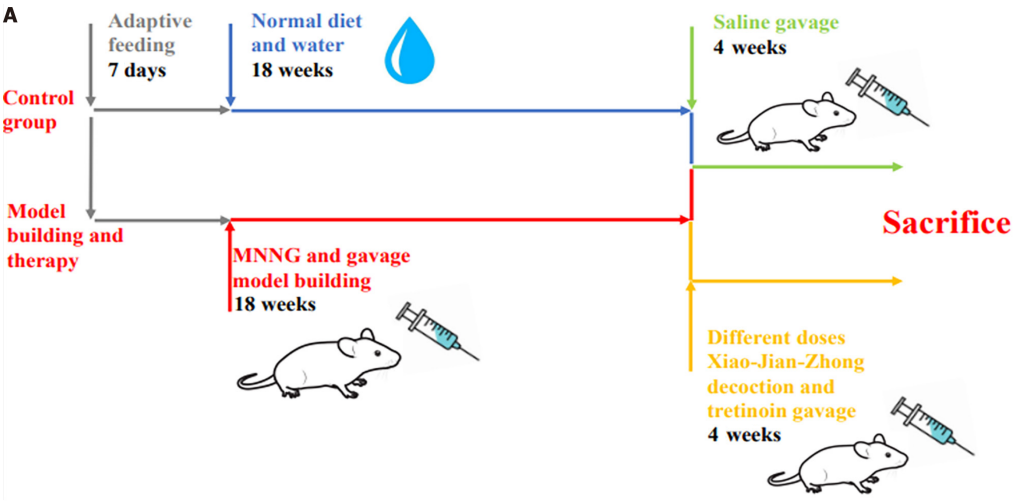
The fixed gastric tissues were dehydrated, paraffin-embedded, and sectioned into 2-μm-thick slices, deparaffinized, and rehydrated using an ethanol gradient. Gastric tissues were stained using hematoxylin-eosin (H&E) and alcian blue-periodic acid-Schiff (AB-PAS) staining. The images were collected using light microscopy.

Serum biochemical test

Serum ALT, Cr, MDA, and SOD were detected using a Rayto automatic biochemical analyzer and a FlexStation3 multimode microplate reader (Molecular Devices) according to the manufacturers' instructions.

Transmission electron microscopy

After the gastric tissues were fixed in glutaraldehyde for 3-4 h, the cells were collected by scraping, centrifuged at 1000 rpm to settle the cells, and placed again in 2.5% glutaraldehyde for 4 h at 4 °C. The cells were rinsed three times for 15 min each in 0.1 M phosphate buffer. Next, 1% osmic acid-phosphate buffer was used to fix the cells at room temperature (20 °C) for 2 h and the ethanol gradient was used to dehydrate the cells for 15 min/time. The cells were infiltrated overnight with a mixture of acetone and 812 embedding medium (1:1), followed by pure 812 embedding medium overnight. After embedding and sectioning, the uranium-lead double staining solution was used to stain the cells for 15 min, and the sections were dried at room temperature overnight. Images were observed and acquired under an electron microscope.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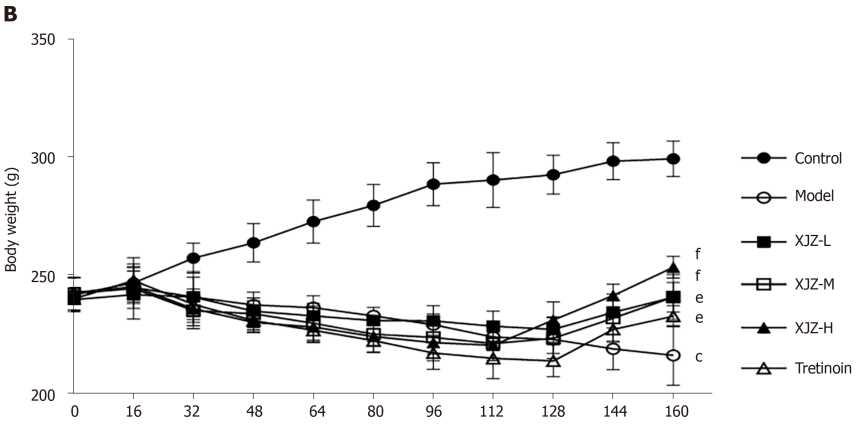
Normal diet and water
18 weeks
Saline gavage
4 weeks
Control group
Model building and therapy
MNNG and gavage model building
18 weeks
Sacrifice
Different doses Xiao-Jian-Zhong decoction and tretinoin gavage
4 weeks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32
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XJZ-M
XJZ-H
Tretinoin
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f
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e
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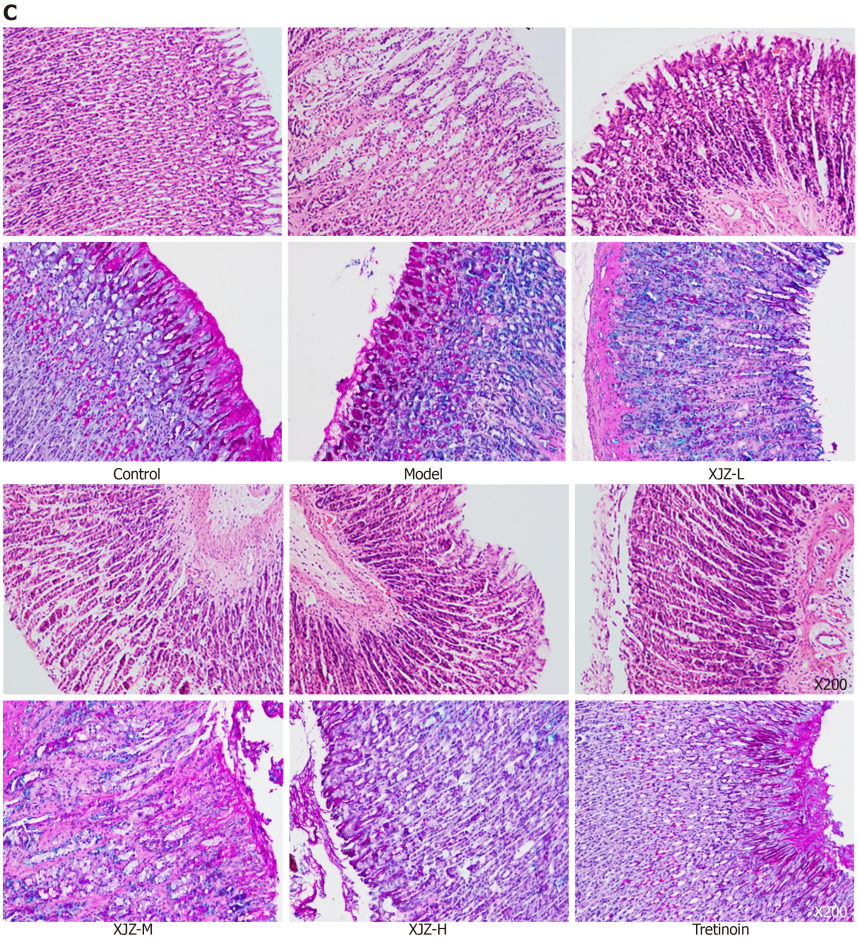
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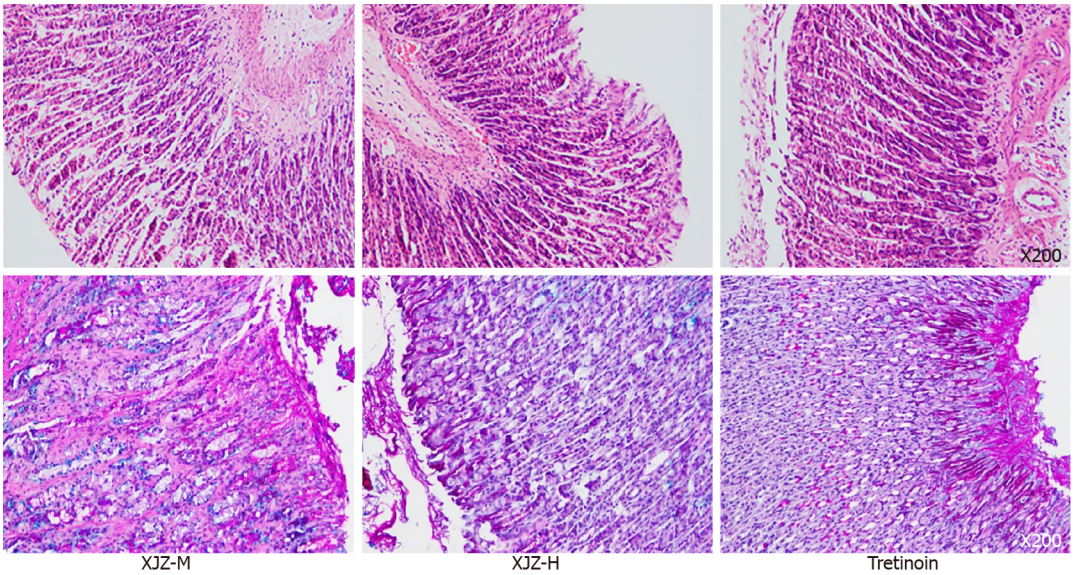
Tretinoin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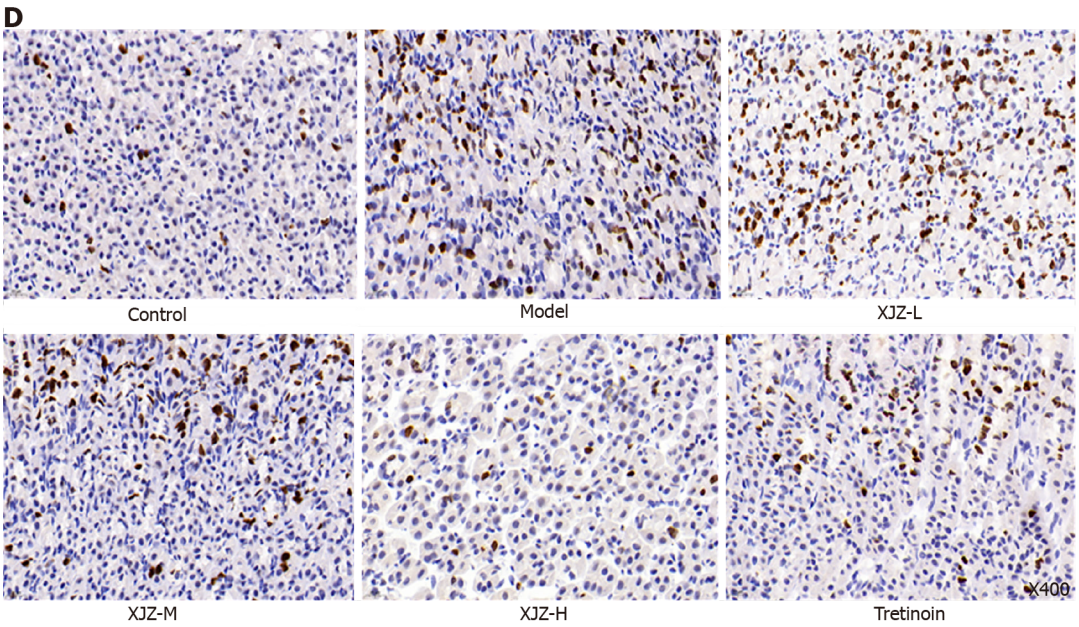
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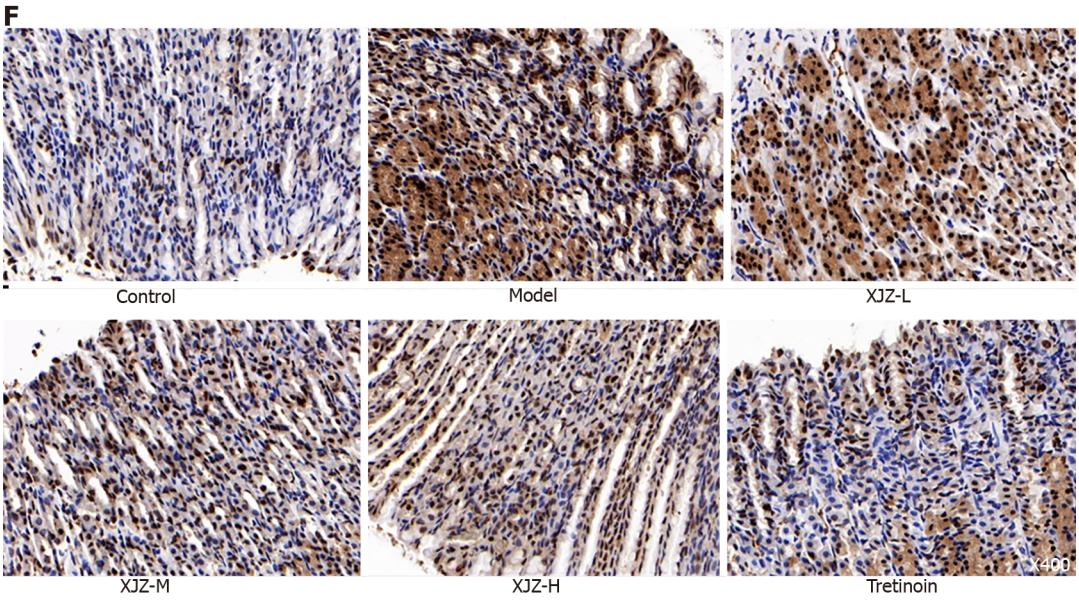
Tretinoin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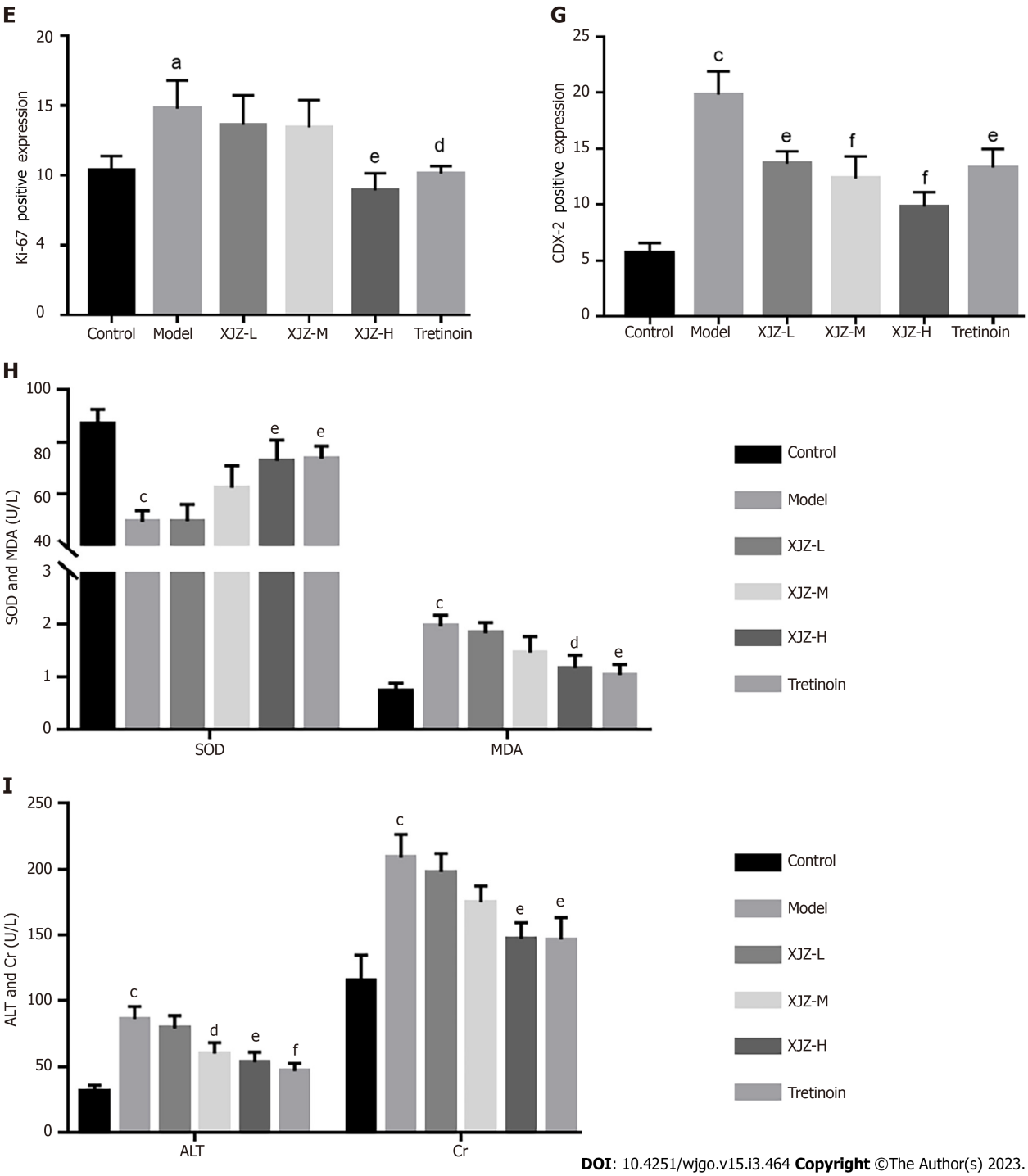

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Figure 1 Effects of Xiaojianzhong decoction on body weight, gastric mucosal pathological conditions and liver kidney toxicity in N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric precancerous lesions rats. A: Schematic diagram of model construction and treatment; B: Schematic diagram of weight change in rats; C: Representative images of hematoxylin-eosin and alcian blue-periodic acid-Schiff staining of gastric tissues; D-G: Ki-67 and caudal-type homeobox protein 2 (CDX-2) expression was determined using immunohistochemistry (n = 5); Ki-67 and CDX-2 positive score was determined using Image J; H: Superoxide dismutase and malondialdehyde levels in serum (n = 3); I: Alanine aminotransferase and creatinine levels in serum (n = 3). Data are expressed as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control group; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* model group. XJZ-L: Low dose of Xiaojianzhong decoction; XJZ-M: Middle dose of Xiaojianzhong decoction; XJZ-H: High dose of Xiaojianzhong decoction; SOD: Superoxide dismutase; MDA: Malondialdehyde; ALT: Alanine aminotransferase; Cr: Creatinine.

Immunohistochemical analysis

Paraffin sections (3 μm thick) were deparaffinized and then rehydrated with an ethanol gradient. The sections were incubated with 3% H_2O_2 to remove endogenous peroxidase and then incubated with Ki-67, CDX-2, p62, and SIRT6 antibodies (1:100) overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies at 37 °C for 30 min. The sections were then stained with hematoxylin and observed under sealed conditions. Images were collected.

Immunofluorescence analysis

Gastric tissue sections were deparaffinized and subjected to antigen retrieval and serum blocking. The sections were then incubated with HIF-1α, Bnip-3, Beclin-1, and CD147 antibodies (1:100) overnight at 4 °C, rinsed with PBST, incubated with diluted fluorescent secondary antibodies for 1 h at 37 °C, and rinsed with PBST. After that, the sections were incubated with DAPI in the dark to stain the nuclei, and the excess DAPI was washed off by PBST. The sections were sealed with a mounting solution containing an anti-fluorescence quencher, and images were collected using a laser confocal fluorescence microscope.

Western blot analysis

Gastric tissue samples were placed in a 2-mL EP tube and lysed and homogenized with 200 μL of lysis buffer. After being fully lysed on ice, the lysate was transferred to a centrifuge tube and centrifuged at 4 °C and 12000 rpm for 5 min, and the supernatant was separated into centrifuge tubes and placed at -20 °C. The protein samples and standard proteins diluted with PBS were added to 96-well plates and incubated for 15 min at 37 °C in the dark. The protein supernatant and buffer were incubated in a boiling water bath. The prepared protein samples and MAKER were added to the loading wells for electrophoresis blocking and membrane transfer. Next, 5% skim milk powder was added and blocked the membrane at room temperature on a shaker for 2 h. The PVDF membrane was then mixed with antibodies against HIF-1α (1:4000), Bnip-3 (1:1000), LC-3 (1:4000), Beclin-1 (1:1000), p-PI3K (1:1000), and PI3K (1:1000), p-AKT (1:3000), AKT (1:2000), p-mTOR (1:5000), mTOR (1:10000), p-p53 (1:1000), p53 (1:1000), p-AMPK (1:1000), AMPK (1:2000), p-ULK1 ser317 (1:1000), p-ULK1 ser555 (1:1000), ULK1 (1:1000), β-actin (1:1000), and Lamin B (1:1000) for overnight incubation at 4 °C. The PVDF membranes were washed well with TBST five times and incubated with secondary antibodies for 2 h. Protein bands were visualized using an ELC reagent followed by exposure on X-ray film (Supplementary material).

Reverse transcription-polymerase chain reaction analysis

The gastric tissue was added with 1 mL of Trizol reagent, ground into a pulp with a homogenizer, and transferred to an RNase-free 1.5-mL EP tube for lysis for 10 min. Next, 200 μL of chloroform was added and mixed well; the mixture was placed at room temperature for 5 min and centrifuged at 4 °C and 12000 rpm for 8 min. The upper aqueous phase was transferred to a new 1.5 mL EP tube, and 400 μL of isopropanol was added. The mixture was mixed well, placed at room temperature for 10 min, and centrifuged at 4 °C and 12000 rpm for 10 min. The supernatant was discarded and 1 mL of RNase-free 75% ethanol was added. The mixture was vortexed and mixed well, followed by centrifugation at 10000 rpm for 5 min at 4 °C. The supernatant was discarded, the RNA precipitate was dried for 5-10 min, and the precipitate was dissolved in 20 μL of DEPC water. Then, 2 μL of dissolved RNA was taken, and the purity and concentration of RNA were calculated using a microspectrophotometer (see Table 1 for primer sequences).

Statistical analysis

All quantitative data are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software Inc., San Diego, CA, United States). Comparisons between the groups were performed using a one-way analysis of variance, whereas multiple comparisons were performed using LSD. Statistical significance was set at $P < 0.05$, $P < 0.01$, or $P < 0.001$.

RESULTS

Chemical profiling of the constituents in XJZ

We analyzed the main components of XJZ using UPLC-Q-Orbitrap HRMS and successfully identified the main components of XJZ, which is recommended by the Chinese Pharmacopeia 2020. The results showed that XJZ samples contained abundant chromatographic peaks (Figure 2A), indicating the presence of a large number of natural constituents, including albiflorin, paeoniflorin, liquiritin, cinnamaldehyde, and 6-gingerol (Figure 2B-F).

XJZ prevents MNNG-induced GPL progression in rats

MNNG is typically used to construct a GPL animal model because it has the advantages of high stability and easy administration[11]. In this experiment, GPL was successfully induced in rats by a compound modeling method of drinking MNNG freely in the dark + 20% ethanol/hot saline-ranitidine by alternating gavage + alternating "satiation-starvation" (Figure 1A). After 4 wk of treatment, we observed that XJZ increased the body weight of rats in a time- and dose-dependent manner (Figure 1B), ameliorated the morphological abnormalities of gastric mucosa in GPL rats in a dose-dependent manner, promoted the restoration of the integrity and regularity of the gastric mucosal gland structures and reduced the secretion of IM acidic mucus in the gastric mucosa (Figure 1C). In addition to the disorder of glandular structure and the secretion of acidic mucus, the gastric mucosa of GPL also shows

Table 1 Primer pair sequences

Gene	Primer	Sequence, 5'-3'	PCR products
B-actin	Forward	CACGATGGAGGGGCCGGACTCATC	240 bp
	Reverse	TAAAGACCTCTATGCCAACACAGT	
Rat PI3K	Forward	GCAACAAGTCCTCTGCCAAA	222 bp
	Reverse	ACGTAATAGAGGAGCTGGGC	
Rat AKT	Forward	GCTCTTCTTCCACCTGTCTCG	186 bp
	Reverse	CACAGCCCGAAGTCCGTTA	
Rat mTOR	Forward	GAGATACGCCGTCATTCCT	199 bp
	Reverse	ATGCTCAAACACCTCCACC	
Rat HIF-1α	Forward	GCGGCGAGAACGAGAAGAAAAATAG	129 bp
	Reverse	GAAGTGGCAACTGATGAGCAAG	
Rat MCT1	Forward	TGTATGCCGGAGGTCCTATC	177 bp
	Reverse	AGTTGAAAGCAAGCCCAAGA	
Rat MCT4	Forward	ACGGCAGGTTTCATAACAGG	162 bp
	Reverse	GCCATACGAGATCCCAAAGA	
Rat Bnip-3	Forward	GGTCAAGTCGGCCAGAAAAT	207 bp
	Reverse	TTGTCAGACGCCTTCCAATG	
Rat LC3	Forward	AAAATGGGGCACGGATGAAG	155 bp
	Reverse	GCAGGTCTTCAAAATGCCCA	
Rat CDX-2	Forward	TCTCCGAGAGGCAGGTTAAA	186 bp
	Reverse	GCAAGGAGGTCACAGGACTC	
Homo ULK1	Forward	TTATCATGTCCCAGCACTACGAT	242 bp
	Reverse	AAATTCATCAAAGTCCATGCGGT	
Rat CD147	Forward	GGCACCATCGTAACCTCTGT	211 bp
	Reverse	CAGGCTCAGGAAGGAAGATG	
Rat Beclin-1	Forward	GAGGTACCGACTTGTTCCCT	225 bp
	Reverse	CCTTTCTCCACGTCCATCCT	

AKT: Protein kinase B; Beclin-1: Moesin-like BCL2-interacting protein 1; Bnip-3: B cell lymphoma/Leukemia-2 and adenovirus E1B19000 interacting protein 3; HIF-1α: Hypoxia-inducible factor-1α; LC-3: Microtubule associated protein 1 light chain 3; PCR: Polymerase chain reaction; PI3K: Phosphatidylinositol 3-kimase; MCT: Monocarboxylate transporter; mTOR: Mammalian target of rapamycin signaling pathway; ULK1: Unc-51 like kinase 1.

the positive expression of related markers (Figure 1D-G). We found that XJZ decreased the positive expression of Dys and IM markers Ki-67 and CDX-2 in gastric mucosa tissues in a dose-dependent manner (Figure 1D-G).

We next investigated the protective effect of XJZ on the gastric mucosa and its effect on the liver and kidney. The results revealed that XJZ reduced serum MDA levels and increased SOD levels in rats, as well as reduced the levels of ALT and Cr, which are serum markers of liver and kidney injury (Figure 1H and I). These results indicate that XJZ can improve the general condition of rats, inhibit IM and Dys, has an antioxidant damage effect, has some protective effects on gastric mucosa, and has little effect on the liver and kidney of rats, making it an ideal drug for inhibiting GPL.

XJZ inhibits autophagy of gastric mucosal cells in GPL rats

As a biological process throughout GPL, autophagy maintains the survival of IM and Dys cells and destroys normal gastric mucosal cells. These effects promote GPL progression. Therefore, we examined autophagy levels in the gastric mucosal cells of rats. Transmission electron microscopy revealed that the formation of crescent-shaped autophagosomes, double-membrane autophagic vesicles, and the number of autolysosomes in the gastric mucosal tissue in the model group were significantly increased

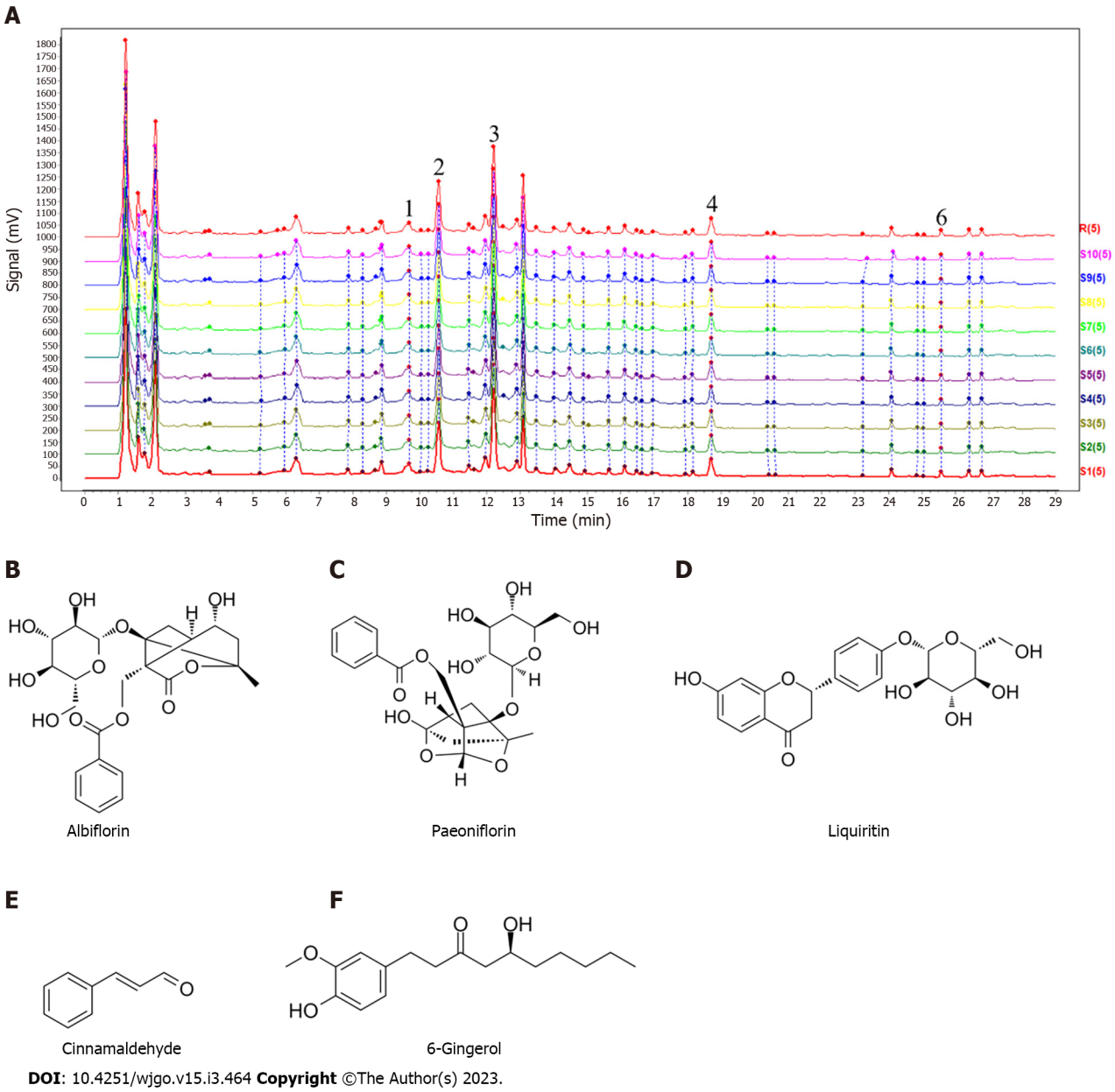

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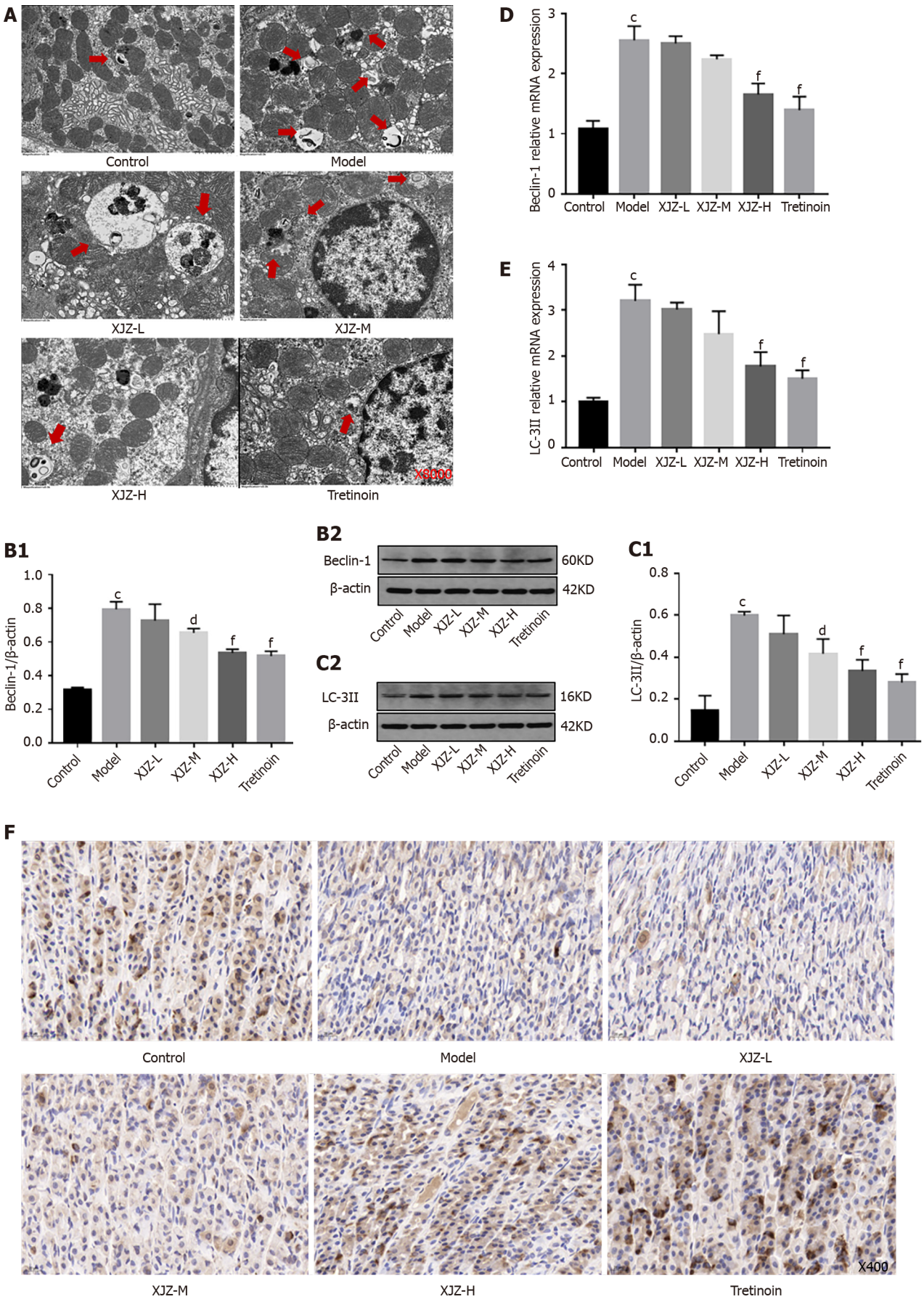
Figure 2 Result of the high-performance liquid chromatography method coupled with triple-quadrupole tandem mass spectrometry assays of the chemical composition of Xiaojianzhong decoction. A: High-performance liquid chromatography chromatogram; B-F: Compounds contained in Xiaojianzhong decoction.

compared with those in the control group, whereas XJZ reduced the formation of all the above autophagy-related structures in the gastric mucosal tissue (Figure 3A). Furthermore, we detected the related proteins involved in autophagy. The results showed that XJZ decreased Beclin-1 and LC-3II protein expressions and transcript levels in a dose-dependent manner (Figure 3B-E), increased p62 expression in gastric mucosal tissues (brown) (Figure 3F and G), and reduced the co-expression of Bnip-3 and Beclin-1 in the gastric mucosa (Figure 3H-J). These findings indicate that XJZ can inhibit autophagosome formation and autophagy level in GPL gastric mucosal tissues.

XJZ promotes the activation of the PI3K/AKT/mTOR signaling pathway to inhibit autophagy

The PI3K/AKT/mTOR signaling pathway is a classical pathway that regulates autophagy, and its inhibition activates autophagy[12]. We found that XJZ promoted PI3K, AKT, mTOR protein phosphorylation, and mRNA expressions in a dose-dependent manner (Figure 4), suggesting that it inhibits autophagy by activating the PI3K/AKT/mTOR signaling pathway. However, autophagy is also regulated by other mechanisms; for example, hypoxia is an important trigger of autophagy. Many studies have confirmed that the inflammatory state of the GPL gastric mucosa increases cellular oxygen consumption and creates a hypoxic microenvironment. Therefore, we explored the effect of XJZ on gastric mucosal hypoxia.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16KD
42KD
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LC-3II/β-actin
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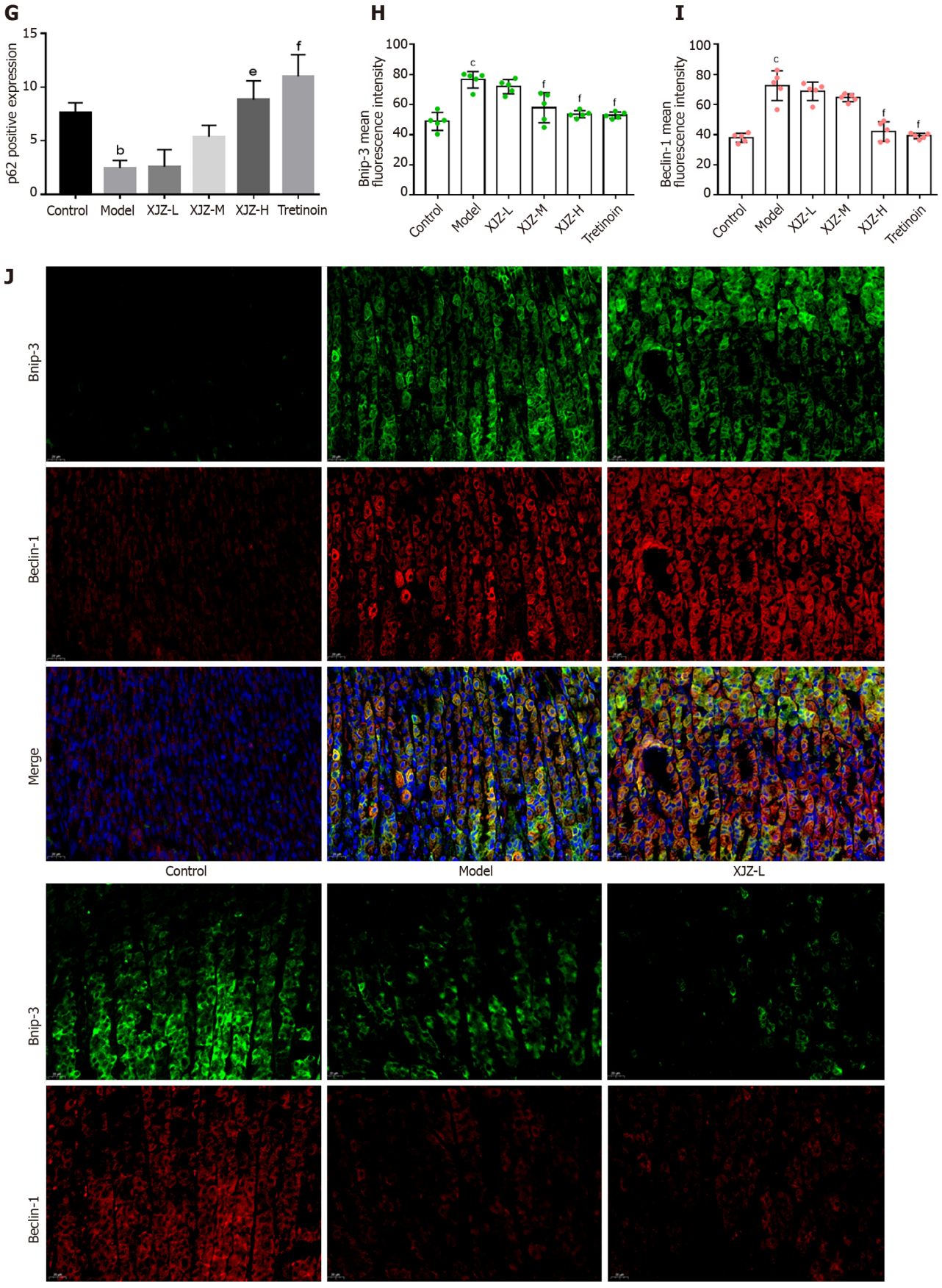
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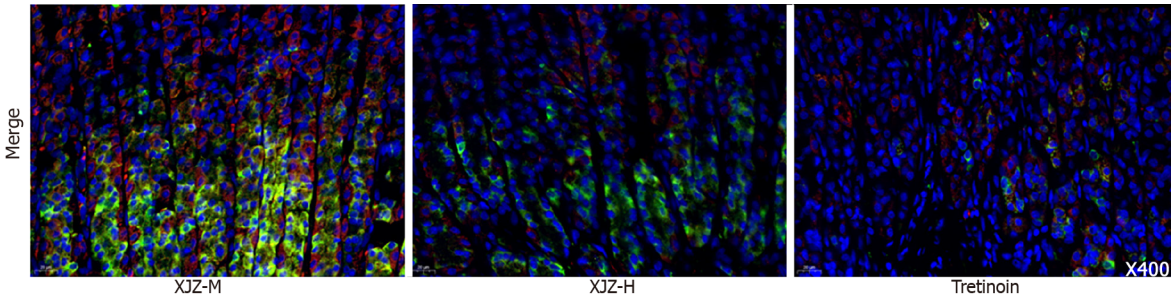

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Figure 3 Effects of Xiaojianzhong decoction on autophagy of gastric mucosal epithelial cells in N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric precancerous lesions rats. A: Observation of autophagy in rat gastric epithelial cells by transmission electron microscope (the red arrows in the figure are autophagosomes or autophagolysosomes); B and C: Western blot analysis was performed to detect BCL2-interacting protein 1 (Beclin-1), microtubule associated protein 1 light chain 3 (LC-3) II protein expression (n = 3); D and E: Beclin-1, LC-3II mRNA expression was determined using real-time polymerase chain reaction analysis (n = 3); F and G: p62 expression was determined using immunohistochemistry (n = 5); p62 positive score was determined using Image J; H-J: B cell lymphoma/leukemia-2 and adenovirus E1B19000 interacting protein 3 (Bnip-3), Beclin-1 proteins expression was determined using immunofluorescence (n = 5); Bnip-3, Beclin-1 porteins positive score was determined using ImageJ. Data are expressed as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control group; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* model group. XJZ-L: Low dose of Xiaojianzhong decoction; XJZ-M: Middle dose of Xiaojianzhong decoction; XJZ-H: High dose of Xiaojianzhong decoction; Beclin-1: BCL2-interacting protein 1; LC-3: Microtubule associated protein 1 light chain 3.

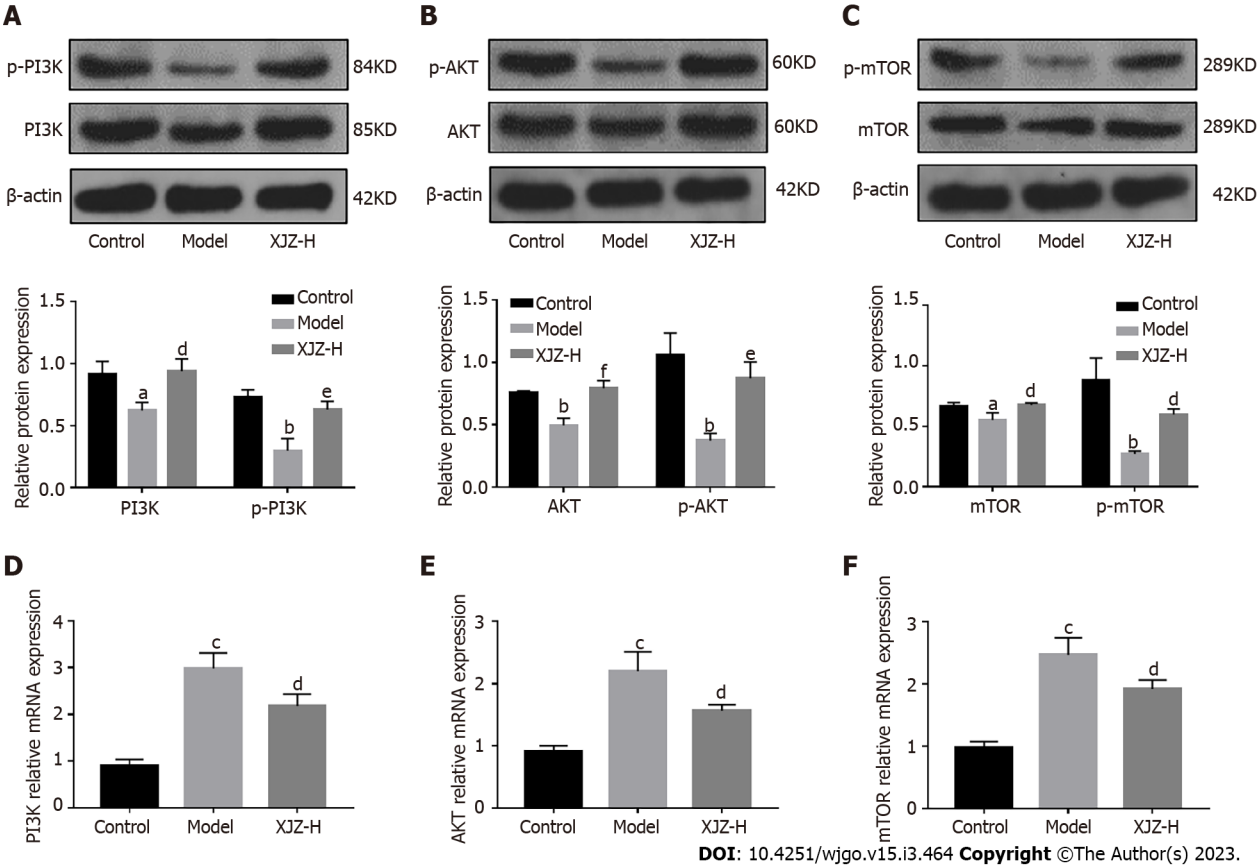

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Figure 4 Xiaojianzhong decoction up-regulates phosphatidylinositol 3-kimase/protein kinase B/mammalian target of rapamycin signaling pathway in N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric precancerous lesions rats. A-C: Western blot analysis was performed to detect phosphorylated-phosphatidylinositol 3-kimase (p-PI3K), phosphorylated-protein kinase B (p-AKT), and phosphorylated-mammalian target of rapamycin (p-mTOR) protein expression (n = 3); D-F: PI3K, AKT, and mTOR mRNA expression was determined using real-time polymerase chain reaction analysis (n = 3). Data are expressed as mean ± standard deviation. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control group; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* model group. XJZ-L: Low dose of Xiaojianzhong decoction; XJZ-M: Middle dose of Xiaojianzhong decoction; XJZ-H: High dose of Xiaojianzhong decoction.

XJZ ameliorates gastric mucosal hypoxia to inhibit autophagy and glycolysis in GPL rats

The hypoxic microenvironment of GPL gastric mucosa promotes the stable expression of HIF-1α, a marker of tissue hypoxia, and HIF-1α can specifically induce autophagy under hypoxia[13]. To investigate whether XJZ could inhibit autophagy by improving gastric mucosal hypoxia, we examined

the activation of HIF-1α and related autophagic pathways. The results revealed that XJZ dose-dependently inhibited HIF-1α and Bnip-3 protein and mRNA expressions (Figure 5A-D) and reduced the co-expression of HIF-1α and Bnip-3 protein in the gastric mucosa (Figure 5E-G). Thus, XJZ can ameliorate hypoxia of gastric mucosa in the GPLs and inhibit the activation of the HIF-1α/Bnip-3 signaling pathway, thereby decreasing autophagy.

In addition to regulating autophagy, hypoxia also causes abnormal glucose metabolism. HIF-1α can directly promote the transcription of glycolytic target genes and change the metabolic mode from aerobic oxidative phosphorylation to anaerobic glycolysis, thereby accelerating the cellular energy supply and promoting dysplastic cell proliferation, and GPL deterioration[14]. We observed that XJZ dose-dependently reduced the co-expression of HIF-1α and CD147 (Figure 6A-C), which assists in lactate transport, in the rat gastric mucosa and inhibited the mRNA transcription of Monocarboxylate transporter (MCT1), MCT4, and CD147 (Figure 6D-F). Immunohistochemistry results revealed that XJZ promoted the expression of glycolysis co-repressor SIRT6 in the gastric mucosa, while the structure of gastric mucosal glands returned to normal (Figure 6G and H). Therefore, gastric mucosal hypoxia and HIF-1α expression, as important factors of glycolysis initiation, may be regulated by XJZ, thereby inhibiting glycolysis and preventing GPL progression.

XJZ inhibits ULK1 activation to improve abnormal glucose metabolism and autophagy

To further elucidate the underlying mechanism of glycolysis inhibition by XJZ, we next investigated the effect of XJZ on the activation of ULK1, a signaling molecule involved in initiating glycolysis. Our experiments revealed that XJZ effectively inhibited the transcriptional level and expression of ULK1 in gastric mucosa (Figure 7A-C), suggesting that the improvement of glucose metabolism by XJZ may be related to ULK1 inhibition.

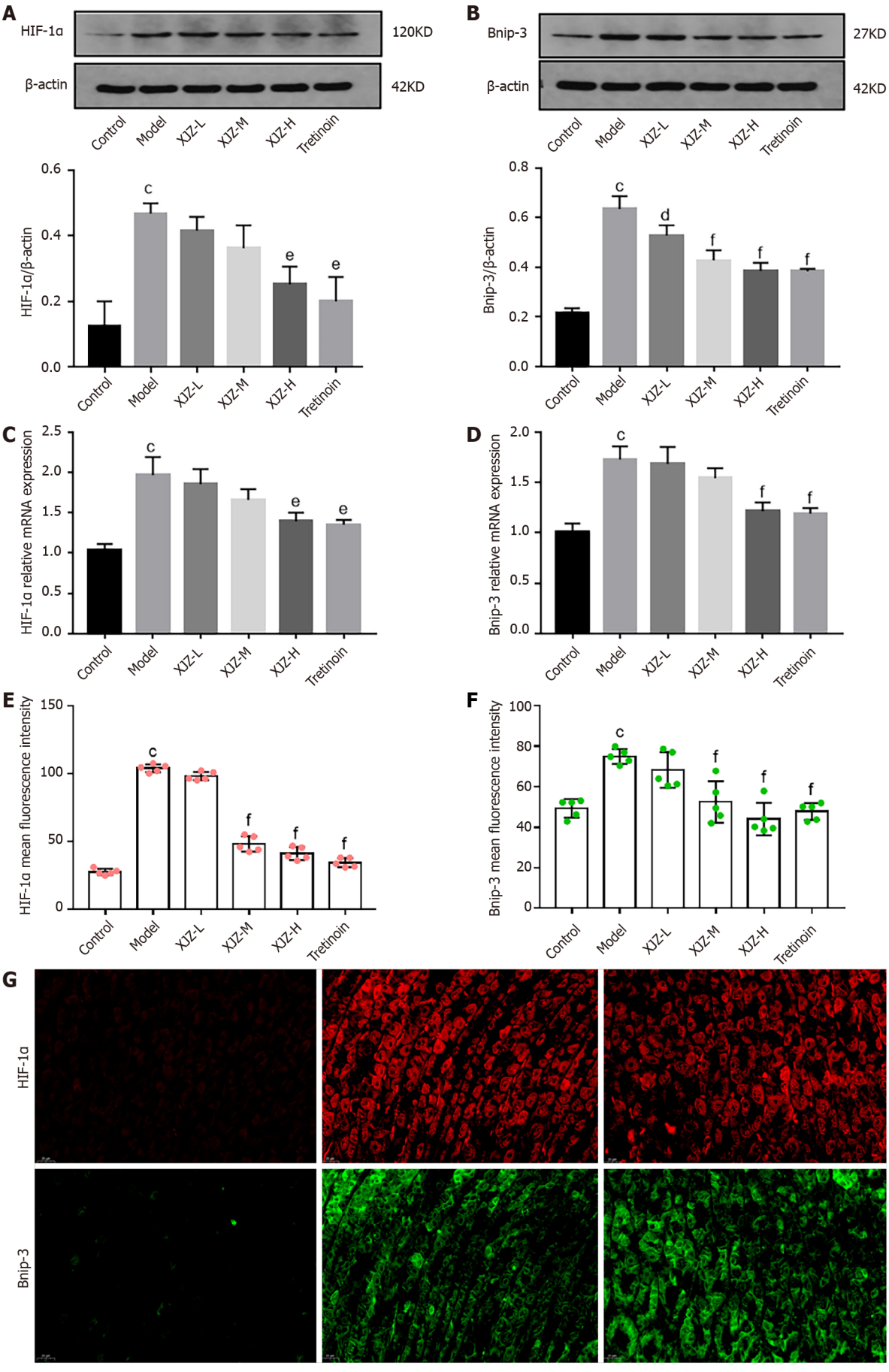
Notably, in addition to affecting glycolysis, ULK1 activation also promotes autophagy initiation. The p53 nucleoprotein upstream of ULK1 promotes the phosphorylation of the ULK1 Ser-317 and Ser-555 sites by activating AMPK, thus activating autophagy[15,16]. We next examined the phosphorylation levels of ULK1 autophagy-related sites and the p53/AMPK signaling pathway. The results showed that XJZ inhibited the phosphorylation of p53 nucleoprotein, AMPK, and ULK1 Ser-317 and Ser-555 sites in a dose-dependent manner (Figure 7D-G) and reduced p53 expression in gastric mucosa (Figure 7H-I). The above results suggest that XJZ may also inhibit autophagy by regulating the phosphorylation levels of the p53/AMPK pathway and ULK1 autophagy-related sites (Figure 8).

DISCUSSION

GPL is inevitable for the occurrence and development of GC. In the past few decades, the incidence of GPLs has been increasing worldwide, predominantly in East Asia, South America, and Central Europe [17,18]. Although the importance of preventing and treating GPL has been clarified, many patients are unable to receive timely and effective treatment, eventually leading to GC. In this study, we reported that the traditional Chinese herbal medicine XJZ has a preventive and therapeutic effect on GPLs and that the possible underlying mechanism is related to inhibiting autophagy and improving the hypoxic environment of gastric mucosa and abnormal glucose metabolism.

When GPL occurs, normal gastric mucosal cells are gradually replaced by IM and Dys cells, a large amount of acidic mucus is secreted, and CDX-2 and Ki-67 are abnormally expressed[19,20]. CDX-2 is a nuclear transcription factor that is mainly expressed in the small intestine and colon. When it is ectopically expressed in the gastric mucosa, it marks the occurrence of IM[21]. Ki-67 is closely related to cell proliferation and ribosome transcription and is often used to assess the level of tumor proliferation. If the gastric mucosa expresses Ki-67, it indicates the occurrence of Dys[22]. XJZ improved the general condition of GPL rats, reduced the degree of gastric mucosal injury and pathology, inhibited CDX-2 and Ki-67 expressions in gastric mucosa, adjusted serum MDA and SOD levels associated with oxidative stress injury, and protected the gastric mucosal barrier. All these pathomechanisms may be related to the inhibition of autophagy by XJZ.

Autophagy is an essential and highly conserved biological process that occurs in various human cells. Autophagy is activated during the GPLs by factors such as hypoxia and signaling molecule disorders and affects the pathological process of GPL. However, few studies have analyzed the role of autophagy in GPLs. Autophagy can remove harmful factors from IM and Dys cells through lysosomes. The increase in its level not only maintains the stability of the intracellular environment and cell survival in dysplastic cells but also exacerbates the gastric mucosal injury and genomic disorders by promoting the autophagic death of normal cells[23,24]. Thus, uncontrolled autophagy further promotes GPL deterioration. Bnip-3 binds to and activates LC3, which exists in two forms: LC3-I and LC3-II, and LC3-II is involved in autophagosome formation, together with autophagy-related protein Beclin-1[25]. In addition, LC3-II can bind the autophagy substrate p62 to autophagosomes to promote the formation and degradation of autophagy-lysosomes[26]. This degradation also degrades the "task-completed" p62 to decrease its expression, so the p62 Level can reflect the autophagy level. XJZ can reduce the formation of autophagosomes and autolysosomes, inhibit the protein expressions and transcription levels of Bnip-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XJZ-M
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Tretinoin
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B
Bnip-3
β-actin
27KD
42KD
Bnip-3/β-actin
C
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D
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G
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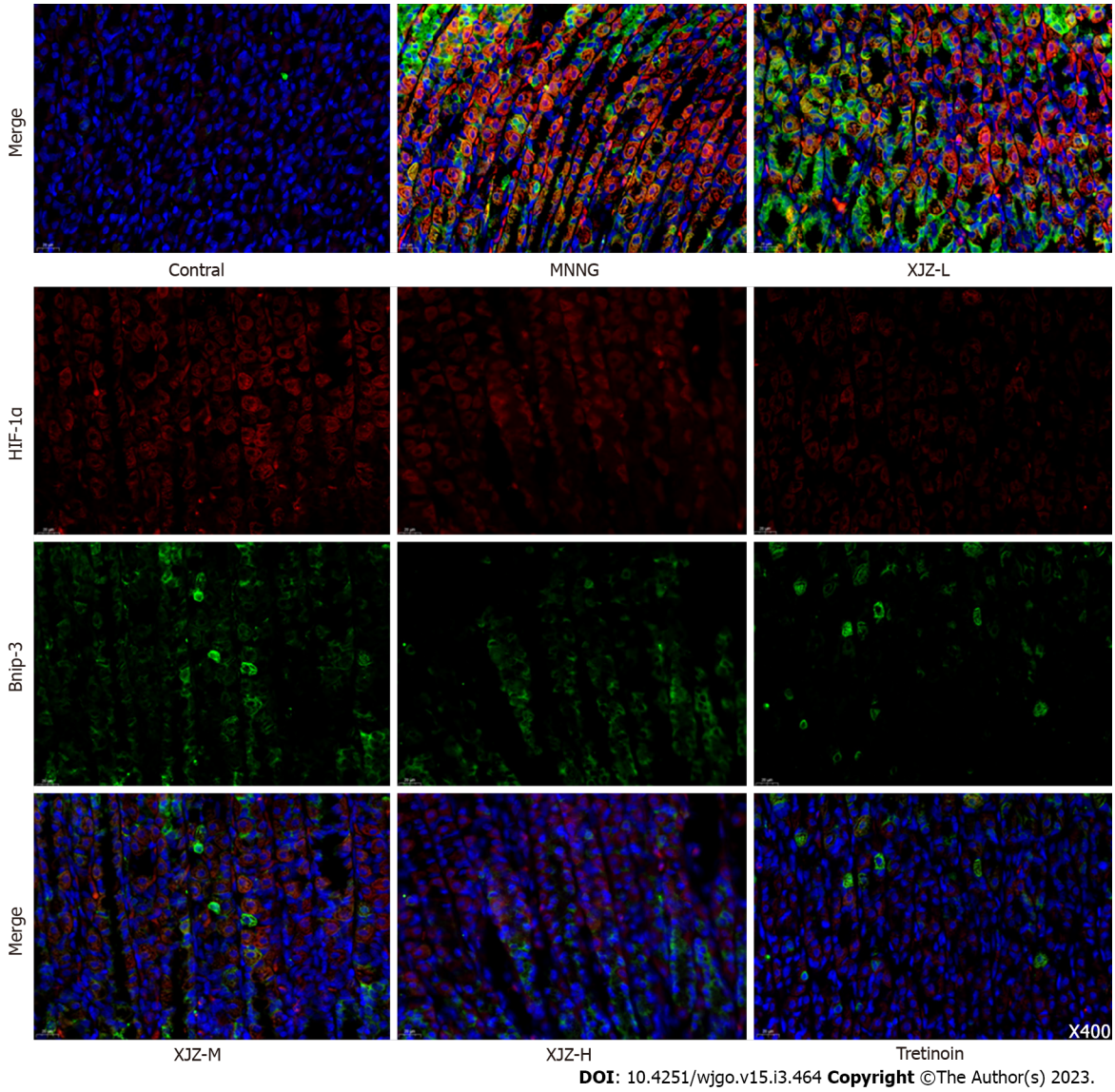

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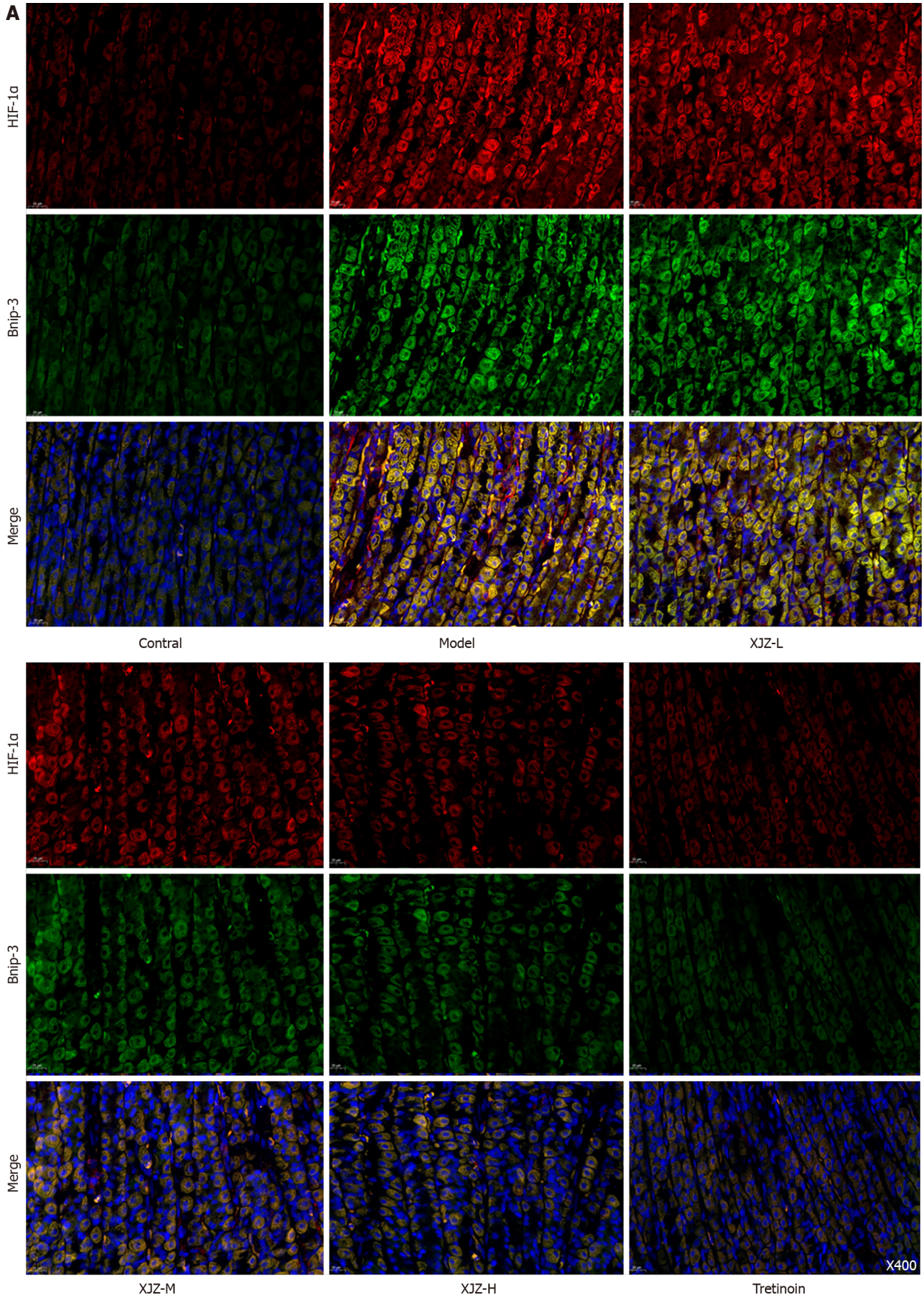
Figure 5 Effects of Xiaojianzhong decoction on gastric mucosal hypoxia in N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric precancerous lesions rats. A and B: Western blot analysis was performed to detect hypoxia-inducible factor 1α (HIF-1α), E1B19000 interacting protein 3 (Bnip-3) protein expression (n = 3); C and D: HIF-1α, Bnip-3 mRNA expression was determined using real-time polymerase chain reaction analysis (n = 3); E-G: Hypoxia-induced autophagy-related protein HIF-1α, Bnip-3 expression was determined using immunofluorescence (n = 5); HIF-1α, Bnip-3 proteins positive score was determined using Image J. Data are expressed as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control group; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* model group. XJZ-L: Low dose of Xiaojianzhong decoction; XJZ-M: Middle dose of Xiaojianzhong decoction; XJZ-H: High dose of Xiaojianzhong decoction; HIF-1α: Hypoxia-inducible factor 1α; Bnip-3: B cell lymphoma/Leukemia-2 and adenovirus E1B19000 interacting protein 3.

3, Beclin-1, and LC-3II; and promote p62 expression in the gastric mucosa of rats with GPL, suggesting that XJZ has a rather comprehensive inhibitory effect on autophagy.

The PI3K/Akt/mTOR pathway is a classical autophagy regulatory pathway. PI3K activation promotes AKT activation, which further binds AKT to the TSC1/2 complex to activate mTOR. The mTOR can form two complexes, mTORC1 and mTORC2. The mTORC1 promotes the phosphorylation of the ribosomal protein P70S6K, and the phosphorylated P70S6K, in turn, activates mTOR to downregulate the expression of ULK1, which is involved in the initiation of autophagy, thereby inhibiting autophagy[27,28]. XJZ can increase the protein phosphorylation and transcription levels of PI3K, Akt, and mTOR in the gastric mucosa, which may be one of the potential mechanisms by which XJZ inhibits autophagy. In addition to the PI3K/Akt/mTOR pathway, hypoxia, a characteristic state of GPL gastric mucosa, also affected the initiation of autophagy.

The gastric mucosa of GPL is filled with a large number of inflammatory cells and inflammatory factors, and their high metabolic levels increase oxygen consumption, leading to gastric mucosal hypoxia and promoting the expression of the hypoxia adaptation regulator HIF-1α[29]. HIF-1α can bind to the autophagy-associated protein Bnip-3 through the hormone response element (HRE) binding domain to initiate autophagy[30], so the regulation of HIF-1α is crucial for inhibiting autophagy. Several studies have demonstrated that HIF-1α is activated in the hypoxic microenvironment of GPL[31,32],


A
HIF-1α
Bnip-3
Merge
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HIF-1α
Bnip-3
Merge
XJZ-M
XJZ-H
Tretinoin
X400

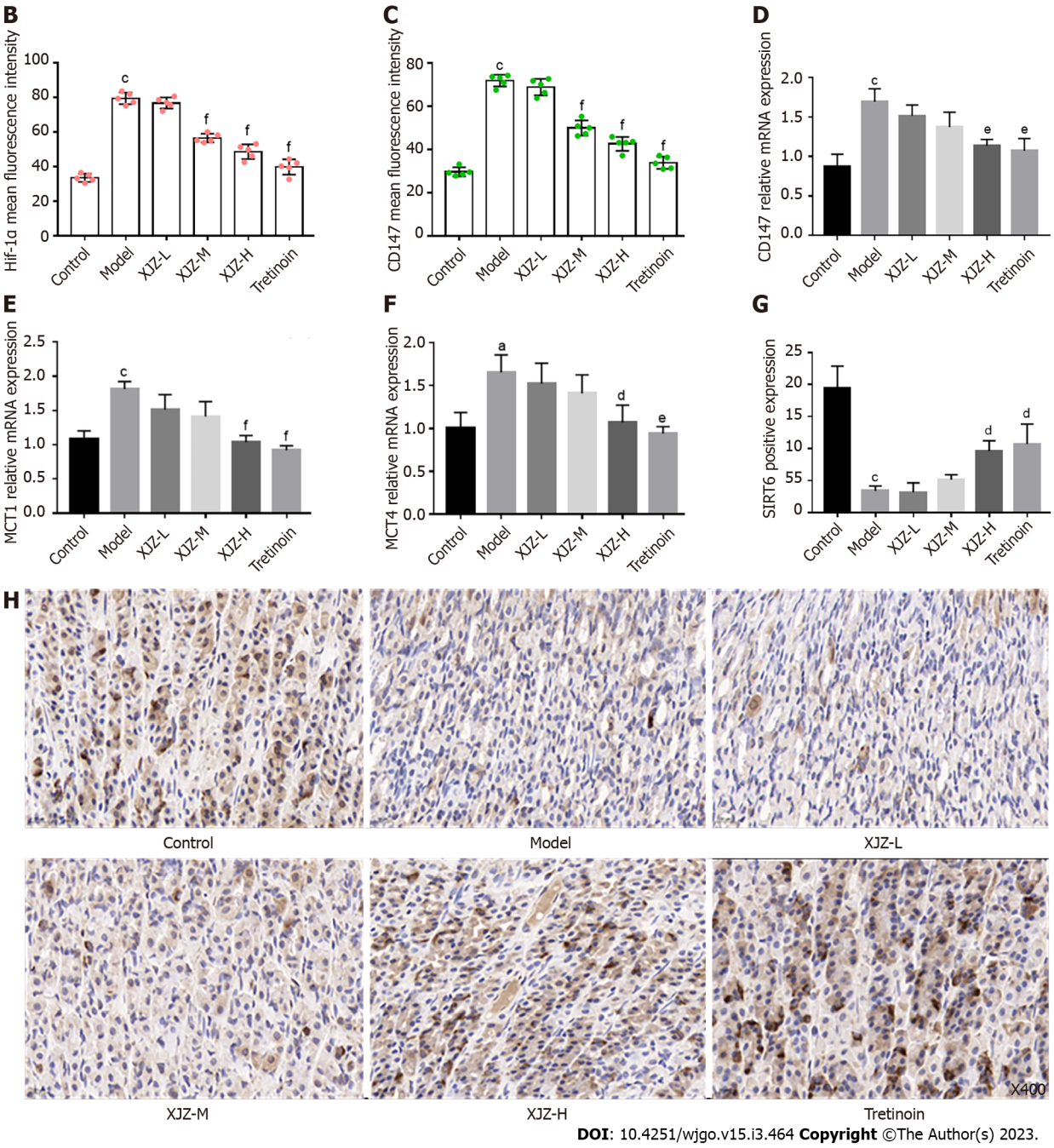
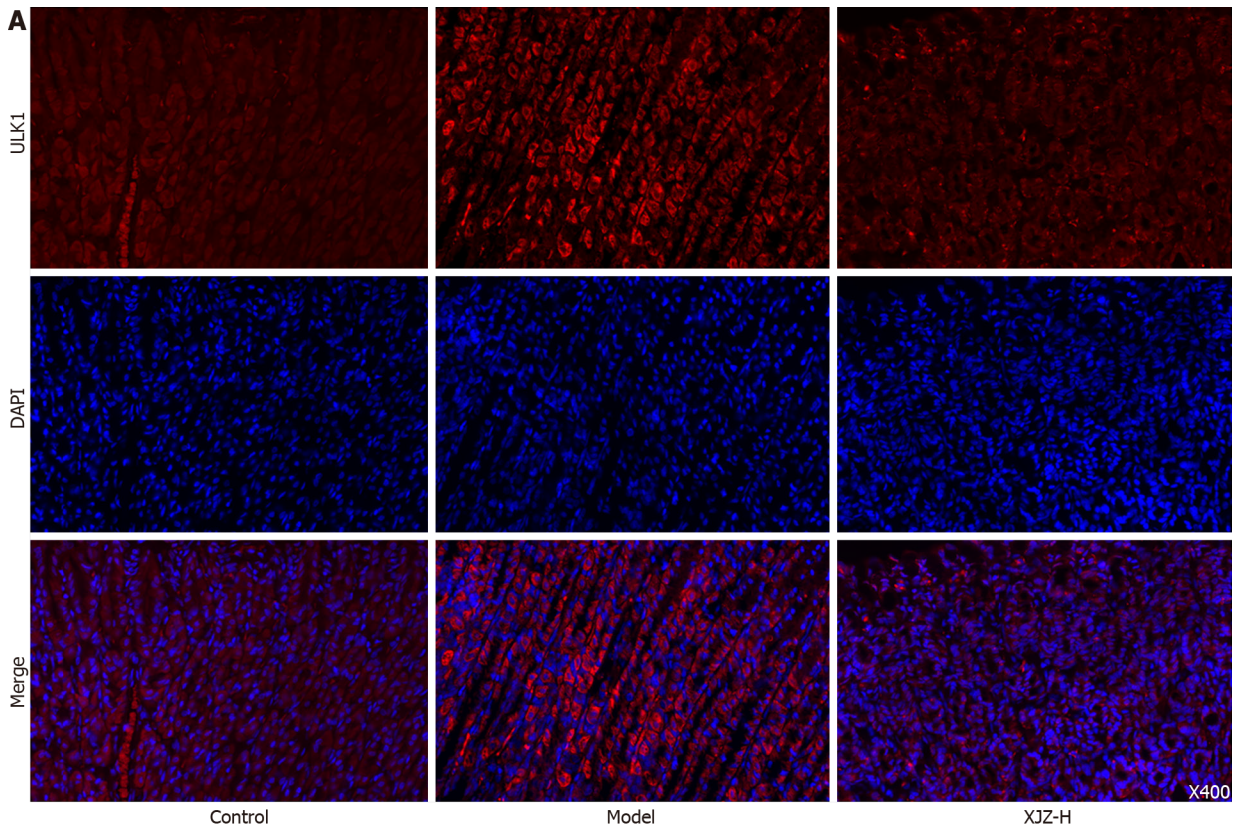


Figure 6 Effects of Xiaojianzhong decoction on hypoxia-induced glycolysis in gastric mucosal epithelial cells. A-C: Hypoxia-induced glycolysis-related protein hypoxia-inducible factor 1α (HIF-1α), CD147 expression was determined using immunofluorescence (n = 5); HIF-1α, CD147 proteins positive score was determined using Image J; D-F: CD147, monocarboxylate transporter (MCT1), and MCT4 mRNA expression was determined using real-time polymerase chain reaction analysis (n = 3); G and H: Sirtuin 6 (SIRT6) expression was determined using immunohistochemistry (n = 5); SIRT-6 positive score was determined using Image J. Data are expressed as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control group; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* model group. XJZ-L: Low dose of Xiaojianzhong decoction; XJZ-M: Middle dose of Xiaojianzhong decoction; XJZ-H: High dose of Xiaojianzhong decoction; HIF-1α: Hypoxia-inducible factor 1α; MCT: Monocarboxylat transporter.

consistent with our results. The increase in HIF-1α protein and transcript levels in the gastric mucosa of rats in the MNNG group indicated that the gastric mucosa was in a hypoxic state. After the intervention with XJZ, the protein expressions and transcription levels of HIF-1α and Bnip-3, as well as their co-expression in the gastric mucosa were reduced, suggesting that XJZ can improve gastric mucosal hypoxia and may inhibit autophagy initiation for this reason.

Interestingly, we observed that XJZ also improved abnormal glucose metabolism in GPL gastric mucosal cells by inhibiting the expression of HIF-1α. In addition to the effect on autophagy, HIF-1α also promotes the shift of cellular energy metabolism from oxidative phosphorylation to glycolysis. Glycolysis often occurs in a hypoxic state, and the stable expression of HIF-1α promotes the transcription of glycolytic target genes, which eventually generate ATP and lactate through a series of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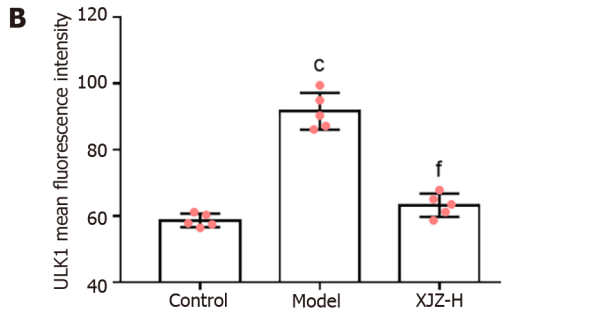
XJZ-H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ULK1 mean fluorescence intensity
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100
80
60
40
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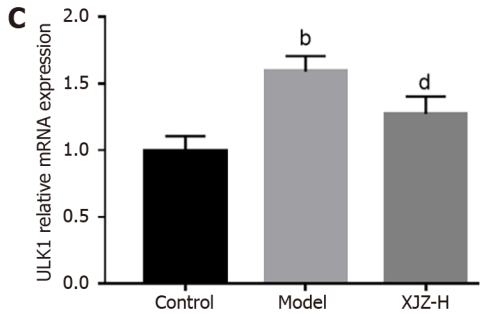
f
Control
Model
XJZ-H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ULK1 relative mRNA expression
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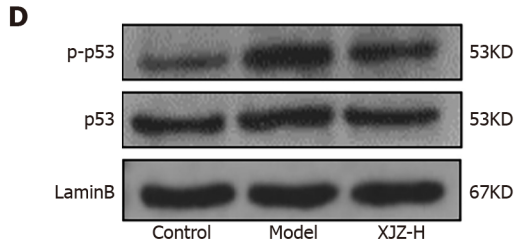
1.5
1.0
0.5
0.0
b
d
Control
Model
XJZ-H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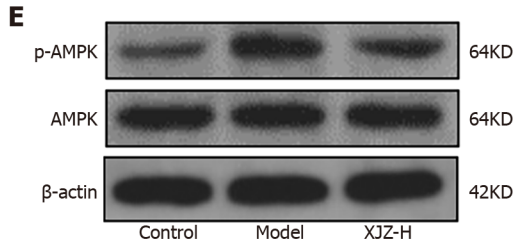
p53
LaminB
53KD
53KD
67KD
Control
Model
XJZ-H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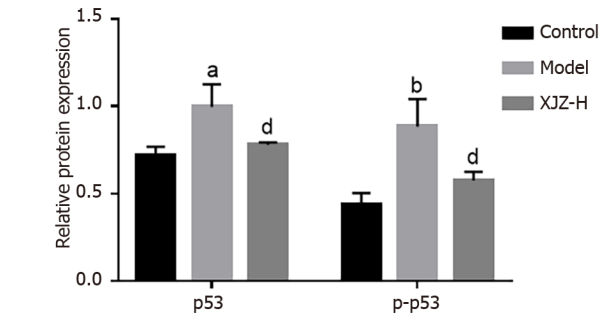
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b
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Control
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XJZ-H
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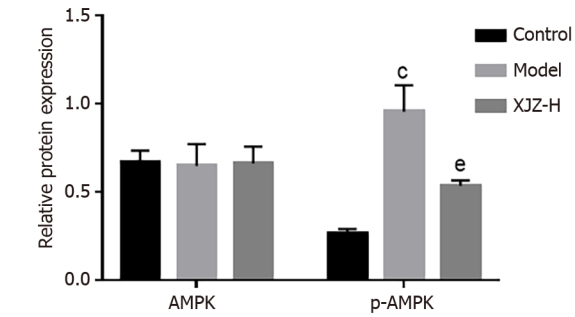
p-p53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0.5
0.0
c
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Control
Model
XJZ-H
AMPK
p-AMPK

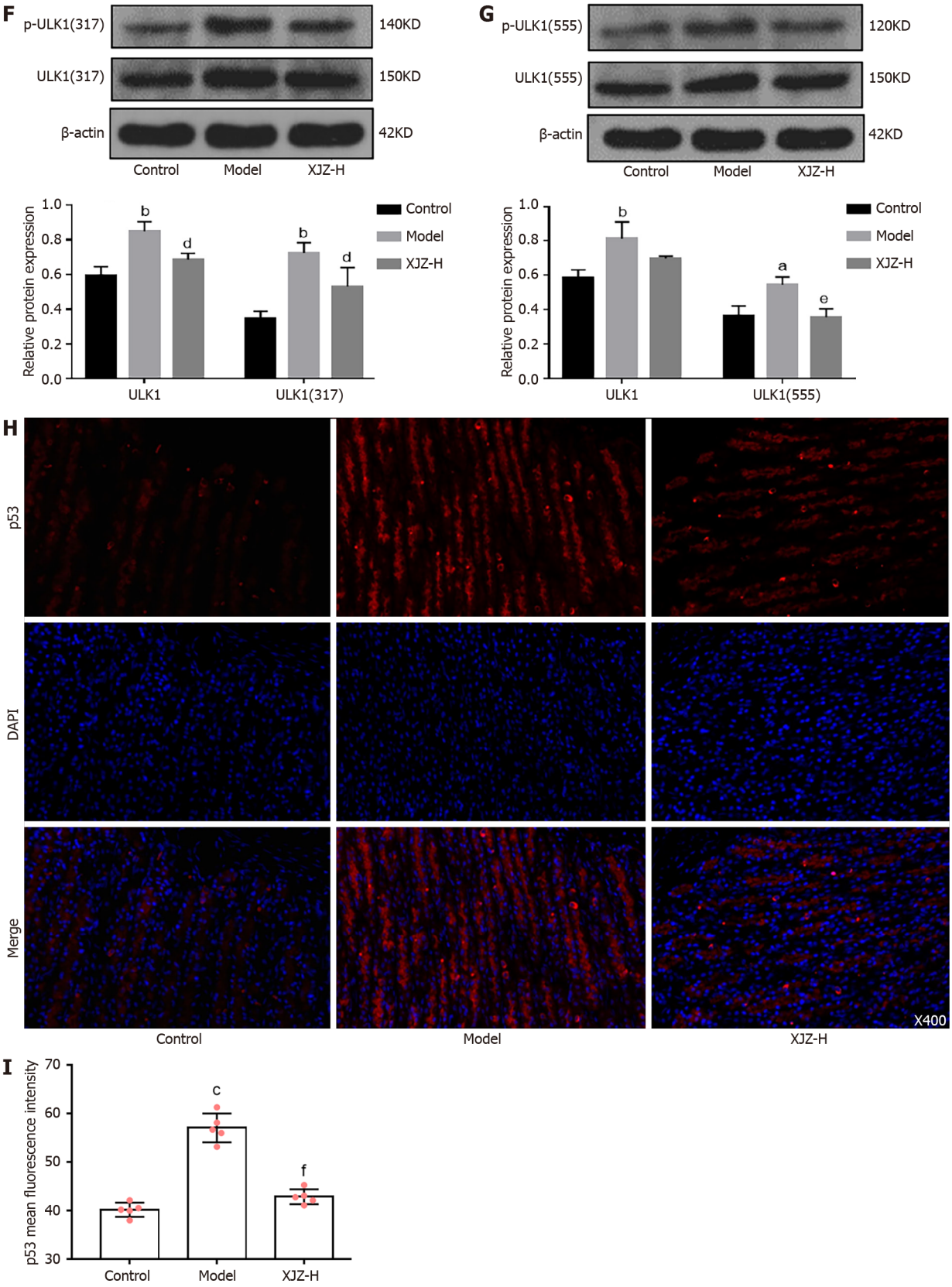

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Figure 7 Xiaojianzhong decoction inhibited Unc-51 Like kinase 1 and p53/AMP-activated protein kinase pathway in N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric precancerous lesions rats. A and B: Unc-51 like kinase 1 (ULK1) and p53 protein expression was determined using immunofluorescence (n = 5); ULK1, p53 proteins positive score was determined using Image J; C: ULK1 mRNA expression was determined using real-time polymerase chain reaction analysis (n = 3); D-G: Western blot analysis was performed to detect p-p53, p-AMP-activated protein kinase, p-ULK1 (Ser555), and p-ULK1 (Ser317) protein expression (n = 3); H and I: ULK1 and p53 protein expression was determined using immunofluorescence (n = 5). Data are expressed as

mean ± SD. [a]*P* < 0.05, [b]*P* < 0.01, [c]*P* < 0.001 *vs* control group; [d]*P* < 0.05, [e]*P* < 0.01, [f]*P* < 0.001 *vs* model group. XJZ-L: Low dose of Xiaojianzhong decoction; XJZ-M: Middle dose of Xiaojianzhong decoction; XJZ-H: High dose of Xiaojianzhong decoction; ULK1: Unc-51 like kinase 1.

metabolic reactions[33]. Glycolysis is common in a variety of tumor diseases, including GC. Its rapid energy production meets the energy demand for tumor cell growth and proliferation, while the increase in efferent lactate also promotes tumor progression[34,35]. CD147 activation assists MCT1 and MCT4 in outward lactate transport and the increased accumulation of extracellular lactate forms an acidic environment suitable for tumor growth[36]. In addition, low SIRT6 expression during high glycolytic activity impedes the deacetylation of H3K9 and promotes the transcription of glycolysis-related genes [37]. Our experiments revealed that XJZ reduced the co-expression of HIF-1α/CD147 in gastric mucosa and the transcription levels of MCT1, MCT4, and CD147. Immunohistochemistry results showed that the SIRT6 expression in the gastric mucosa was upregulated with the increase of XJZ dose and the GPL-related pathological manifestations were ameliorated. These suggest that XJZ may decrease abnormal glucose metabolism by preventing gastric mucosa from hypoxia. Glycolysis has been a hotspot in the research of tumor-related diseases in recent years, but its effects on GPL remain unclear. Our results indicated the adverse effects of higher glycolysis level on GPL and the culprit may be closely related to gastric mucosal hypoxia. In addition to hypoxia, the initiation of glycolysis is also affected by various factors, so we continued to explore the potential mechanism of XJZ in regulating glycolysis in gastric mucosal cells.

ULK1 is an important signaling molecule that affects glycolysis level and is highly expressed in the gastric mucosa during the GPL stage. More specifically, it improves the glycolysis level by promoting the transcription of key glycolysis enzyme genes such as hexokinase 2 (HK2) and phosphofructokinase-1[38]. It has previously been revealed that XJZ reduces the expression and transcription levels of ULK1 in gastric mucosa, which suggests that the inhibition of ULK1 expression could be a potential mechanism by which XJZ regulates the glycolysis level. As mentioned above, ULK1 is also involved in the regulation of autophagy, principally due to the different regulatory roles of its upstream PI3K/AKT/mTOR and p53/AMPK signaling pathways. Notably, p53 is an important metabolic regulator known to affect both autophagy and glycolysis in multiple ways[39]. On the one hand, the activation of nucleoprotein, p53, prompts AMPK to phosphorylate the 317 and 555 sites of ULK1, thereby activating autophagy[40,41]. It has also been determined that p53 can induce mitochondrial autophagy by increasing the Bnip-3 level[42] or promote its target, TP53INP1, to bind the LC-3 and Autophagy-related protein 8 (ATG8) proteins, leading to the excessive activation of autophagy or even autophagic cell death[43]. On the other hand, p53 activates cyclooxygenase 2 (COX-2) to promote the production of reactive oxygen species (ROS), while ROS, as an important product of glycolysis, stabilizes the expression of HIF-1α and maintains glycolytic activity[44]. In the present study, we focused on examining the effects of XJZ on the activation of the p53/AMPK pathway and ULK1 autophagy-related sites. Our results revealed that XJZ inhibited the expression of p53 in gastric mucosa and the phosphorylation of the nucleoprotein p53, AMPK, and the ULK1 317 and 555 sites. This indicates that the inhibition of the p53/AMPK signaling pathway and the phosphorylation of the ULK1 317 and 555 sites may also represent a potential mechanism for the regulation of autophagy by XJZ in GPL. Moreover, the inhibition of ULK1 and the nucleoprotein p53 by XJZ may be involved in the dual regulation of autophagy and glycolysis in GPL. In addition, this result suggests that the inhibition of autophagy by XJZ may stem from the co-regulation of the PI3K/AKT/mTOR and p53/AMPK/ULK1 pathways.

Notably, a recent study on Escherichia coli found that HIF-1α activation can also activate autophagy by promoting ULK1 expression[45]; however, this result has not been confirmed in GPL-related studies. Our data showed that XJZ inhibited the expressions of HIF-1α and ULK1 in the gastric mucosa of GPL rats. It is speculated that XJZ may also affect autophagy by ameliorating gastric mucosal hypoxia to downregulate ULK1 expression.

Little is currently known about the effects of the main components of XJZ on both autophagy and glycolysis in gastric mucosal cells. Some prior studies have determined that the cinnamaldehyde found in XJZ can inhibit autophagy through the AMPK/mTOR/ULK1 signaling pathway in esophageal cancer cells, which suggests the possibility of adjusting autophagy to a level that can maintain intracellular homeostasis[46]. Moreover, paeoniflorin has been found to inhibit the autophagy level by inhibiting the expression of Beclin-1 and LC-3II in cardiomyocytes, thereby protecting the cardiomyocytes[47]. The 6-gingerol has been observed to inhibit autophagy by targeting the Notch signaling pathway in order to block breast cancer cell growth[48]. In addition, licorice chalcone A was found to inhibit the glycolysis level through the PI3K/AKT signaling pathway in mouse melanoma B16F10 cells[49]. These studies of the main components of XJZ in other disease models may provide useful insights into the specific components of XJZ that act in gastric mucosal cells.

In the present study, we determined that XJZ was able to effectively prevent the progression of MNNG-induced GPL in rats, and it may prevent the further deterioration of GPL by regulating autophagy, glycolysis, and gastric mucosal hypoxia. However, this study did not verify the relevant signaling molecules and pathways to further clarify the specific mechanism of XJZ in the treatment of

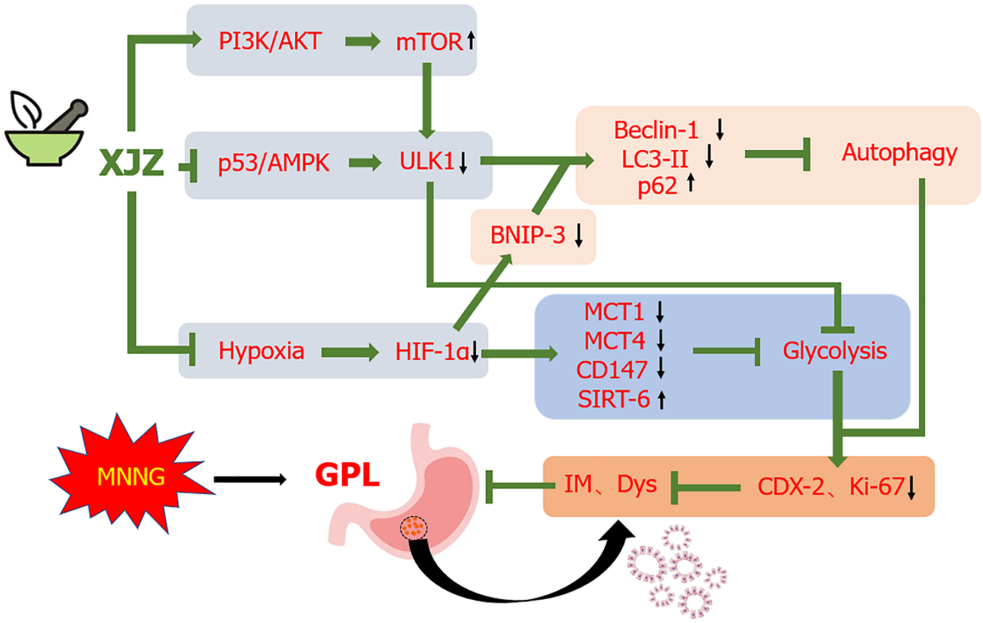

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Figure 8 Scheme summarizing the protective effects of Xiaojianzhong decoction on N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric precancerous lesions rats *via* regulation of the p53/AMP-activated protein kinase/Unc-51 like kinase 1, phosphatidylinositol 3-kimase/protein kinase B/mammalian target of rapamycin axis and hypoxia state. AKT: Protein kinase B; AMPK: AMP-activated protein kinase; Bnip-3: B cell lymphoma/Leukemia-2 and adenovirus E1B19000 interacting protein 3; Dys: Dysplasia; GPL: Gastric precancerous lesions; HIF-1α: Hypoxia-inducible factor-1α; IM: Intestinal metaplasia; MCT: Monocarboxylate transporter; MNNG: N-methyl-N'-nitro-N-nitrosoguanidine; PI3K: Phosphatidylinositol 3-kimase; mTOR: Mammalian target of rapamycin signaling pathway; SIRT6: Sirtuin 6; ULK1: Unc-51 like kinase 1.

GPL. Additionally, we did not perform an in-depth investigation of the specific components of XJZ that are effective against GPL.

CONCLUSION

The observed effect of XJZ against GPL in rats may be due to its ability to inhibit both autophagy and glycolysis in gastric mucosal cells. Furthermore, the intrinsic mechanism of XJZ may be related to the regulation of the PI3K/AKT/mTOR and p53/AMPK/ULK1 signaling pathways and the improvement in gastric mucosal hypoxia. Given these findings, our experiments provide new ideas for the prevention and treatment of GPL, in addition to providing a theoretical basis for the clinical application of XJZ in GPL. The results of our experiments also indicate that XJZ has great potential in the development of functional foods able to protect gastric mucosa and prevent GPL progression. In future studies, we will verify and enrich the existing research results through cellular experiments, multi-omics, and other means. We will also clarify the specific components of XJZ that exert therapeutic effects in order to render its use more precise.

ARTICLE HIGHLIGHTS

Research background

In recent years, more and more attention has been paid to the research of gastric precancerous lesions. Gastric precancerous lesions (GPLs) are an important histopathological stage before the occurrence of gastric cancer (GC). Effective treatment and reversal of it is an important means to prevent GC.

Research motivation

Xiaojianzhong decoction (XJZ) is a classic prescription in ancient China, and it is mostly used clinically to treat gastrointestinal diseases such as chronic atrophic gastritis. This study aimed to explore the *in vivo* mechanism of XJZ in treating GPL.

Research objectives

To study the effect and mechanism of XJZ on autophagy and glycolysis of GPL gastric mucosal

epithelial cells, and provide a theoretical basis for the clinical prevention and treatment of GPL.

Research methods

Using the N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) compound modeling method to construct a model of gastric precancerous lesions in rats, intervene with XJZ, and use a variety of methods to detect the effects of XJZ on autophagy, glycolysis and gastric precancerous lesions in rats.

Research results

The treatment of XJZ can promote the weight gain of rats and improve the pathological manifestations of GPL-related tissues; the formation of autophagosomes and autolysosomes in gastric tissue is reduced, and the expressions of B cell lymphoma/leukemia-2 and adenovirus E1B19000 interacting protein 3, moesin-like BCL2-interacting protein 1, and microtubule associated protein 1 light chain 3 are reduced. The expression of p62 increased, and the level of autophagy was fully inhibited. XJZ inhibited the level of autophagy by activating the phosphatidylinositol 3-kimase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway; while improving gastric mucosal hypoxia, it could also inhibit autophagy and improve abnormal glucose metabolism in gastric mucosal cells. XJZ inhibited the expression of Unc-51 Like kinase 1 (ULK1) to improve abnormal glucose metabolism in gastric mucosa, and prevented the increase of autophagy level by inhibiting p53/AMP-activated protein kinase (AMPK) pathway and ULK1 Ser317555 phosphorylation.

Research conclusions

Xiaojianzhong Decoction has a therapeutic effect on MNNG-induced gastric precancerous lesions, which may be achieved through the joint regulation of PI3K/AKT/mTOR, p53/AMPK/ULK1 signaling pathways and gastric mucosal hypoxia.

Research perspectives

This study mainly explores Xiaojianzhong Decoction regulates PI3K/AKT/mTOR, p53/AMPK/ULK1 signaling pathways and gastric mucosal hypoxia to improve gastric precancerous lesions in rats.

FOOTNOTES

Author contributions: Zhang JX performed the experiments, sample detection, data analysis and wrote the manuscript; Bao SC, Chen J, and Chen T helped with the performing and sample collection of the animal experiment; Yan SG, Li JT, Wei HL, and Zhou XY conceived and supervised the experiments and finalized the manuscript; all authors reviewed the manuscript.

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Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Regulations for the Care and Use of Laboratory Animals in Shaanxi University of Chinese Medicine (Approval No. SCXK 2019-0004).

Conflict-of-interest statement: The authors declare that there is no conflict of interest in this study.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at ysg2002. student@sina.com upon reasonable request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

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ORIGINAL ARTICLE

Basic Study

F-box and leucine-rich repeat 6 promotes gastric cancer progression *via* the promotion of epithelial-mesenchymal transition

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Abstract

BACKGROUND

F-box and leucine-rich repeat 6 (FBXL6) have reportedly been associated with several cancer types. However, the role and mechanisms of FBXL6 in gastric cancer (GC) require further elucidation.

AIM

To investigate the effect of FBXL6 in GC tissues and cells and the underlying mechanisms.

METHODS

TCGA and GEO database analysis was performed to evaluate the expression of FBXL6 in GC tissues and adjacent normal tissues. Reverse transcription-quantitative polymerase chain reaction, immunofluorescence, and western blotting were used to detect the expression of FBXL6 in GC tissue and cell lines. Cell clone formation, 5-ethynyl-2′-deoxyuridine (EdU) assays, CCK-8, transwell migration assay, and wound healing assays were performed to evaluate the malignant biological behavior in GC cell lines after transfection with FBXL6-shRNA and the overexpression of FBXL6 plasmids. Furthermore, *in vivo* tumor assays were performed to prove whether FBXL6 promoted cell proliferation *in vivo*.

RESULTS

FBXL6 expression was upregulated more in tumor tissues than in adjacent normal tissues and positively associated with clinicopathological characteristics. The outcomes of CCK-8, clone formation, and Edu assays demonstrated that FBXL6 knockdown inhibited cell proliferation, whereas upregulation of FBXL6 promoted proliferation in GC cells. Additionally, the transwell migration assay revealed that FBXL6 knockdown suppressed migration and invasion, whereas the overex-

pression of FBXL6 showed the opposite results. Through the subcutaneous tumor implantation assay, it was evident that the knockdown of FBXL6 inhibited GC graft tumor growth *in vivo*. Western blotting showed that the effects of FBXL6 on the expression of the proteins associated with the epithelial-mesenchymal transition-associated proteins in GC cells.

CONCLUSION
Silencing of FBXL6 inactivated the EMT pathway to suppress GC malignancy *in vitro*. FBXL6 can potentially be used for the diagnosis and targeted therapy of patients with GC.

Key Words: Gastric cancer; F-box and leucine-rich repeat 6; Invasion; Epithelial-mesenchymal transition; Metastasis

Core Tip: F-box and leucine-rich repeat 6 (FBXL6) is up-regulated in gastric cancer (GC) cell lines and tissues, which is correlated with tumor size, grade of differentiation, and TNM stage. Knockdown of TRIM55 in GC cells suppressed proliferation, migration and invasion of cells and affected the expression of cell epithelial-mesenchymal transition-related proteins. Our study provides novel evidence that FBXL6 contributes the growth and metastasis of GC.

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INTRODUCTION

Globally, gastric cancer (GC) is the second most common cause of cancer-related deaths and the fourth most common cancer[1,2]. Approximately 950000 new cases of patients with GC are diagnosed worldwide each year; however, a decline in incidence and mortality rates has been observed in recent years[3]. As traditional treatment strategies for gastric cancer, surgical resection, chemotherapy, and radiotherapy continue to show shortcomings; this is the main reason for the < 30% 5-year overall survival (OS) for patients with GC[4,5]. Therefore, further research is warranted to help researchers elucidate the underlying molecular mechanisms and identify effective therapeutic avenues to enhance survival in GC.

F-box and leucine-rich repeat 6 (FBXL6) is an FBXL protein that is closely associated with the degradation of ETV6, which is involved in nucleoplasm formation in the intercellular phase through the ubiquitin-proteasome system[6]. Recent studies have reported that FBXL6 activates the estrogen receptor by promoting its transcription and mediating its protein hydrolysis[7]. Furthermore, FBXL6 expression is reportedly associated with the occurrence of tumors in humans. To illustrate, Li *et al*[8] reported that FBXL6 is a unique prognostic marker, and it demonstrates the occurrence of the malignant progression of renal cell carcinoma. Other studies found that FBXL6 is upregulated and connected with poor prognosis in CRC, in which FBXL6 targets phosphorylated p53 to regulate its polyubiquitination and degradation in cases of colorectal cancer. However, it is unclear whether FBXL6 is closely related to the progression and function in GC.

Epithelial-mesenchymal transition (EMT) has been identified as a vital factor in promoting metastasis in multiple tumors[9,10]. Several EMT-related factors are abnormal, including E-cadherin, vimentin and N-cadherin, which is a biological phenomenon in the EMT progression[11]. The findings of previous studies indicated that the AKT signaling pathway suppresses GSK3β-mediated phosphorylation of β-catenin, thereby causing the β-catenin-mediated transcription of EMT[12]. Furthermore, Song *et al*[13] reported that HOXA10 mediates EMT to promote gastric cancer metastasis through TGF-B2/Smad/METTL3 signaling axis. The fact that EMT progression may contribute to the poor survival prognosis of GC patients has been reported extensively[14,15]; however, information regarding the underlying mechanisms is limited. Thus, performing an investigation of the regulatory mechanisms of EMT in GC development is essential to understand the malignant progression of GC.

Here, we described the relationship between FBXL6 and clinicopathological characteristics and the potential role of FBXL6 in GC cell proliferation and invasion *in vitro* and *in vivo*. Our results showed that FBXL6 is significantly upregulated in GC tissues and that high expression of FBXL6 in patients is commonly associated with poor OS. Therefore, FBXL6 can evidently be considered a novel prognostic biomarker and direction of treatment in GC.

MATERIALS AND METHODS

Patients tissue samples

At the first afflation hospital of Anhui Medical University, 68 pairs of GC tissues and precancerous tissue samples of patients were gathered between January 2020 and December 2020. The clinical samples were stored at -80 °C for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), extraction of proteins, or embedded in paraffin for immunohistochemistry. This study had been approved by the Ethics review committee of the First Affiliated Hospital of Anhui Medical University (approval: Quick-PJ 2019-10-11) and written informed consent was obtained from all cancer patients.

Bioinformatic analysis

The RNA-seq expression files for GC were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) and GEO database was used to compare FBXL6 expression between tumors and normal tissue in GC. The correlation relation between FBXL6 expression and (OS) in GC was analyzed using the Kaplan-Meier plots (http://kmplot.com/), and their statistical significance was obtained by the log-rank test.

Cell culture and stable transfection

Three gastric cancer cell lines (MKN-45, HGC-27, and MGC-803) were purchased from Fenghui Biotechnology Co., Ltd (Hunan Province, China), and gastric mucosal epithelial cell line (GES-1) was maintained in laboratory. Cell cultured was performed using standard media supplemented 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C. Transfections were performed using Fugene® HD (Promega) as per manufacturer instructions. Two short hairpin RNAs (sh-FBXL6#1 and sh-FBXL6#2) and sh-NC negative control and overexpressed FBXL6 plasmid were obtained from GeneChem company (Shanghai, China). the sequences of shRNA were as follows: sh-FBXL6#1: 5′-CACCGGCATCAACCGTAATAG-3′; sh-FBXL6#2: 5′-TGGAGTGGCTTATGCCCAATC-3′; infection of HGC-27 and MKN45 cell lines with shFBXL6#1 and shFBXL6#2 and MGC-803 with OE-FBXL6, and screening with puromycin (10 μg/mL) was performed for 1 wk to establish stable FBXL6 knockdown and overexpression cell lines.

Western blotting

Tissues and cells were processed by performing lysis in the RIPA reagent containing 1 mmol/L PMSF, and the protein was then prepared and quantified through bicinchoninic acid (BCA) analysis. The 20-μg protein sample was separated *via* SDS-PAGE (8%-10%); the isolated proteins were then placed onto polyvinylidene fluoride membranes (Millipore). Blocking was performed with 3% bovine serum albumin (BSA) for 2 h, and the membrane was subsequently incubated at 4 °C for 12 h with the following primary antibody: FBXL6 (1:1000; Abcam, United Kingdom) or anti-GAPDH (ZSGB, China), Vimentin, E-cadherin, and N-cadherin (1:1000, Proteintech, China), matrix metalloproteinase-2 (MMP-2; 1:500, Abcam, United Kingdom), and MMP9 (1:500, Abcam, United Kingdom). The membranes were then incubated with goat anti-rabbit and goat anti-mouse HRP (1:10000, Proteintech, China) for 2 h at 25 °C. Finally, the protein membranes were visualized using the ECL system (Tanon, Shanghai, China).

RT-qPCR

RT-qPCR was performed as described previously[16]. Total RNA was extracted from cells and sample tissues using Trizol (Life Technologies, United States). The reverse transcriptase enzyme was used to conduct reverse transcription of 2 μg of purified (Yeasen, Shanghai, China). RT-qPCR was conducted with the appropriate primers using 2 × SYBR Green PCR Master Mix (Yeasen, Shanghai, China) and using the light cycler 96 qPCR System (Roche, United States), and GAPDH as the control. The primer sequences are as follows: FBXL6 forward, 5′-GGAGACCGCATTCCCTTGG-3′; reverse, 5′-AAA-ACCGATTGGGCATAAGCC-3′.

Immunofluorescence

Tissue and cellular immunofluorescence were performed as described in protocol. In this study, GC cells were inoculated in 24-well plates (containing crawlers) and reached the appropriate density. The cells on crawl sheets were then washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. FBXL6-specific antibodies (1:100, Abcam, United Kingdom) were used overnight and FBXL6-stained samples were incubated with secondary antibodies (Proteintech, China). Following this, DAPI staining of cell nuclei was performed.

IHC

IHC experimental methods are identical to those reported in the literature[16]. The Intensity scores were analyzed as follows: 0: No staining; 1: Low staining; 2: Medium staining; and 3: High staining. Proportional scores were further classified as 0: 0%; 1: 1%-25%; 2: 26%-50%; 3: 51%-75%; and 4: > 75%. Immunoreactivity scores were calculated by multiplying the intensity and percentage scores. Staining

results were compared and scored by two independent pathologists.

5-Ethynyl-2'-deoxyuridine assay

Stably transfected GC cells were incubated in 12-well plates. Then 50 μM 5-ethynyl-2'-deoxyuridine (EdU, Beyotime, Shanghai, China) was added to each well and incubated at 37 °C; however, the incubation time varied on a cell-to-cell basis. Then the cells were fixated, permeabilized, and processed with 200 μL of Hoechst 33342 to achieve nuclear staining. A fluorescence microscope was used to capture images to determine the proportion of EdU-positive cells.

Cell proliferation and cell clone formation assay

Transfected GC cells were digested when their content reached 80%; these cells were then inoculated into 96-well culture plates at 2×10^3 cells per well, cultured in 96-well contain standard medium, and cell viability was assessed at 0 h, 24 h, 48 h, and 72 h applying the CCK-8 kit (Beyotime, Shanghai, China). The absorbance value was then detected at 450 nm. For the cell clone formation assay, the stably transfected cells were seeded into 6-well plates. After 2 wk, the cell debris was washed, clone cells were fixed and stained with crystal violet (Beyotime, Shanghai China), and the number of clone cells was calculated by the camera.

Wound healing assay

Transfected GC cells were prepared into a 6-well plate, and when their density reached 80% and confluence and were sectioned with a 200 mL pipette. The surface of the cells was washed once with a serum-free medium, and the cell debris was removed. The cells were then recorded and captured under a microscope with a 100-fold magnification, and their position in the photograph was recorded. Subsequently, each group of cells was then continued to incubate at 37 °C for 24 h. Finally, photographs were captured and recorded, and the migration area of each group was calculated.

Transwell migration and invasion assay

The Transwell cell assay was used to evaluate the migration and invasion abilities of tumor cells. Typically, invasion assays were constructed using BD Matrigel (Corning, United States) and covered the upper chamber and GC cells (1×10^5) were starved for 24 h in a medium without FBS, and the bottom plate supplemented standard medium. After 24 h, the upper cells were removed and adhered to the membrane's lower surface, fixed, and stained with crystal violet. The cells visible in the field of view were recorded and counted.

Animal study and in vivo tumorigenic assays

BALB/c female mice (age, 4 wk) were obtained from SLAC Laboratory Animal Company (Shanghai, China). All nude mice were randomly raised and grouped in an SPF environment. Subcutaneous injection of stable knockout sh-FBXL6#1 and sh-NC HGC-27 cells in nude mice. Furthermore, the length and width of the tumor was recorded and calculated every 4 days using vernier calipers. Finally, the weight of the tumor (mg) was recorded after the mice were euthanized. All animal experiments were approved by the Experimental Animal Ethics Committee of Anhui Medical University (Approval: LLSC2020513).

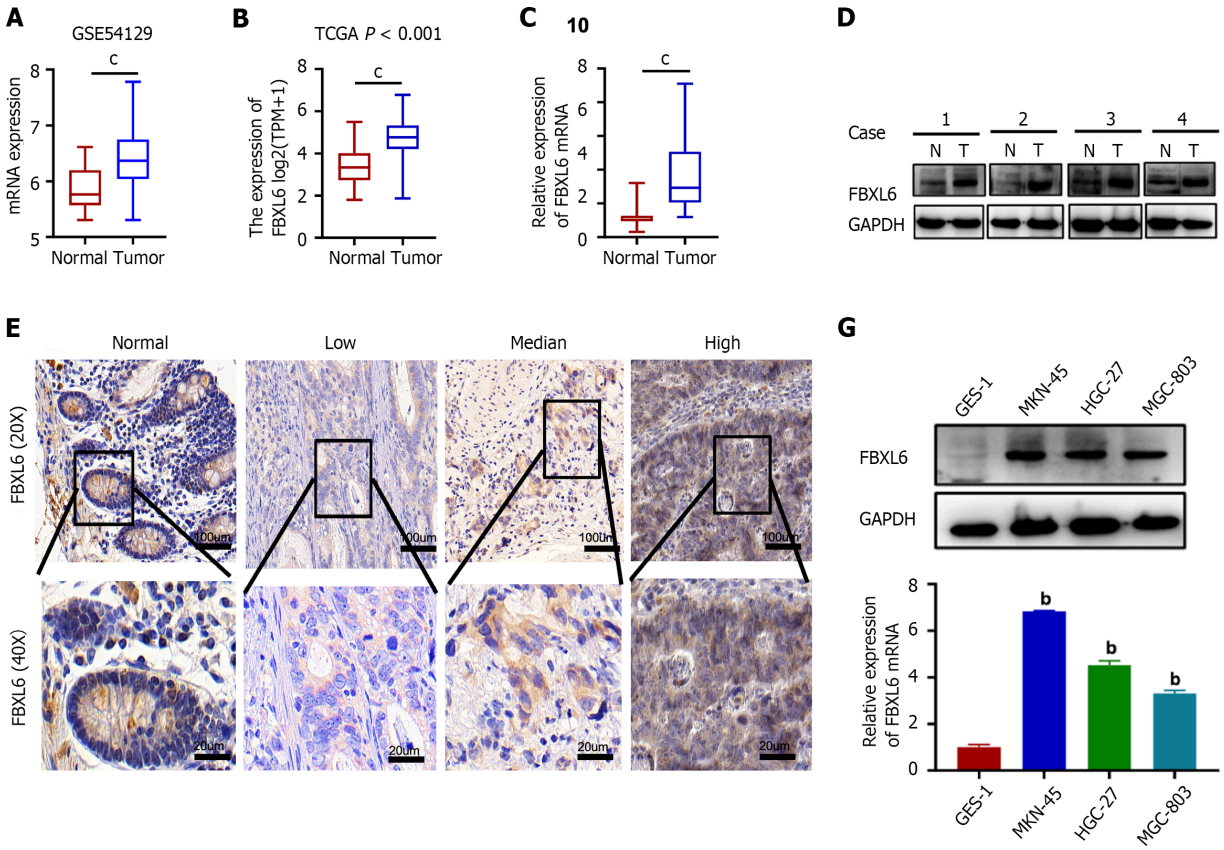
Statistical analysis

The Student's two-tailed *t*-test was used to perform comparisons between the two groups, and comparisons between multiple groups were performed using a one-way ANOVA. Associations between FBXL6 levels and GC clinical data were analyzed using logistic analysis. All statistical analyses were carried out using GraphPad Prism 8.0 software (GraphPad Software, United States) and SPSS 22.0 (IBM Corp). All data are mean ± SD. $^aP < 0.05$, and $^bP < 0.01$ indicated statistical significance.

RESULTS

FBXL6 is highly expressed in GC samples and GC cell lines

To identify potential FBXL6 associations with GC, our analysis of the TCGA and GSE54129 datasets revealed that FBXL6 expression was upregulated considerably more in GC sample than in adjacent normal samples (Figure 1A and B, $P < 0.001$). Additionally, RT-qPCR and western blotting were used to measure the expression of FBXL6 in primary GC samples. FBXL6 mRNA levels were significantly elevated in GC tumors (Figure 1C, $P < 0.001$) and FBXL6 protein was significantly more enriched in GC than in the normal tissue (Figure 1D, $n = 4$). IHC analysis showed that FBXL6 expression was dramatically more abundant in GC tissues than in normal adjacent tissues (Figure 1E). To analyze the relationship between FBXL6 and the clinical data of GC, As shown in Figure 1F, FBXL6 expression was strongly associated with histological grade [3.711 (1.315-10.471), $P = 0.013$], pathological stage [11.250 (3.566-35.496), $P < 0.001$], T grade [4.444 (1.589-12.457), $P = 0.047$], and tumor size [5.111 (1.813-14.408), P



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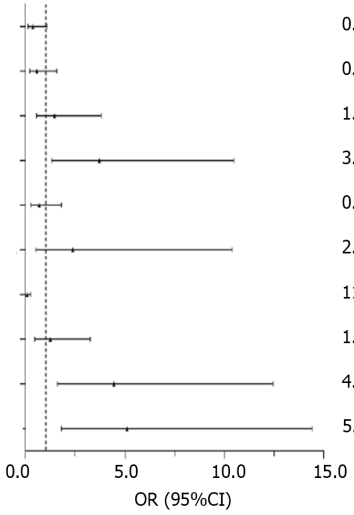
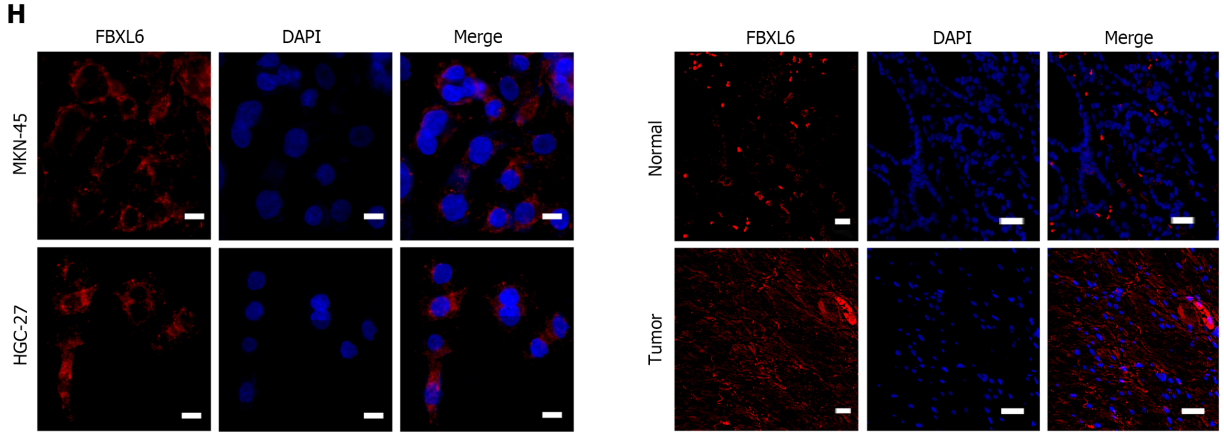
	OR (95%CI)	*P* value
Age (yr)	0.380 (0.135-1.072)	*P* = 0.068
Sex	0.580 (0.213-1.575)	*P* = 0.285
Alcohol	1.453 (0.550-3.805)	*P* = 0.447
Histological grade	3.711 (1.315-10.471)	*P* = 0.013
CEA level	0.696 (0.267-1.812)	*P* = 0.458
Lymph node metastasis	2.370 (0.541-10.387)	*P* = 0.252
Pathological	11.250 (35.66-35.496)	*P* < 0.001
HP	1.255 (0.482-3.265)	*P* = 0.064
T classification	4.444 (1.589-12.457)	*P* = 0.047
Tumor size (cm)	5.110 (1.813-14.408)	*P* = 0.002





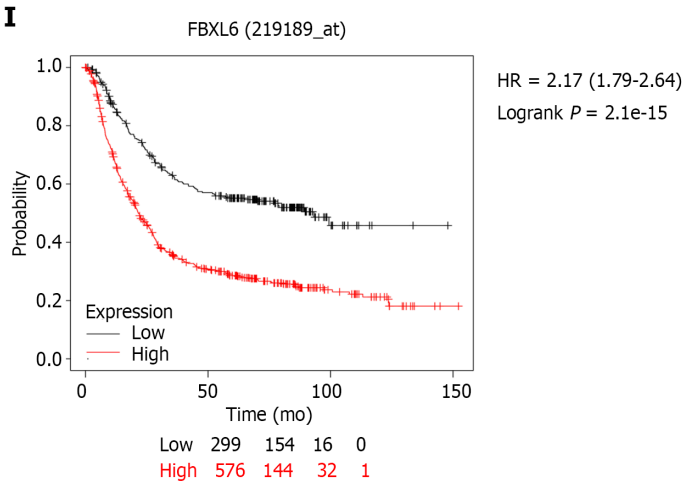


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Figure 1 F-box and leucine-rich repeat 6 is highly expressed in gastric cancer samples and gastric cancer cell lines. A and B: Higher expression of F-box and leucine-rich repeat 6 (FBXL6) was found in gastric cancer (GC) samples than the matched normal tissues (based on GSE54129 and TCGA database); C: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of FBXL6 mRNA expression in 68 pairs of GC patient samples; D: Western blot analysis was performed using an antibody against FBXL6 in 4 pairs of GC patients' samples; E: Outcomes of immunohistochemical staining was performed using an antibody against FBXL6 and representative photographs of FBXL6 in GC patients. Scale bar: 100 μm; F: Relationship between FBXL6 expression and clinical parameters in GC by logistic analysis; G: RT-qPCR and Western blot analysis show the expression of FBXL6 in different GC cell lines (MKN-45, MGC-803 and HGC-27) and GES-1; H and I: Immunofluorescence staining examine the expression and localization of FBXL6 were using in GC cells (H) and tissues (I); J: Kaplan-Meier plots of overall survival and progression-free survival for GC samples from the K-M Plotter database. $^{b}P < 0.01$; $^{c}P < 0.001$. FBXL6: F-box and leucine-rich repeat 6.

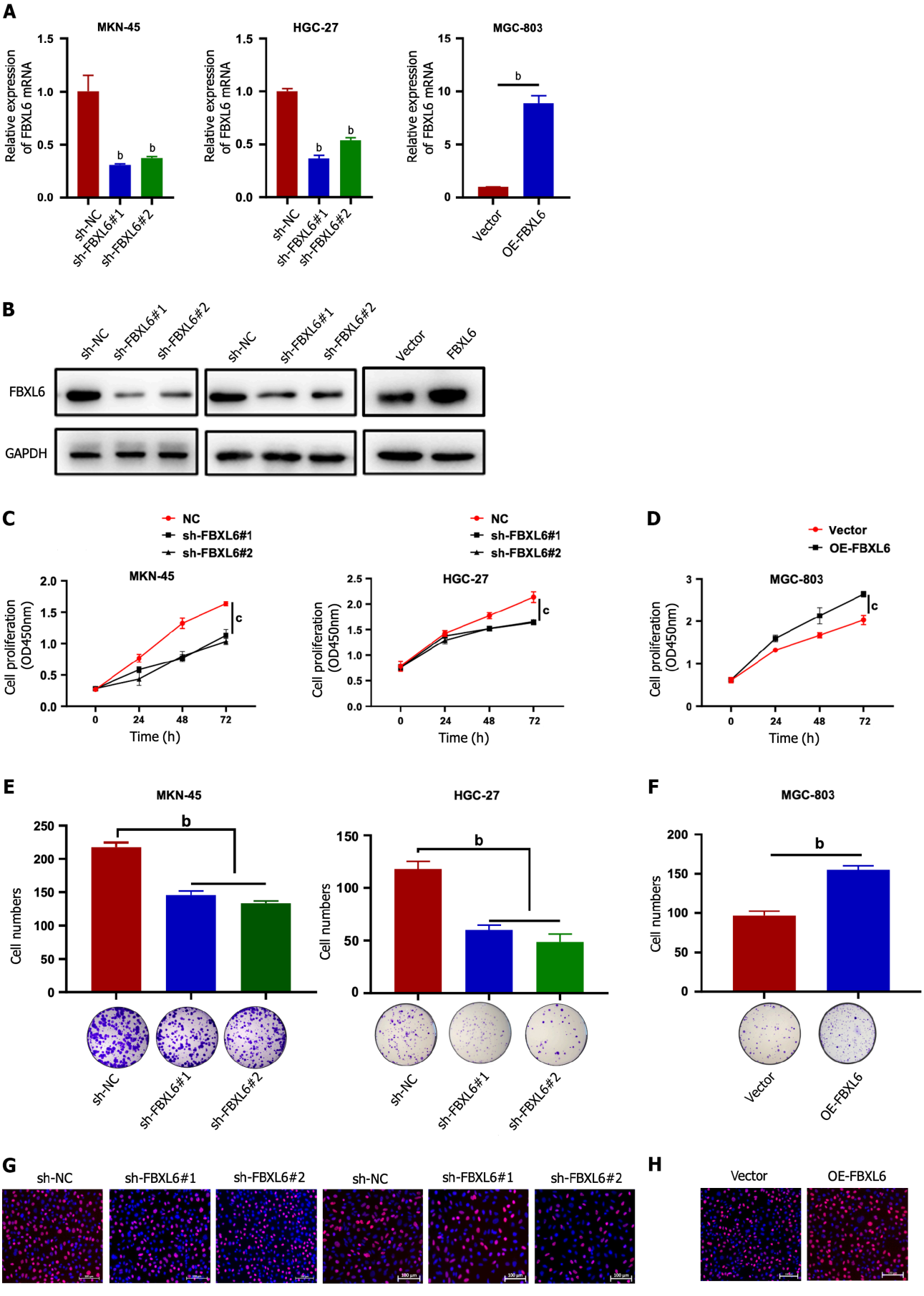
= 0.002]. However, this correlation was not observed in terms of age [0.380 (0.135-1.072), $P = 0.068$], sex [0.580 (0.213-1.575), $P = 0.285$], alcohol [1.453 (0.550-3.805), $P = 0.447$], CEA levels [0.696 (0.267-1.812), $P = 0.458$), lymph node metastasis [2.370 (0.541-10.387), $P = 0.252$], and *Helicobacter pylori* (*H. pylori*) infection [1.255 (0.482-3.265), $P = 0.642$]. We subsequently investigated the FBXL6 protein and mRNA expression levels in three GC cell lines. FBXL6 mRNA and protein (Figure 1G) were higher in the HGC-27, MGC-803, and MKN-45 cell lines than in the GES-1 cell line. We consequently performed IF staining of the GC tissues and cells. In MKN-45 and HGC-27 cell lines, FBXL6 was predominantly located in the cytoplasm and nucleus (Figure 1H); FBXL6 protein staining in normal tissues was relatively lower than that in tumor tissues (Figure 1I). Notably, according to the Kaplan-Meier online database, patients with higher FBXL6 Levels had shorter OS (Figure 1J, HR = 2.7, $P = 2.1e-15$). Furthermore, these results strongly suggest high expression and clinical relevance in GC patents.

FBXL6 promotes gastric cancer cell proliferation

In order to investigate the underlying role of FBXL6 on gastric cancer cells, a stable knockdown FBXL6 was constructed in MKN-45 and HGC-27 cells and overexpression in MGC-803 cells. The knockdown could be effectively detected *via* RT-qPCR and western blot (Figure 2A and B, $P < 0.01$). Knockdown of FBXL6 could suppress the proliferation ability of both HGC-27 and MKN-45 cells; notably, similar results were observed in colony formation assays (Figure 2C-F, $P < 0.01$). In particular, this was noted when knocking down FBXL6 resulted in a decrease in the number of clones. Furthermore, outcomes of the EdU experiment indicated that FBXL6 knockdown suppressed the viability of GC cells (Figure 2G, $P < 0.01$), whereas its overexpression promoted GC cell viability (Figure 2H, $P < 0.01$). These results demonstrated that FBXL6 accelerated the proliferation of GC cells.

FBXL6 regulates gastric cancer cell migration

To accurately determine whether FBXL6 has an effect on the development of migratory and invasive phenotypes cells, The findings of the wound healing assays demonstrated that the migration rate was slower in the stable knockdown MKN-45 and HGC-27 cells (Figure 3A and B, $P < 0.01$); however, we noted that FBXL6 overexpression promoted cell migration in MGC-803 (Figure 3C, $P < 0.001$). To evaluate the impact of FBXL6 on the transwell assays were performed using both MGC-803 and MKN45, and HGC-27 cells. The experimental results revealed that the knockdown of FBXL6 significantly reduced migration and invasion of HGC-27 and MKN-45 cells (Figures 3D and E, $P < 0.001$), whereas the overexpression of FBXL6 increased migration in MGC-803 (Figure 3F, $P < 0.001$). Therefore, the outcomes of the present study suggested that FBXL6 could promote metastasis ability in GC cells.


A
MKN-45
HGC-27
MGC-803
Relative expression of FBXL6 mRNA
sh-NC
sh-FBXL6#1
sh-FBXL6#2
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OE-FBXL6
B
FBXL6
GAPDH
C
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Cell numbers
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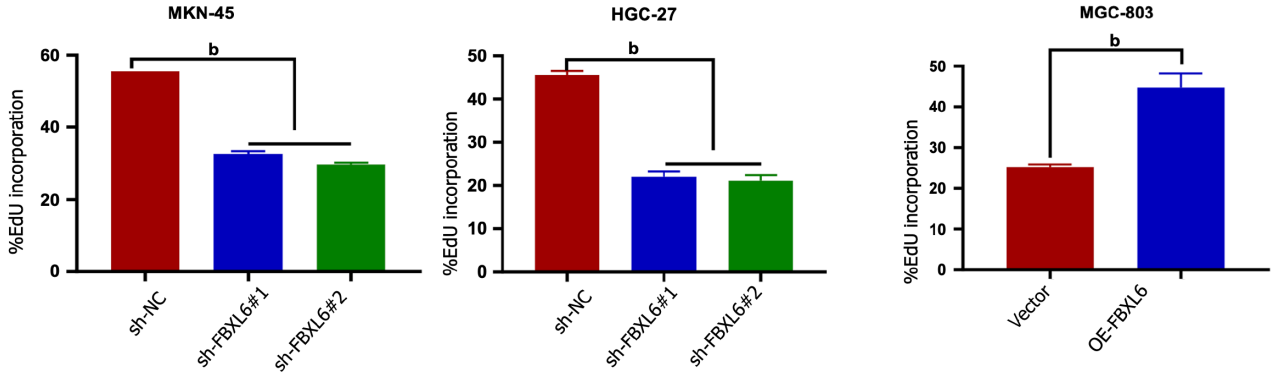

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Figure 2 F-box and leucine-rich repeat 6 promotes gastric cancer cell proliferation. A and B: Reverse transcription-quantitative polymerase chain reaction and Western blot analysis of MKN45 and HGC-27 stably transfected with F-box and leucine-rich repeat 6 (FBXL6) knockdown and overexpression of FBXL6 in MGC-803 cells; C and D: CCK8 assay analyzed the proliferation of stable knockdown in MKN-45 and HGC-27 cell lines and overexpression in MGC-803 cell; E and F: Colony formation assays were used to detect cell growth of knockdown FBXL6 in MKN-45 and HGC-27 or overexpression FBXL6 in MGC-803 cells; G and H: 5-ethynyl-2'-deoxyuridine assays help in the analysis of the viability of knockdown of FBXL6 in MKN-45 and HGC-27 cell or overexpression of FBXL6 in MGC-803; $^{b}P < 0.01$; $^{c}P < 0.001$. FBXL6: F-box and leucine-rich repeat 6.

Knockdown of FBXL6 in GC cells suppressed tumorigenesis in vivo

The potential effects of FBXL6 on tumor growth in nude mice were evaluated that the HGC-27 cells were stably transfected with sh-NC, and sh-FBXL6#1 was injected subcutaneously into each BALB/c nude mouse. Figure 4A-C shows that the subcutaneous tumors in the sh-FBXL6#1 knockout group are significantly lower in terms of volume and mean weight than the sh-NC group. Additionally, H&E staining indicated that the sh-FBXL6#1 group had a lower nuclear malignancy than the sh-NC group (Figure 4D, $P < 0.001$). Notably, IHC staining results revealed that FBXL6, N-cadherin, vimentin, and Ki-67 expression were reduced in the sh-FBXL6#1 group. However, the expression levels of E-cadherin appeared to increase (Figure 4E and F, $P < 0.001$). Therefore, our result proposed that the knockdown of FBXL6 could reduce tumorigenesis and proliferation *in vivo*.

FBXL6 promotes the progression of GC cells through the EMT pathway

EMT has been recognized as a key factor present in all types of tumor metastases. These results showed that the EMT pathway was dramatically inhibited, which led to an increase in the expression of E-cadherin protein and a decrease in that of vimentin and N-cadherin protein in FBXL6-silenced HGC-27 cells. However, overexpression of FBXL6 showed the opposite effect in MGC-803 cells (Figure 5A and B, $P < 0.001$). MMP-9 and MMP2 are essential MMPs associated with EMT and cell metastasis. Our results demonstrated that MMP2 and MMP-9 expression was reduced in HGC-27 cells following the knockdown of FBXL6, and were increased following the overexpression of FBXL6 in MGC-803 cells (Figure 5C and D, $P < 0.001$). These studies demonstrated that FBXL6 promotes GC cell invasion and metastasis by inducing EMT.

DISCUSSION

Identifying genes that are imperative to ensure GC development and progression or its behavioral processes is of paramount importance to successfully explore potentially effective treatments. FBXL6 is a member of the F-box protein (FBP) family and is reported to promote colon cancer progression and play an anti-metastatic role in colon cancer[8]. Additionally, FBXL6 facilitates the stabilization and activation of c-Myc protein through the prevention of HSP90AA1 degradation, which combines directly with the FBXL6 promoter region to enhance mRNA expression in patients with hepatocellular carcinoma[17]. However, the effects of FBXL6 on GC remain unclear and require further elucidation. A new finding from our results indicated that FBXL6 protein and mRNA levels were higher in GC tissues than those normal tissues. Furthermore, the interaction between FBXL6 expression and clinicopathological features was assessed in the current study and a positive correlation was identified between FBXL6 and tumor size, histological grade, and TNM stage. Noteworthy, The Kaplan-Meier Plotter database showed that FBXL6 expression was associated with a potentially poor prognosis of GC. Our results suggested that FBXL6 is a prognostic factor and oncogenic gene in GC patients.

The FBPs are substrate receptors for the SCF E3 ubiquitin ligase and play a critical role in recognizing and recruiting polyubiquitinated substrate proteins[18]. Several studies have reported that FBPs are strongly associated with human cancers and activity of the pertinent oncogenes[19,20]. Reportedly, FBXL16 mechanistically promotes cell growth and migration through the antagonization of the activity

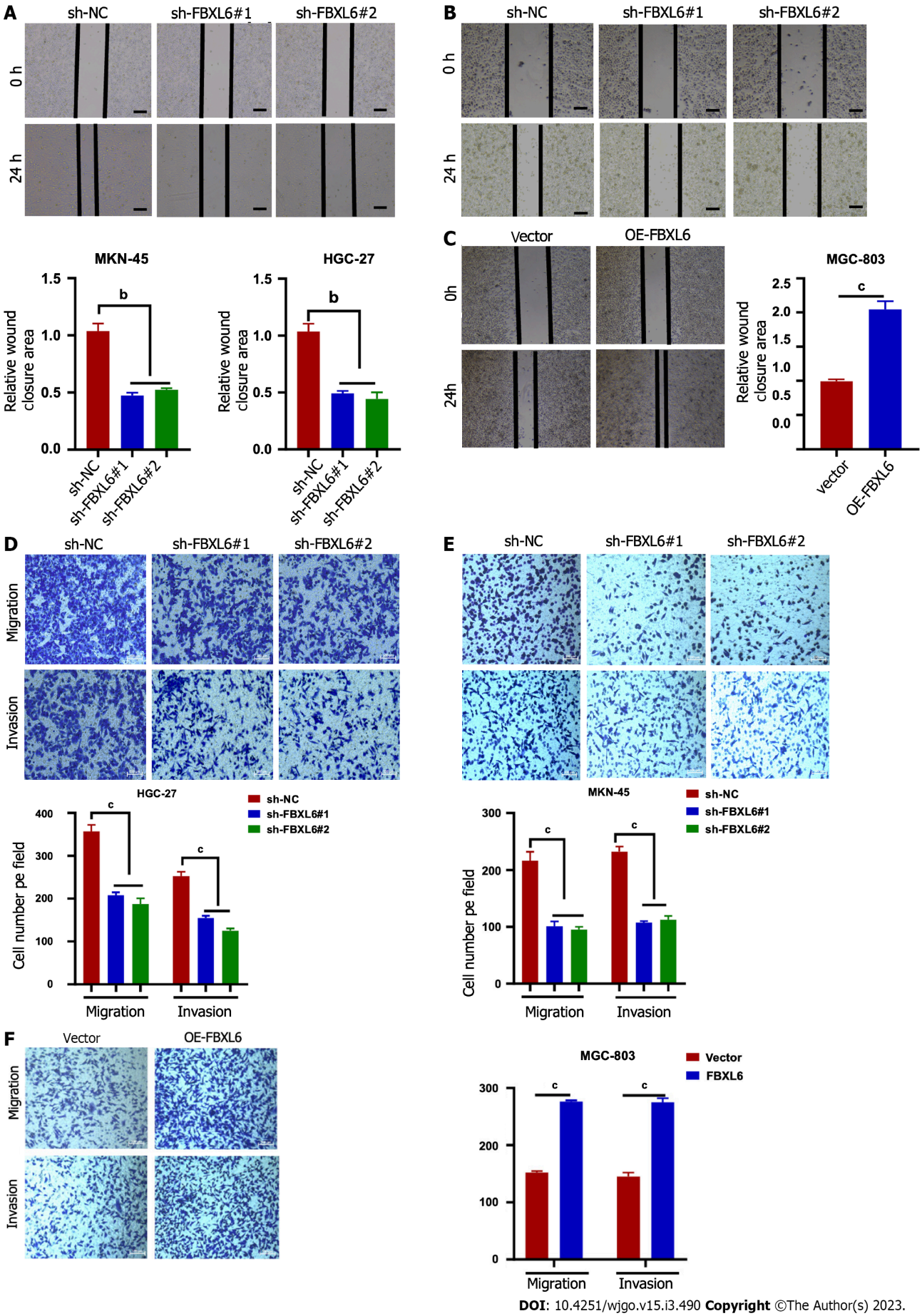

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Figure 3 F-box and leucine-rich repeat 6 regulates gastric cancer cell migration. A and B: Wound healing analysis to assess the migration of F-box

and leucine-rich repeat 6 (FBXL6) knockdown stable cell lines or overexpression of FBXL6 in MGC-803 cells at 0 h and 24 h; C and D: Cell migration and invasion of gastric cancer cells with (C) knockdown of FBXL6 or (D) overexpression of FBXL6 by transwell assay. E and F: The experimental results revealed that the knockdown of FBXL6 significantly reduced migration and invasion of MKN-45 cells (E), whereas the overexpression of FBXL6 increased migration in MGC-803 (F). $^{b}P < 0.01$; $^{c}P < 0.001$. FBXL6: F-box and leucine-rich repeat 6.

of FBW7 and enhancement of the stability of c-Myc[21]. Furthermore, Yang *et al*[22] also noted that FBXO39 was highly expressed in invasive cervical squamous cell carcinoma and that patients with high FBXO39 expression presented with poorer disease prognosis than low expression patients.

In the past, studies have reported that the loss of FBXL6 reduces the growth and induces apoptosis in ccRCC cells[23]. Consistent with our results related to GC cells, silencing of FBXL6 may inhibit the proliferation and colony-forming ability, whereas FBXL6 overexpression demonstrated the opposite effect. These findings were coherent with the effect of FBXL6 *in vivo* assays. In addition, FBXL6 expression knockout decreased the migration viability of GC cells, whereas the overexpression of FBXL6 enhanced cell migration ability. Therefore, our results suggested that FBXL6 acts as an oncogene in gastric cancer by enhancing cell proliferation and invasion ability *in vitro* and *in vivo*. Nonetheless, the molecular mechanisms through which FBXL6 regulates proliferation, migration, and invasion require elucidation. Evidently, EMT is believed to play a critical influence in cancer invasion and metastasis, with the main effects on the expression of E-and N-cadherin proteins[24]. Furthermore, EMT also has been widely recognized as an essential factor in GC metastasis[25,26]. In this research, we determined that silencing FBXL6 downregulated the N-cadherin and vimentin proteins, and increase E-cadherin protein expression. Conversely, FBXL6 overexpression demonstrated the opposite results in GC cells. Recent studies suggest that FBPs play a vital part in tumorigenesis and metastasis, and the research on F-box proteins and EMT factors in cancers is increasing annually. To illustrate, FBXO22 protein significantly reduced RCC cell metastasis ability by reversing EMT and inhibiting MMP-9 expression *in vitro*[27]. Li *et al*[8] identified the fact that FBXW7 regulated MMP-2 and MMP-9 expression and suppresses RCC metastasis through the EMT single pathway. The activation of MMP9 has been implicated in the invasion and metastasis of GC[28]. Furthermore, MMP2 and MMP-9 are dramatically associated with cell invasion and metastasis in particular; this progression may occur through the digestion of the extra-cellular matrix in the basement membrane[29]. Therefore, the outcomes have proved that FBXL6 expression downregulation reduced MMP2 and MMP9 protein expression and inhibited GC invasion and metastasis ability. To summarize, these results provide mechanistic insight into the role of FBXL6 in GC metastasis.

The fact that we did not further investigate the mechanism of action of FBXL6 in GC is a limitation of this study. Owing to the limitations related to follow-up time, no clinical data were recorded that did not allow for the use of statistics to elucidate the survival of the patients. Additionally, Whether or not FBXL6 can bind other genes to induce the ubiquitination process in GC remain unknown. Future studies warranted to investigate the potential mechanisms of FBXL6-mediated regulation of the expression of EMT.

CONCLUSION

In conclusion, this study reported that FBXL6 expression was significantly enhanced in GC tissues and GC cells, and the positive of FBXL6 expression was significantly associated with the prognostic significance of GC patients and correlation between its expression and clinical features. Notably, FBXL6 promoted growth, migration, and invasion through the EMT signaling pathway of GC cells. Therefore, future research needs concern that FBXL6 may have potential as an important prognostic indicator and therapeutic destination for GC.

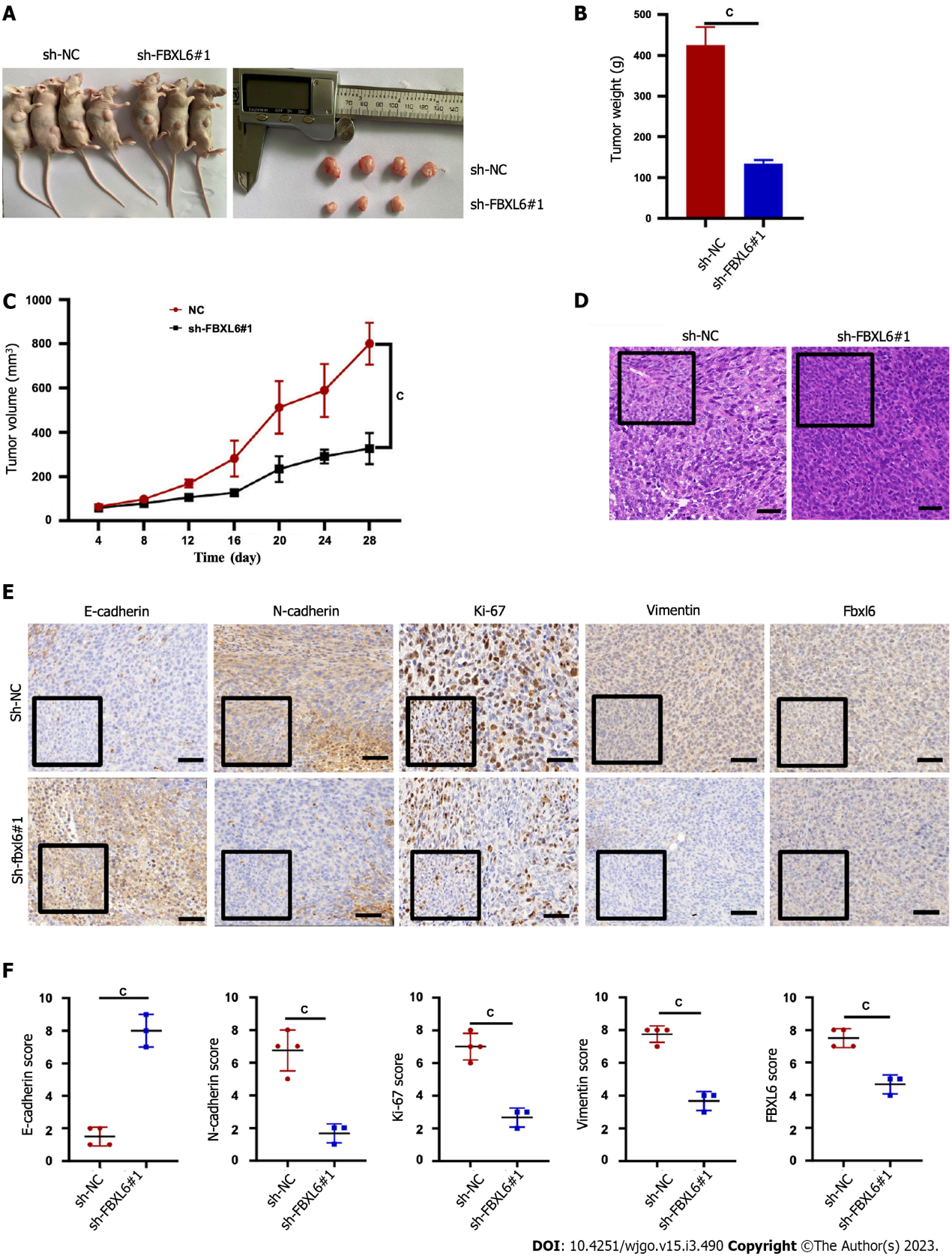

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Figure 4 Knockdown of F-box and leucine-rich repeat 6 in gastric cancer cells suppressed tumorigenesis *in vivo*. A: Images of tumor formation in nude BALB/c mice after injection of sh-NC and sh-F-box and leucine-rich repeat 6 (FBXL6) #1 of HGC-27 cells into their subcutaneous; B: Tumor shape of shRNA and sh-NC groups after dissection; C: The tumor growth curve in sh-FBXL6#1 and sh-NC groups; D: Hematoxylin and eosin staining detected tumor in sh-FBXL6#1 and sh-NC groups; E and F: Immunohistochemical staining was performed to determine the tumor's expression of FBXL6, E-cadherin, N-cadherin, Vimentin, and Ki-67. $^{b}P < 0.01$; $^{c}P < 0.001$. FBXL6: F-box and leucine-rich repeat 6.

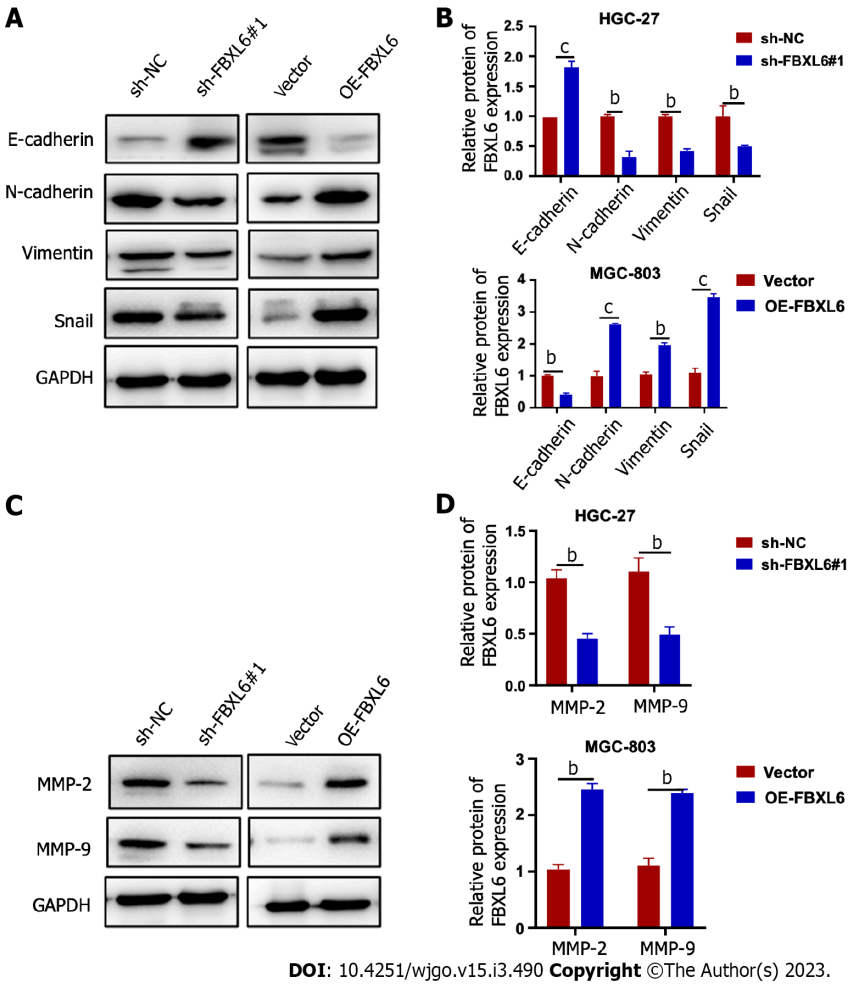

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Figure 5 F-box and leucine-rich repeat 6 promotes the progression of gastric cancer cells through the epithelial-mesenchymal transition pathway. A and B: The expression of epithelial-mesenchymal transition-associated markers (E-cadherin, N-cadherin and Vimentin) and Snail is shown using western blot analysis; C and D: The expression of MMP-2 and MMP-9 was detected in the transfected gastric cancer cells by western blot. $^{b}P < 0.01$; $^{c}P < 0.001$. FBXL6: F-box and leucine-rich repeat 6.

ARTICLE HIGHLIGHTS

Research background

F-box and leucine-rich repeat 6 (FBXL6) have reportedly been associated with several cancer types. However, the role of FBXL6 in the proliferation and epithelial-mesenchymal transition (EMT) of gastric cancer (GC) remains to be investigated.

Research motivation

To investigate the effect of FBXL6 on the proliferation of GC cells and to find new therapeutic targets for the treatment of GC.

Research objectives

The present study to clarify the effect of FBXL6 on the prognosis of GC patients and the proliferation and EMT of GC cells.

Research methods

The expression of FBXL6 expression in GC tissues and cells was detected using RT-qPCR and Western blotting. *In vitro*, stable FBXL6 knockdown and overexpressed GC cell lines were cultured, and the proliferation, clone formation, migration and invasion ability of GC cells were examined using cholecystokinin-8 assay, clone formation assay, wound healing assay and transwell assay, respectively. *In vivo* tumor assays were performed to prove whether FBXL6 promoted cell proliferation *in vivo*. Western blotting was used to detect the association of FBXL6 protein with EMT-related protein expression levels.

Research results

FBXL6 expression is elevated in GC cells and tissues, and FBXL6 expression levels correlated with histological grade, pathological stage, T grade, and tumor size. *In vitro*, endogenous silenced of FBXL6 suppressed GC cell proliferation, migration, invasion and EMT. *In vivo*, knockdown of FBXL6 inhibited subcutaneous graft tumor growth in nude mice.

Research conclusions

FBXL6 expression is increased in GC tissues and cell lines. FBXL6 promotes the proliferation migration, invasion, and epithelial-mesenchymal transition of GC cells.

Research perspectives

FBXL6 may have potential as an important prognostic indicator and therapeutic destination for GC. Further search for potential cancer-promoting mechanisms of FBXL6 is needed in the future.

FOOTNOTES

Author contributions: Meng L collected the data and wrote the manuscript; Hu YT participated in the discussion; Xu AM reviewed the manuscript and provided funding support; all authors contributed to the article and approved the submitted version.

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Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the First Affiliated Hospital of Anhui Medical University (Approval No. Quick PJ 2019-10-11).

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by Anhui Medical University Laboratory Animal Ethics Committee (Approval No. LLSC20200513).

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

Conflict-of-interest statement: There is no conflict of interest in this study.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

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Basic Study

Possible mechanisms associated with immune escape and apoptosis on anti-hepatocellular carcinoma effect of Mu Ji Fang granules

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) is one of the most common digestive system cancers with high mortality rates worldwide. The main ingredients in Mu Ji Fang Granules (MJF) are alkaloids, flavonoids, and polysaccharides. MJF has been used in the clinical treatment of hepatitis, cirrhosis and HCC for more than 30 years. Few previous studies have focused on the mechanism of MJF on tumor immunology in the treatment of HCC.

AIM

To explore the mechanism of action of MJF on tumor immunology in the treatment of HCC.

METHODS

The absorbable ingredients of MJF were identified using Molecule Network related to High Performance Liquid Chromatography-Electron Spray Ionization-Time of Flight- Mass Spectrometry, and hub potential anti-HCC targets were

screened using network pharmacology and pathway enrichment analysis. Forty male mice were randomly divided into the Blank, Model, and MJF groups (1.8, 5.4, and 10.8 g/kg/d) following 7 d of oral administration. Average body weight gain, spleen and thymus indices were calculated, tumor tissues were stained with hematoxylin and eosin, and Interferon gamma (IFN-γ), Tumor necrosis factor α (TNF-α), Interleukin-2, aspartate aminotransferase, alanine aminotransferase, alpha-fetoprotein (AFP), Fas, and FasL were measured by Enzyme-linked Immunosorbent Assay. Relevant mRNA expression of *Bax* and *Bcl2* was evaluated by Real Time Quantitative PCR (RT-qPCR) and protein expression of Transforming growth factor β1 (TGF-β1) and Mothers against decapentaplegic homolog (SMAD) 4 was assessed by Western blotting. The HepG2 cell line was treated with 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL of MJF, and another 3 groups were treated with TGF-β1 inhibitor (LY364947) and different doses of MJF. Relevant mRNA expression of TNF-α, IFN-γ, *Bax* and *Bcl2* was evaluated by RT-qPCR and protein expression of TGF-β1, SMAD2, p-SMAD2, SMAD4, and SMAD7 was assessed by Western blotting.

RESULTS

It was shown that MJF improved body weight gain and tumor inhibition rate in H22 tumor-bearing mice, protected immune organs and liver function, reduced the HCC indicator AFP, affected immunity and apoptosis, and up-regulated the TGF-β1/SMAD signaling pathway, by increasing the relative expression of TGF-β1, SMAD2, p-SMAD2 and SMAD4 and decreasing SMAD7, reducing immune factors TNF-α and IFN-γ, decreasing apoptosis cytokines Fas, FasL and *Bcl2*/*Bax*, and inhibiting the effect of LY364947 in HepG2 cells.

CONCLUSION

MJF inhibits HCC by activating the TGF-β1/SMAD signaling pathway, and affecting immune and apoptotic cytokines, which may be due to MJF adjusting immune escape and apoptosis.

Key Words: Mu Ji Fang granules; Hepatocellular carcinoma; Transforming growth factor β1/Mothers against decapentaplegic homolog; Immune escape; H22 tumor-bearing mice; HepG2 cells

Core Tip: Mu Ji Fang Granules (MJF), a Chinese patent medicine, has been used in hepatitis, cirrhosis and hepatocellular carcinoma (HCC) for more than 30 years. MJF was identified with Molecule Network related to High Performance Liquid Chromatography-Electron Spray Ionization-Time of Flight- Mass Spectrometry, and hub potential anti-HCC targets were screened using network pharmacology and pathway enrichment analysis in H22 tumor-bearing mice and HepG2 cells. It was shown that MJF improved body weight gain and tumor inhibition rate, protected immune organs and liver function, affected immunity and apoptosis, up-regulated the Transforming growth factor β1(TGF-β1)/ Mothers against decapentaplegic homolog(SMAD) signaling pathway, and inhibited the effect of TGF-β1 inhibitor (LY364947).

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INTRODUCTION

In the World Health Organization 2018 World Cancer Report, liver cancer is the sixth most common cancer and the fourth most common cause of cancer death worldwide, and the age-standardized rates in Asia and Africa were 2 to 3 times those in America, Europe, and Oceania[1]. In the latest report on cancer epidemiology in China, liver cancer was the third and seventh most common malignancy in males and females, with an incidence rate of 12.74% and 5.40%, and the second and third highest mortality rate of 16.36% and 9.79%, respectively[2].

Mu Ji Fang granules (MJF, also known as Fufang Mu Ji Granules), a Chinese patent medicine produced by Dandong Pharmaceutical Group Co., Ltd (Liaoning, China), derived from a folk prescription of the Manchu medicine, consists of 4 herbs including Sophorae Tonkinensis Radix[3], Cuscutae Semen[4], Juglans mandshurica Maxim[5] and Coriolus versicolor[6] (Table 1), processed into

Table 1 Four herbs in Mu Ji Fang granules

Herb	Latin name	Family and genus	Plant	Medicinal parts
Sophorae tonkinensis Radix	Sophorae tonkinensis Radix et rhizoma	Leguminosae	Sophora tonkinensis Gapnep	Root
Cuscutae semen	Cuscutae semen	Convolvulaceae	Cuscuta australis R. Br. or Cuscuta chinensis Lam	Seed
Juglans mandshurica Maxim	Juglans mandshurica Maxim	Juglandaceae	Juglans mandshurica	Bark
Coriolus versicolor	Coriolus versicolor	Basidiomycetes	Coriolus versicolor (L. ex Fr.) Quel	Fruit body

proprietary Chinese medicine, has been used in patients with hepatitis, cirrhosis and hepatocellular carcinoma (HCC) for many years and has a considerable curative effect in clinical practice[7]. Previous studies of MJF mainly focused on its clinical efficacy in hepatitis B[8,9] and fundamental pharmacological effects[10], but few reported the mechanism of action on tumor immunology in the treatment of HCC. Our research group previously established the High Performance Liquid Chromatography (HPLC) fingerprint chromatogram of MJF, combined with HPLC-Electron Spray Ionization-Time of Flight- Mass Spectrometry(HPLC-ESI-TOF-MS), and identified the main compounds in MJF to be alkaloids, flavonoids, and polysaccharides. Based on these findings we hypothesize that MJF might be able to promote tumor cell apoptosis, anti-inflammatory activity and enhance immunity.

In this study, we used Molecule Network (MN) coupled with UPLC-ESI-TOF-MS to analyze the compounds in mouse plasma and a chemical profile was built based on the structures of these compounds. Combined with the compounds from MN, network pharmacology was adopted to analyze the anti-HCC pharmacological mechanisms of MJF. H22 tumor-bearing mice and HepG2 cells were introduced as experimental support for verification, Enzyme Linked Immunosorbent Assay (ELISA), Real Time Quantitative PCR (RT-qPCR), and Western blotting assays were used to evaluate the expression levels of target genes *in vivo* and *in vitro*. Our data indicated that MJF had an anti-HCC effect by regulating immune escape and promoting apoptosis.

MATERIALS AND METHODS

Establishment of H22 tumor-bearing mouse models and sample preparation

Eight-week-old Institute of Cancer Research (ICR) male mice weighing 18-22 g, (Experimental Animal Center of Liaoning Changsheng Biological Technology Co., Ltd., License No. SCXK Liao 2015-0001), were adaptively raised in a controlled-environment animal experiment room (temperature 20°C-25°C, humidity 45%-65%, light/dark cycle for 12 h/12 h) for 7 d with free access to food and tap water. All experimental procedures were performed with the approval of the animal guidelines and protocols established by the Medicine Ethics Review Committee of Animal Experiments of Liaoning University of Traditional Chinese Medicine (TCM) and The Affiliated Hospital of Liaoning University of TCM. Mouse HCC H22 cell strain (Jiangsu Chi Scientific Co., Ltd.) was cultured in RPMI-1640 culture medium (Gibco, Thermo Fisher Scientific, Waltham, MA, United States) containing 1% streptomycin and 10% fetal bovine serum (100 μg/mL penicillin, 100 μg/mL streptomycin) (Gibco Inc. United States) in an incubator at 37 °C, 5% CO_2 and 95% of relative saturation humidity. H22 cells in the logarithmic growth period were adjusted to 1 × 10^7/mL with saline for intraperitoneal injection into mice at 0.2 mL each animal. Five days later, the mice developed ascites due to the growth of H22 cells in the abdominal cavity. We extracted abdominal ascites from the mice and adjusted them to 1 × 10^7/mL with saline as the first generation ascites and repeated the above intraperitoneal injection twice in healthy mice. The third generation ascites were harvested and adjusted to 1 × 10^7/mL with saline as the final implant cell line. An axillary vaccination was conducted in each mouse using 0.2 mL H22 diluted ascites. Three days later, nodules were observed in the right armpit of the mouse, indicating that the established model was successful.

Forty mice were randomly divided into the Model group, cyclophosphamide (CTX) group (cyclophosphamide, 2.7 mg/mL), and 3 MJF groups (M-L received a low MJF dose of 1.8 g/kg, M-M received a mid MJF dose of 5.4 g/kg, M-H received a high MJF dose of 10.8 g/kg) with 8 mice in each group. A further 8 ICR male mice (weighing 18-22 g) were included in the Blank group. All mice were administered the drugs orally once a day (the Blank and Model groups received saline) for 7 d, no food was allowed 12 h before the experiment, but the animals had free access to water. Thirty minutes after the final drug administration, all mice were euthanized under deep anesthesia. Body weight was measured and blood was collected for analysis. After sacrifice, intact thymus, spleen, and tumor tissues were removed and weighed (Figure 1). The thymus index, spleen index, and tumor inhibitory rates in

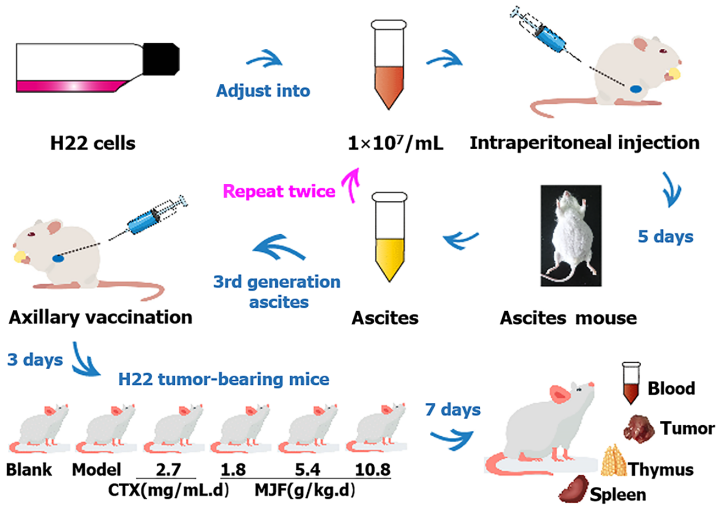

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Figure 1 Establishment of H22 tumor-bearing mice. CTX: Cyclophosphamide; MJF: Mu Ji Fang Granules.

each mouse were calculated according to the following formulas.

Thymus index = thymus weight (mg)/ body weight (g) × 10

Spleen index = spleen weight (mg)/ body weight (g) × 10

Tumor inhibitory rate (%) = (1 - average tumor weight in the drug administration group/ average tumor weight in the Model group) × 100%

Drug and plasma sample preparation for HPLC-ESI-TOF-MS and ingredient identification

Five gram MJF granules (#20190725334, provided by Dandong Pharmaceutical Group Co., Ltd., Dandong China) were weighed and added to 30 mL anhydrous methanol (HPLC grade, Merck Co. Ltd., Darmstadt, Germany), ultrasonically extracted for 30 min, and then filtered through a micropore membrane (0.22 mm; Jinteng Corp., Tianjin, China) before use. Seventeen accurately weighed reference substances were mixed and dissolved in 10 mL of methanol to obtain a solution at the concentration of 21.00 μg/mL for protocatechuic acid, 24.54 μg/mL for ellagic acid, 18.96 μg/mL for kaempferol-3-O-rutinoside, 17.45 μg/mL for rutin, 15.09 μg/mL for hyperoside, 20.64 μg/mL for isoquercitrin, 18.44 μg/mL for astragalin, 17.42 μg/mL for kaempferol, 18.40 μg/mL for isorhamnetin, 20.62 μg/mL for marine, 23.36 μg/mL for sophocarpine, 18.43 μg/mL for gallic acid, 16.48 μg/mL for naringenin, 22.72 μg/mL for cytisine, 21.53 μg/mL for caffeate, 21.34 μg/mL for quercetin (Sichuan Victory Biological Technology Co., Ltd., Sichuan, China) and 18.92 μg/mL for ferulic acid (HPLC grade, Tianjin Kermel Chemical Co., Tianjin, China). 50 μL of thawed frozen plasma samples of MJF high dose (M-H) were placed into 1 mL centrifuge tubes, and then thoroughly mixed with 100 μL methanol and vortexed for 1 min. The solutions were centrifuged at 8000 rpm/min and 4 °C for 10 min. The supernatants were filtered through a 0.22 μm membrane for HPLC-MS analyses.

The HPLC–MS analysis was performed using an Agilent 1290 HPLC system (Agilent Technologies, Inc., CA, United States) in tandem with an Agilent 6550 quadrupole-time of flight with mass spectrometry (Agilent Co., United States). The analysis system consisted of an Agilent Proshell SB-C18 column (100 mm × 3 mm, 2.1 μm) (Agilent Technologies, Inc., CA, United States) and was used with the column temperature maintained at 35 °C; the flow rate of the mobile phase was 0.4 mL/min and the injection volume was 0.6 μL, the mobile phase consisted at 0.1% formic acid in water of A and acetonitrile of B, and the column was eluted with a linear gradient of 3%–8% B over initial to 5.0 min, 8%–15% B over 5.0-9.0 min, 15%-22% B over 9.0-12.0 min, 22%-55% B over 12-15 min, 55%-70% over 15-20 min,70%–95% B over 20.0–24.0 min, returned to 3% B for 1.0 min and then held for 2.5 min at an eluent flow rate of 0.5 mL/min. Optimal conditions for HPLC-Q-TOF-MS analysis were as follows: ion source was Dual AJS ESI, both positive and negative ion mode detection was adopted, drying gas flow of 13 L/min, drying gas temperature maintained at 350 °C, a capillary voltage of 3500 V, nebulizer pressure of 310.28 kPa, frag mentor voltage of 125 V, OCT 1RF Vpp voltage of 750 V, collision energy of 40 eV and mass spectrometric data were acquired in the mode from 1000 to 1000 m/z with an acquisition rate of 1.5 spectra/s. Data were corrected and obtained during acquisition using a correction mixed solution (Agilent Technologies, Inc., CA, United States, m/z = 112.985587, m/z = 1033.988109).

Molecular networking establishment of MJF and the identification of absorbable ingredients

For MN data processing, MS/MS data on MJF extraction and MJF high dose were collected and converted into mzXML format using Proteo Wizard software (www.proteowizard.sourceforge.net, Proteo Wizard, Palo Alto, CA, United States) and then uploaded separately into the GNPS platform (

https://gnps.ucsd.edu, UCSD, San Diego, CA, United States) (accessed on 16 November 2021) (Figure 2A). The GNPS parameters were as follows: mass error of less than 0.02 Da, matched peaks greater than 3, and cosine score greater than 0.50. After analysis, graphic format files were generated from the GNPS platform, and the files were then downloaded and imported into Cytoscape software v 3.7.0 (www.cytoscape.org, NRNB, Hill St, San Diego, CA, United States) to build the molecular network. According to Wang *et al*'s study[11], MN of MJF extraction and plasma after MJF oral administration were merged, and the absorbed constituents of MJF in plasma were obtained. All constituents were identified using reference substances on HPLC-Q-TOF-MS.

Network pharmacology construction and pathway enrichment analysis of MJF

One thousand two hundred and thirty-two HCC-related genes were extracted from DisGeNET[12] (https://www.disgenet.org) and two liver cancer databases Liverome[13] (http://Liverome.kobic.re.kr/index.php) and OncoDB. HCC[14] (http://oncodb.hcc.ibms.sinica.edu.tw). Validated and predicted targets of 17 ingredients screened from MN of MJF were collected from Swiss Target Prediction[15] (http://www.swisstargetprediction.ch), an online tool to predict the macromolecular targets (proteins from human, mouse, and rats) of small bioactive molecules. All the ingredient-related genes were mapped to HCC-related genes to obtain a total of 221 shared targets, which were then uploaded to the STRING database[16] (https://string-db.org) to acquire the protein-protein interaction (PPI) network. Cytoscape software and the Network Analyzer App were employed for topological analysis of the PPI network, and the medians of each index were calculated. Nodes of a degree over 19, betweenness centrality over 0.00283, and closeness centrality over 0.51485 were gathered as predicted targets and were uploaded to the Metascape database[17] (https://metascape.org/) for possible Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment. The final results of the ingredient-pathway-target network were demonstrated by Cytoscape.

Histopathological analysis and biochemical analysis

Mouse tumor tissues were immediately fixed in 4% paraformaldehyde (Beijing Solaibao Technology Co., Ltd., Beijing, China). Following paraffin embedding, the tissues were cut into 4 μm slices, stained with hematoxylin and eosin (HE), and morphological changes were observed with a microscope (× 200 high power visual field).

Plasma samples from all five groups were centrifuged at 8000 rpm/min and 4 °C for 10 min. The supernatants were then used for Interferon gamma (IFN-γ), Tumor necrosis factor α (TNF-α), Interleukin-2 (IL-2), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alpha-fetoprotein (AFP), Fas, and FasL analyses by ELISA in accordance with the kit instructions (Shanghai Langton Biotechnology Co., Ltd., Shanghai, China), respectively.

HepG2 cell culture and drug administration

The HepG2 cell strain (Jiangsu Chi Scientific Co., Ltd, Jiangsu, China) was cultured in DMEM culture medium containing 1% streptomycin and 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, United States) (100 μg/mL penicillin, 100 μg/mL streptomycin) in an incubator at 37 °C, 5% CO_2 and 95% of relative saturation humidity. Cells at the density of 1 × 10^5/mL were inoculated into 6-well plates, and after culture for 24 h the medium was removed. 4.0 g MJF was added to 20 mL 50% methanol and treated with ultrasonic extraction for 20 min (700 W, 40 kHz), and the liquid was filtered and dried. 1.0 g MJF were mixed with 0.5 mL DMSO, diluted with DMEM culture medium to 10 mg/mL, 20 mg/mL, 30 mg/mL, and 40 mg/mL, and then added to a 6-well plate with 1 mL of MJF in each well (M1, M2, M3, and M4). The Transforming growth factor β1(TGF-β1) inhibitor Ly364947 (#HY-13462, MedChem Express Co., Ltd., NJ, United States) was adjusted to 1 μM and 2.5 μM, respectively, and added to 6-well plates with 1 mL of LY364947 in each well. M2 + LY-1 and M4 + LY-2 groups contained 20 mg/mL and 40 mg/mL of MJF each and 1 μM Ly364947. 1 mL culture medium was added to the Blank group. All groups were cultured for 8 h.

Quantitative RT-qPCR analysis

Tumor tissue was placed in TRlzol reagent to obtain a homogenate, and total RNA was extracted according to the manufacturer's instructions. The reverse transcription kit (Trans Script One-Step gDNA Removal and cDNA Synthesis Super Mix, Beyotime Biotechnology Co., Ltd., Shanghai, China) was used to reverse transcribe the extracted RNA into cDNA and processed as previously described[18]. The total RNA in HepG2 cells in each group was processed in the same way. The primer sequences for RT-qPCR of tumor tissue and HepG2 cells are listed in Tables 2 and 3.

Western blotting assay for target protein detection

Tumor tissue proteins from the mice in each group were extracted with RIPA reagent. Proteins were separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Beyotime Biotechnology Co., Ltd., Shanghai, China). The membranes were washed, blocked with BSA blocking solution for 2 h, and then incubated overnight with anti-β-actin (#20536-1-AP), anti-TGF-β1 (#21898-1-AP), anti- Mothers against decapentaplegic homolog(SMAD) 4 (#10231-1-AP), anti-SMAD7 (#25840-1-AP) (Proteintech

Table 2 Primer sequences for Real Time Quantitative PCR of tumor tissue

Name of primers	Sequences
Bax-F	*5′-AGGATGCGTCCACCAAGAA-3′*
Bax-R	*5′-CAAAGTAGAAGAGGGCAACCAC-3′*
Bcl-2-F	*5′-CAACACTCCCTCTTGACCTATGC-3′*
Bcl-2-R	*5′-GAAAATGTTCCCAAGTGAGTTAGA-3′*
β-actin-F	*5′-GTCCCTCACCCTCCCAAAAG-3′*
β-actin-R	*5′-GCTGCCTCAACACCTCAACCC-3′*

Table 3 Primer sequences for Real Time Quantitative PCR of HepG2 cells

Name of primers	Sequences
TNF-α-F	*5′-ATCGGTCCCAACAAGGAGGAGAAGT-3′*
TNF-α-R	*5′-ACGTGGGCTACGGGCTTGTCACTC-3′*
IFN-γ-F	*5′-ACAACCAGGCCATCAGCAACAACATA-3′*
IFN-γ-R	*5′-CTGTGGGTTGTTCAGCTCGAACTT-3′*
Bax-F	*5′-AAACTGGTGCTCAAGGCCC-3′*
Bax-R	*5′-AAAGTAGGAGAGGAGGCCGTC-3′*
Bcl-2-F	*5′-CAGGATAACGGAGGCTGGGATG-3′*
Bcl-2-R	*5′-TTCACTTGTGGCCCAGATAGG-3′*
β-actin-F	*5′-GGACCTGACTGACTACCTC-3′*
β-actin-R	*5′-TACTCCTGCTTGCTGAT-3′*

TNF-α: Tumor necrosis factor α; INF-γ: Interferon gamma.

Group, Inc., Rosemont, IL, United States), anti-SMAD2 (#5339S), and anti-phosphorylated-SMAD2 (#3108S, Cell Signaling Technology, Danvers, MA, United States) at 4 °C. After washing with TBST 3 times for 10 min each time, the membranes were incubated with secondary antibodies (Goat Anti-Rabbit IgG H + L) (#SA00001-2, Proteintech Group, Inc., Rosemont, IL, United States) for 2 h at room temperature. Immunoreactivity was determined using ECL glow (Beyotime Biotechnology Co., Ltd., Shanghai, China). Data analysis was conducted *via* Image J 1.8.0 software (National Institutes of Health, Bethesda, MD, United States). Protein in HepG2 cells from each group was processed using the same steps as above.

Statistical analysis

Statistical analyses were performed with SPSS 25.0 software (IBM Corporation, Armonk, NY, United States) using one-way ANOVA and data were expressed as the mean ± SD. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered very significant. RT-qPCR data were analyzed by the $2^{-\Delta\Delta Ct}$ algorithm[19].

RESULTS

MJF ingredient identification in plasma after oral administration

A comprehensive MN based on LC-MS/MS spectra in order to reveal absorbable ingredients in MJF was performed as shown in Figure 2B. The MN map contained 933 precursor ions, including 137 clusters (nodes > 2) and 352 single nodes. A total of 17 main prototype components (P1-17) were screened out by the GNPS MS database, based on the mass measurements and fragmentation patterns confirmed by reference substances. These compounds were identified as cysteine, gallic acid, matrine, sophocarpine, protocatechuic acid, caffeate, hyperoside, quercetin, isoquercitrin, rutin, kaempferol-3-o-rutinoside, astragalin, naringenin, kaempferol, ellagic acid, ferulic acid, and isorhamnetin (Figure 2, Table 4). These compounds were of three main types: Polyphenol acids, flavonoids, and alkaloids, all of which were selected and used for the following target prediction in network pharmacology analysis.

Table 4 Compound identification in Mu Ji Fang Granules by High Performance Liquid Chromatography-Electron Spray Ionization-Time of Flight- Mass Spectrometry with reference substances

No.	RT (min)	[M-H/M+FA]-	Actual-M	Formula	Proposed compound
1	3.412	189.1291	189.1290	$C_{11}H_{14}N_2O$	Cytisine
2	3.585	169.0149	169.0145	$C_7H_6O_5$	Gallic acid
3	3.607	249.2185	249.2179	$C_{15}H_{24}N_2O$	Matrine
4	3.615	247.2023	247.2020	$C_{15}H_{22}N_2O$	Sophocarpine
5	4.997	153.0922	153.0914	$C_7H_6O_4$	Protocatechuic acid
6	7.109	178.0506	179.0349	$C_{15}H_{24}N_2O_2$	Caffeate
7	9.003	463.0878	463.0873	$C_{21}H_{20}O_{12}$	Hyperoside
8	9.271	303.0512	303.0508	$C_{15}H_{10}O_7$	Quercetin
9	9.832	464.3002	464.2997	$C_{21}H_{20}O_{12}$	Isoquercitrin
10	10.183	610.3217	610.3213	$C_{27}H_{30}O_{16}$	Rutin
11	10.452	594.2133	594.2131	$C_{27}H_{35}O_5$	Kaempferol-3-O-rutinoside
12	10.793	476.3361	476.3364	$C_{21}H_{20}O_{11}$	Astragalin
13	12.033	271.0689	271.0681	$C_{15}H_{12}O_5$	Naringenin
14	15.525	288.3132	288.3128	$C_{15}H_{10}O_6$	Kaempferol
15	17.542	302.3295	302.3287	$C_{14}H_6O_8$	Ellagic acid
16	18.413	194.0819	194.0823	$C_{10}H_{10}O_4$	Ferulic acid
17	20.414	318.3255	318.3251	$C_{16}H_{12}O_7$	Isorhamnetin

RT: Real time.

Network pharmacology construction of the anti-HCC targets of MFJ and pathway enrichment analysis

Seventeen ingredients related 1232 genes to HCC related genes were mapped, a total of 166 targets were obtained and an ingredients-anti-HCC-target network was established. The degree of each node was measured by the Network Analyzer App in Cytoscape (Figure 3A). The degree of one node indicates the number of nodes that have direct interactions with it, the higher degree one node has, the more biological processes it participates in, and the more biological importance it possesses.

From the topological analysis of 177 ingredients-anti-HCC-targets through the PPI network generated by the STRING database, 72 predicted targets were acquired. By uploading all these predicted targets into the Metascape database, we obtained the GO and KEGG pathways to enrich the analysis. Figure 3B lists the top 5 GO pathways to enrich the analysis indicating that MJF could respond to inorganic substances and xenobiotic stimulus *etc*. of Biological Processes (BP), kinase binding and transcription factor binding *etc*. of Cellular Components (CC), and membrane raft, vesicle lumen *etc*. of Molecular Functions (MF). Among the top 15 KEGG pathways which enriched the analysis (Figure 3B), only 6 of them were related to HCC including pathways in cancer, hepatitis B, proteoglycans in cancer, hepatitis C, HCC, and the TGF-β signaling pathway. From the heatmap in Figure 3B, 14 genes with the most significantly different abundance related to the 6 KEGG pathways of HCC were clustered, including FAS, *BCL2*, TNF, IL2, *BAX*, IFNG, TGFB1, SMAD4, MMP9 SRC, EGF, STAT1, IGF2, and IGF1R. A comprehensive predicted ingredient-anti-HCC-pathway-target network is shown in Figure 3C, where 17 potentially effective compounds related to the 6 pathways of HCC, from which, we clustered 8 hub-targets had a greater degree (above the median of 34.763) from the topological analysis of the network including TNF, *BAX*, *BCL2*, TGFB1, IFNG, FAS, IL2 and SMAD4 (Figure 3D).

Regulatory effects of MJF on H22 tumor-bearing mice

As displayed in Figure 4A, except for the Model group, the average body weight in the other groups all showed varying rapid gain. Compared with the Blank group, H22 tumor-bearing mice in the Model group had a much higher spleen index and lower thymus index ($P < 0.05$), due to H22 bearing tumors causing splenomegaly and thymic atrophy in mice. The CTX and MJF groups (M-L, M-M and M-H) revealed a significantly reduced spleen index and increased thymus index relative to the Model group, and in the MJF groups these levels progressed with increased dose ($P < 0.05$, $P < 0.01$). Figure 4B indicates that the concentration of AFP (HCC indicator), ALT and AST (liver function indicators), and TNF-α, IFN-γ, and IL-2 (immune factors) in the Model group markedly increased compared with the

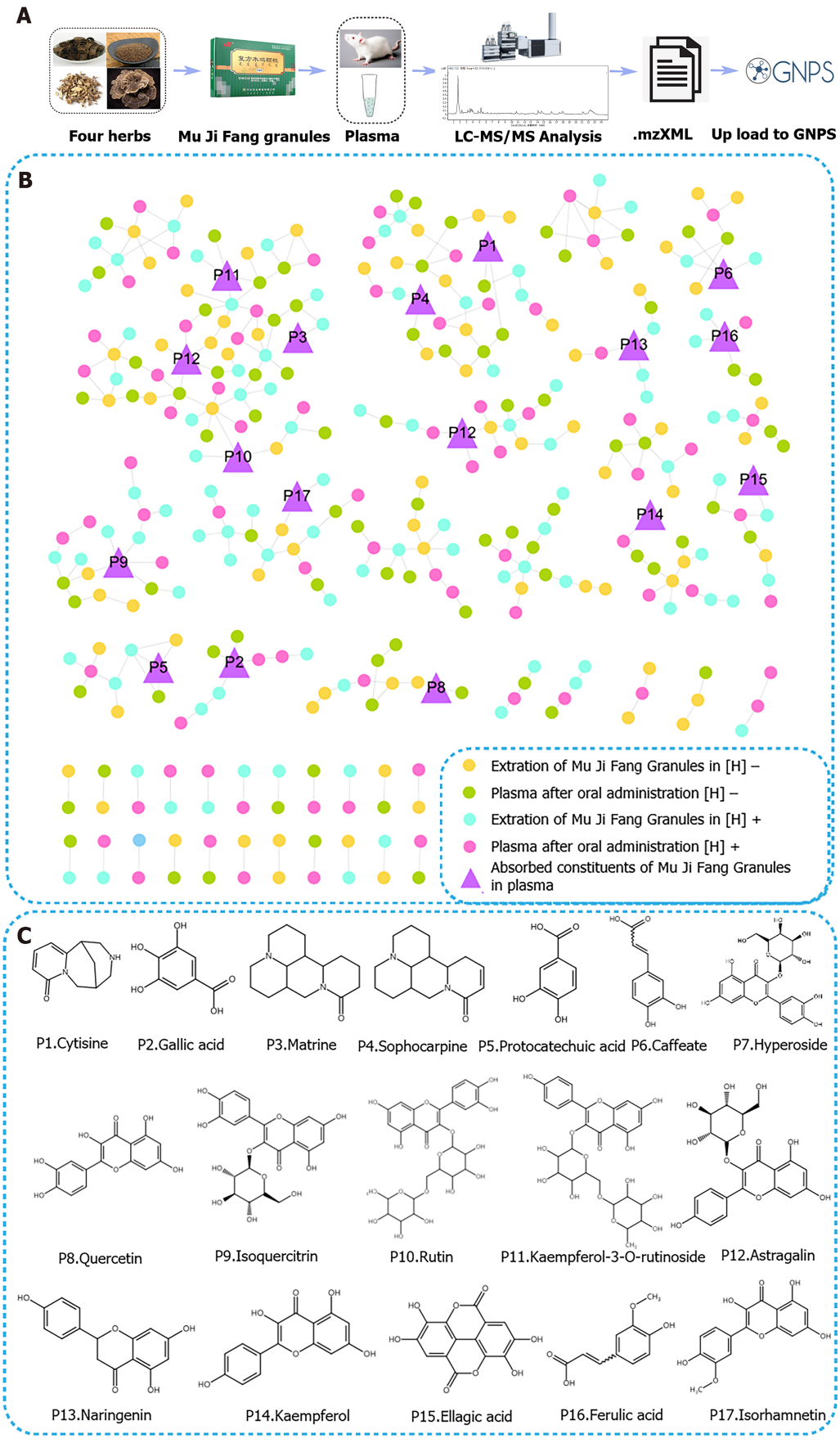

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Figure 2 Identification of the absorbable ingredients in Mu Ji Fang Granules using molecular networking. A: Workflow of the molecular networking approach; B: Whole molecule networking profile of Mu Ji Fang Granules after oral administration; C: Absorbable ingredients identified using reference substances. mzXML: A kind of format for Mass spectrometry data file. GNPS: Website of Global Nature Products Social Molecular Networking; LC-MS/MS: Liquid

Chromatograph Mass Spectrometer.

Blank group ($P < 0.05$), which confirmed that H22 tumors may affect immune action and inflammation in the liver and reduce liver function in mice. Compared with the Model group, CTX and MJF effectively reduced the concentration of AFP, ALT, AST, TNF-α and IFN-γ, and increased the concentration of IL2. With an increase in dose, the MJF mid and high dose groups (M-M and M-H) showed significant enhancement in adjusting the concentration of the above indicators ($P < 0.05$, $P < 0.01$).

As shown in Figure 4C, the size of the tumor in the Model group was larger and the color was darker, blood and vessels were clearly observed on the surface, while the tumors in the other groups were pale, and the size of the tumor and the average inhibitory rate in the 3 MJF groups (M-L, M-M and M-H) gradually changed with increased dose, respectively. Figure 4D shows the microscopic images and local enlarged images of HE stained sections of tumor tissue from each group. The tumor cells in the Model group were densely arranged with high cell density and large nuclei, and necrotic cells and cytoplasm were rarely seen. In the CTX group, tumor cells were loosely arranged, necrotic cells showed nucleus necrosis and rupture, and the cytoplasm was condensed and shrunken. In contrast, the three MJF groups all had different degrees of necrosis and apoptotic cell areas. The cytoplasm was highly agglutinated and condensed. In the MJF high dose group (M-H), the number of dead cells and round apoptotic bodies were more numerous, the size of the cells varied, and the connective tissue was more obvious than in the other two MJF groups (M-L and M-M).

We evaluated the concentration of the cell apoptotic factors Fas and FasL, and the mRNA expression of *Bax* and *Bcl2*, two cytokines that can form homologous or heterologous dimers to regulate apoptosis, in order to determine the effect of MJF in improving the apoptosis of cancer cells. As shown in Figure 5A, the concentrations of Fas and FasL in the Model group were markedly increased relative to the Blank group, whereas, CTX and MJF (M-L, M-M and M-H) reduced the concentrations, respectively ($P < 0.01$). In addition, CTX and MJF also up-regulated the relative mRNA expression of *Bax* and down-regulated that of *Bcl2* compared to the Model group ($P < 0.01$) (Figure 5B). Moreover, Western blot analysis was performed to determine the protein level of TGF-β1 and SMAD4, and it was demonstrated that CTX and MJF significantly increased the expression of TGF-β1 and SMAD4, and the expression levels in the MJF mid and high dose groups were even higher than that in the CTX group ($P < 0.01$) (Figure 5C).

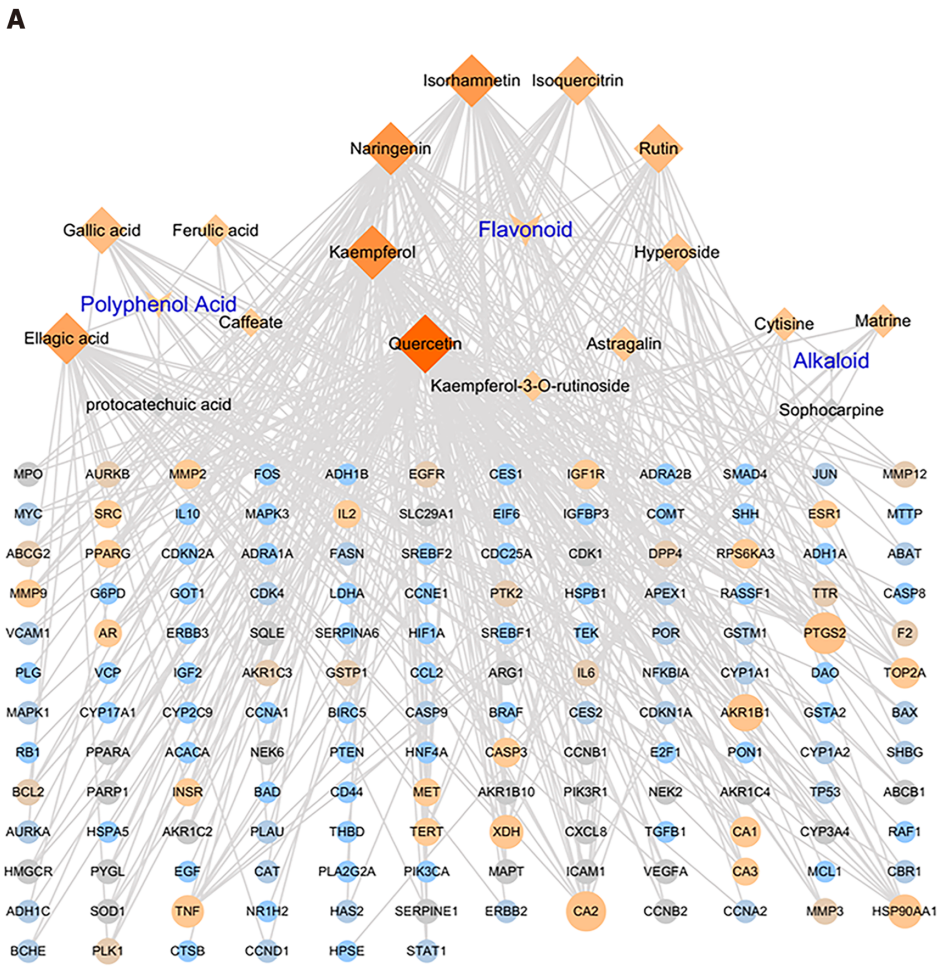
MJF modulated the TGF-β1/SMAD pathway, immune and apoptotic cytokines in HepG2 cells

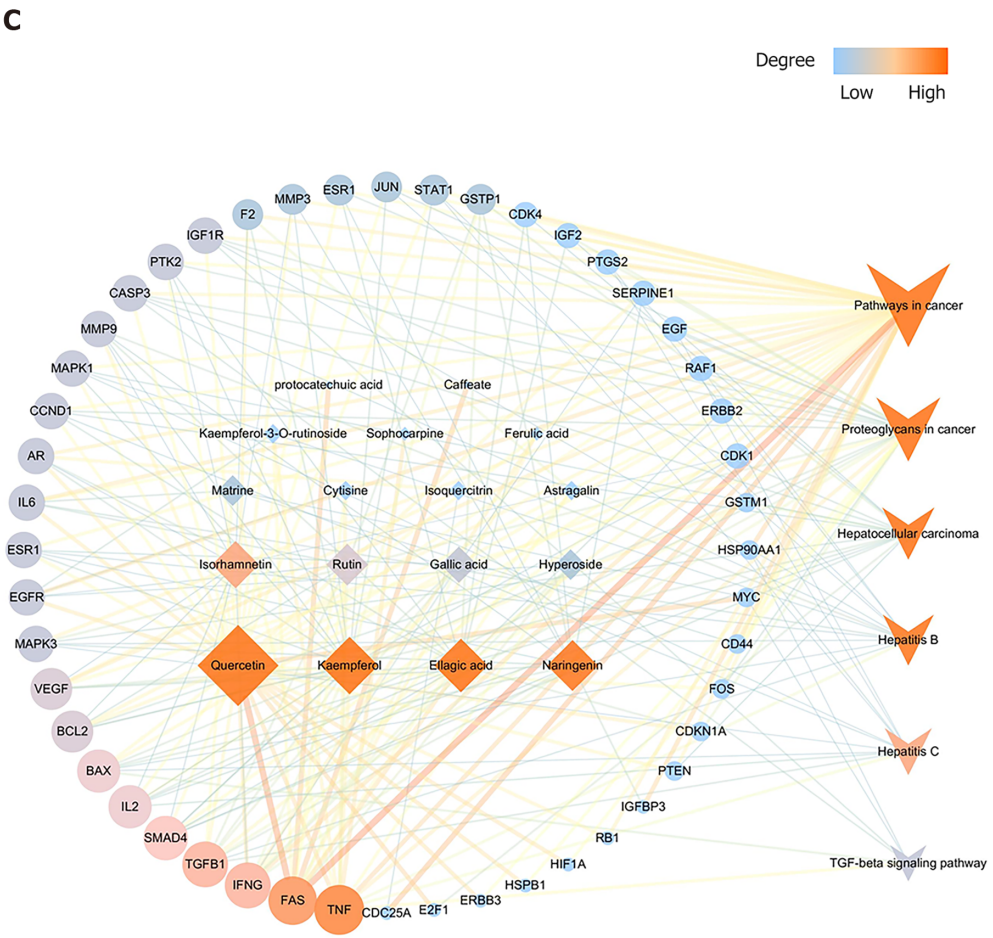
Western blot analysis was used to determine the protein expression of TGF-β1, SMAD2, p-SMAD2, SMAD4, and SMAD7, and we evaluated the effectiveness of MJF in regulating immune escape factors and promoting apoptosis in HepG2 cells, as the TGF-β1/SMAD signaling pathway plays an essential role in the development and invasion of HCC. The results showed that MJF (10 mg/mL to 40 mg/mL effectively increased the expression of TGF-β1, SMAD2, p-SMAD2 and SMAD4, and reduced the expression of SMAD7 protein relative to the levels in the Control group (MJF 0 mg/mL), respectively ($P < 0.05$) (Figure 6A). Furthermore, the results also showed that, compared with the Control group (MJF 0 mg/mL), mRNA expression of the immune cytokines, TNF-α and IFN-γ, in MJF (10 mg/mL to 40 mg/mL) was markedly decreased, and mRNA expression of the apoptosis cytokine, *Bax* in MJF (10 mg/mL to 40 mg/mL) was significantly increased and that of *Bcl2* was reduced ($P < 0.05$) (Figure 6B and C).

MJF alleviated the modulation of TGF-β1 inhibitor (LY364947) on the TGF-β1/SMAD pathway, and immune and apoptotic cytokines in HepG2 cells

To evaluate the regulation of MJF on the TGF-β1/SMAD signaling pathway and both immune and apoptotic cytokines in HepG2 cells, LY364947, a TGF-β1 inhibitor, was added to the cell culture. As demonstrated in Figure 7A, relative to the Control group (LY364947 0 μM and MJF 0 mg/mL), LY364947 effectively inhibited the expression of TGF-β1, SMAD2, p-SMAD2 and SMAD4 protein and promoted that of SMAD7 (LY364947 2.5 μM and MJF 0 mg/mL), while, MJF (LY364947 2.5 μM, MJF 20 and 40 mg/mL) reduced the effect of LY364947 by improving the expression of TGF-β1, SMAD2, p-SMAD2 and SMAD4, and down regulating SMAD7 ($P < 0.05$).

With regard to the immune cytokines, TNF-α and IFN-γ, compared to the Control group (LY364947 0 μM and MJF 0 mg/mL), LY364947 significantly increased mRNA expression of TNF-α and IFN-γ (LY364947 2.5 μM and MJF 0 mg/mL), and MJF (LY364947 2.5 μM, MJF 20 and 40 mg/mL) showed a marked decrease in these two cytokines ($P < 0.05$) (Figure 7B). Moreover, compared with the Control group LY364947 0 μM and MJF 0 mg/mL), the expression of *Bax* mRNA was inhibited and that of *Bcl2* was improved after treatment with LY364947 (LY364947 2.5 μM and MJF 0 mg/mL), yet, MJF (LY364947 2.5 μM, MJF 20 and 40 mg/mL) weakened this effect by increasing *Bax* and reducing *Bcl2*, respectively ($P < 0.05$) (Figure 7C).


A
Isorhamnetin
Isoquercitrin
Naringenin
Rutin
Gallic acid
Ferulic acid
Flavonoid
Kaempferol
Hyperoside
Polyphenol Acid
Caffeate
Ellagic acid
Quercetin
Astragalin
Cytisine
Matrine
Alkaloid
Kaempferol-3-O-rutinoside
protocatechuic acid
Sophocarpine
MPO AURKB MMP2 FOS ADH1B EGFR CES1 IGF1R ADRA2B SMAD4 JUN MMP12
MYC SRC IL10 MAPK3 IL2 SLC29A1 EIF6 IGFBP3 COMT SHH ESR1 MTTP
ABCG2 PPARG CDKN2A ADRA1A FASN SREBF2 CDC25A CDK1 DPP4 RPS6KA3 ADH1A ABAT
MMP9 G6PD GOT1 CDK4 LDHA CCNE1 PTK2 HSPB1 APEX1 RASSF1 TTR CASP8
VCAM1 AR ERBB3 SQLE SERPINA6 HIF1A SREBF1 TEK POR GSTM1 PTGS2 F2
PLG VCP IGF2 AKR1C3 GSTP1 CCL2 ARG1 IL6 NFKBIA CYP1A1 DAO TOP2A
MAPK1 CYP17A1 CYP2C9 CCNA1 BIRC5 CASP9 BRAF CES2 CDKN1A AKR1B1 GSTA2 BAX
RB1 PPARA ACACA NEK6 PTEN HNF4A CASP3 CCNB1 E2F1 PON1 CYP1A2 SHBG
BCL2 PARP1 INSR BAD CD44 MET AKR1B10 PIK3R1 NEK2 AKR1C4 TP53 ABCB1
AURKA HSPA5 AKR1C2 PLAU THBD TERT XDH CXCL8 TGFB1 CA1 CYP3A4 RAF1
HMGCR PYGL EGF CAT PLA2G2A PIK3CA MAPT ICAM1 VEGFA CA3 MCL1 CBR1
ADH1C SOD1 TNF NR1H2 HAS2 SERPINE1 ERBB2 CA2 CCNB2 CCNA2 MMP3 HSP90AA1
BCHE PLK1 CTSB CCND1 HPSE STAT1


C
Degree
Low
High
MMP3 ESR1 JUN STAT1 GSTP1 CDK4 IGF2 PTGS2 SERPINE1 EGF RAF1 ERBB2 CDK1 GSTM1 HSP90AA1 MYC CD44 FOS CDKN1A PTEN IGFBP3 RB1 HIF1A HSPB1 ERBB3 E2F1 CDC25A TNF FAS IFNG TGFB1 SMAD4 IL2 BAX BCL2 VEGF MAPK3 EGFR ESR1 IL6 AR CCND1 MAPK1 MMP9 CASP3 PTK2 IGF1R F2
Pathways in cancer
Proteoglycans in cancer
Hepatocellular carcinoma
Hepatitis B
Hepatitis C
TGF-beta signaling pathway
protocatechuic acid
Caffeate
Kaempferol-3-O-rutinoside
Sophocarpine
Ferulic acid
Matrine
Cytisine
Isoquercitrin
Astragalin
Isorhamnetin
Rutin
Gallic acid
Hyperoside
Quercetin
Kaempferol
Ellagic acid
Naringenin

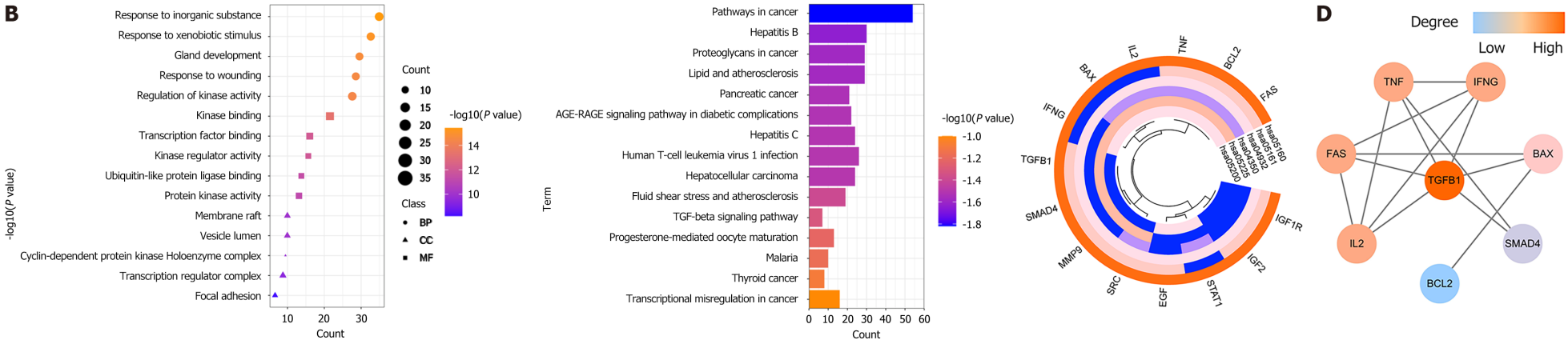


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Figure 3 Network pharmacology construction and pathway enrichment analysis of Mu Ji Fang Granules. A: Ingredients-anti-hepatocellular carcinoma (HCC)-targets network of Mu Ji Fang Granules (MJF) (diamond represents ingredients and ellipse represents targets). The color of the nodes from blue to red indicate the degree from low the high; B: Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and heatmap of the 14 most significantly different abundant genes related to the 6 KEGG pathways of HCC (BP: Represents Biological Process; CC: Represents Cellular Component; and MP: Represents Molecular Function); C: Ingredient-anti-HCC-pathway-target network of MJF (diamond represents ingredients, octagon represents targets and V represents pathway). The color of the nodes from red to yellow to green to blue indicates the degree from high to low; D: Hub targets of MJF most closely related to HCC. TNF: Tumor necrosis factor; TGF: Transforming growth factor; SMAD4: Mothers against decapentaplegic homolog 4; IL: Interleukin; FAS: TNF superfamily receptor 6.

DISCUSSION

HCC is a malignant tumor with high morbidity and mortality worldwide[20]. At present, the main clinical treatments for malignant liver tumors are surgery, radiation, and chemotherapy[21]. Common chemotherapy drugs have side effects such as cytotoxicity, multiple drug resistance, *etc*., and have different degrees of impact on liver function and the immune system[22]. TCM has a particular function in cancer treatment and prevention due to its high multi-target biological activity and low toxicity[23]. The long history and extensive study of TCM indicate its potential advantages in alleviating symptoms and improving quality of life in different patterns and stages of the disease[24]. As a Chinese patent medicine, MJF has been used in the treatment of liver cancer since the 1980s, with the exception of HCC, and has also been proved to have anti-inflammatory effects, enhances immunity, and improves liver function. Thus, in the present study, we mainly focused on exploring the mechanism of action of MJF.

In a recent study, the MN technique associated with HPLC-ESI-TOF-MS was employed in studies of natural products derived from plants, microorganisms, marine organisms, and other biological sources [25]. As a visual computational strategy, MN can be intuitively implemented based on a comparison of the theoretical MS/MS spectra to test MS/MS spectra and establish the relative network by clustering similar structures with similar mass spectra for compound identification[26]. In this study, we imported this recent approach, and established the MN of MJF solution and plasma after oral administration, and by merging the two MNs, the shared compounds were thought to be the absorbable constituents of MJF. To verify this, we also used reference substances to identify the 17 ingredients obtained from MN.

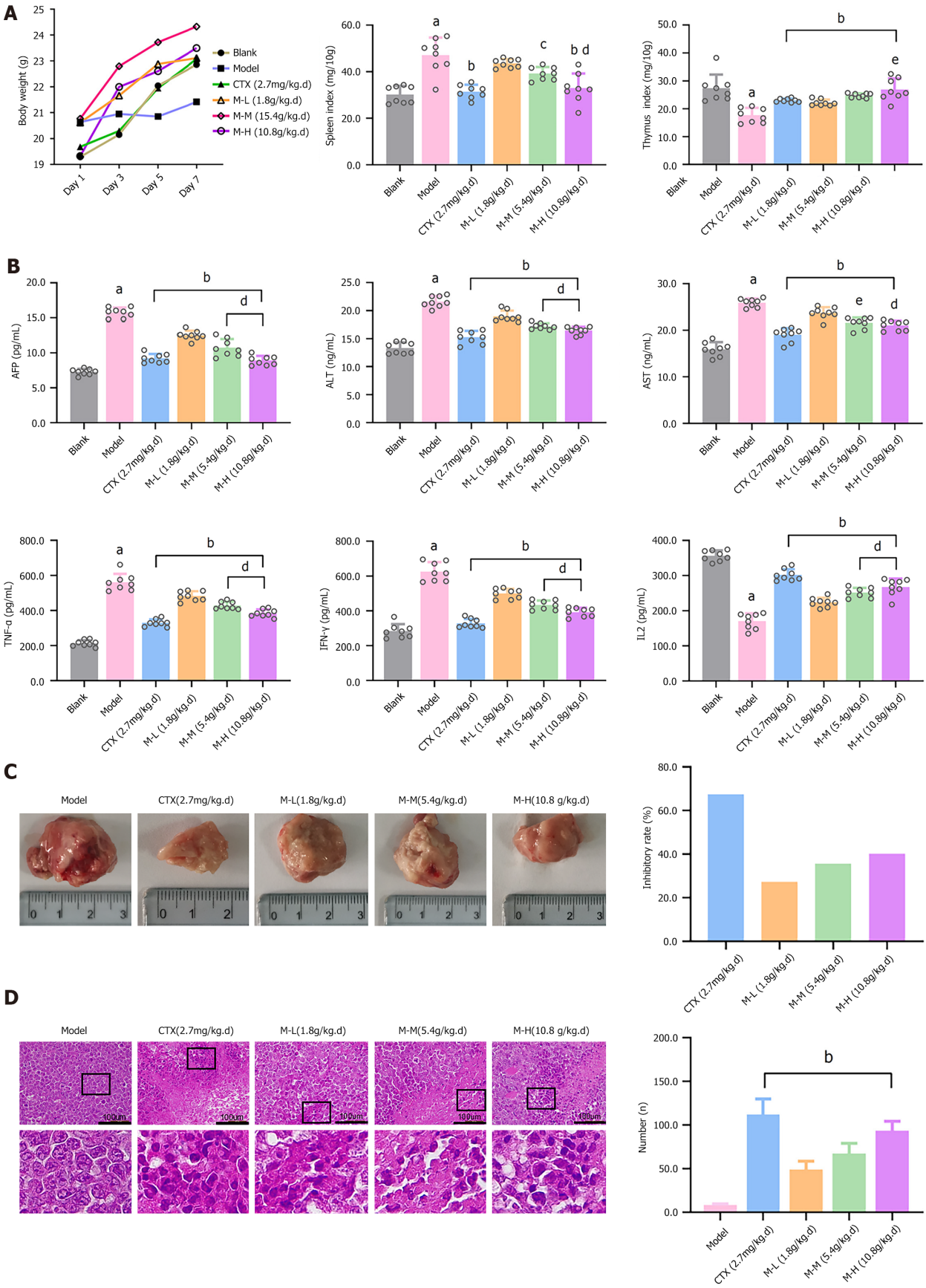

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Figure 4 Mu Ji Fang Granules improved average body weight, spleen and thymus indices, decreased the concentration of hepatocellular carcinoma indicators, liver function indicators and immune factors. A: Average body weight, spleen index and thymus index (n = 8); B: Concentrations

of hepatocellular carcinoma indicator alpha-fetoprotein, liver function indicators alanine aminotransferase and aspartate aminotransferase and immune factors Tumor necrosis factor α, Interferon gamma and Interleukin-2 in serum (n = 8); C: Tumor tissue from H22 tumor-bearing mice and the tumor inhibitory rate (n = 8); D: Hematoxylin and eosin staining of tumor tissue pathology sections from H22 tumor-bearing mice (n = 3). Data are shown as the mean ± standard deviation. [a]P < 0.01 *vs* Blank. [b]P < 0.01 *vs* Model. [c]P < 0.05 *vs* Model. [d]P < 0.01 *vs* M-L. [e]P < 0.05 *vs* M-L.

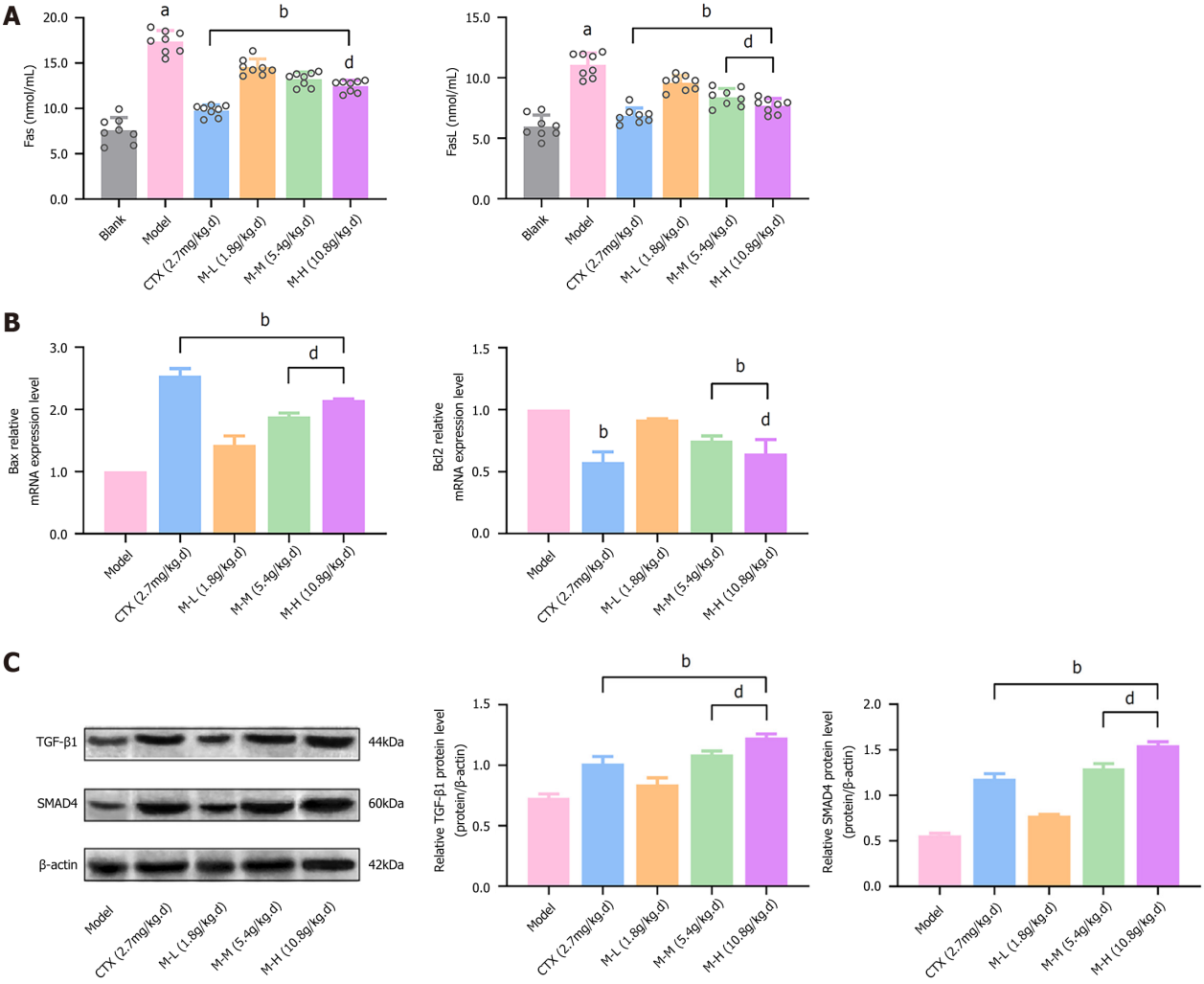

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Figure 5 Mu Ji Fang Granules improved apoptosis and regulated the Transforming growth factor β1/ Mothers against decapentaplegic homolog signaling pathway in H22 tumor-bearing mice. A: The concentration of Fas and FasL in serum (n = 8); B: Relative expression of *Bax* and *Bcl2* mRNA in tumor tissue (n = 3); C: Relative expression of Transforming growth factor β1 and Mothers against decapentaplegic homolog 4 protein in tumor tissue (n = 3). Data are shown as the mean ± standard deviation. [a]P < 0.01 *vs* Blank. [b]P < 0.01 *vs* Model. [d]P < 0.01 *vs* M-L.

We then introduced network pharmacology to establish an image of the function and behavior of the biological network of MJF, identify potential targets and pathways of the drugs related to the disease using topology and computational methods, and explored the topology parameters of the 17 ingredients and their related targets in anti-HCC among all the ingredients-anti-HCC-pathways-targets. As a consequence, targets including TGFB1, SMAD4, TNF, IFNA1, IL2, *BAX*, *BCL2*, and FAS (TNF superfamily receptor 6) were screened out.

The TGF-β1/SMAD signaling pathway plays an important role in the development and invasion of HCC[27,28]. Cytoplasmic protein SMAD is the most critical and important signal transduction factor in the TGF β signaling pathway. When the TGF-β pathway is activated, phosphorylated SMAD2 combines with SMAD4 to form a complex, and then enters the nucleus to activate the expression of downstream target genes. SMAD 7 is a TGF-β signaling inhibitory factor, which is localized in the cell nucleus, and can regulate the activity of TGF-β[29]. IL-2 is an important cytokine which regulates immune function, promotes the proliferation and activation of T cells, and stimulates the proliferation of natural killer cells [30,31]. TNF-α and IFN-γ are essential cellular immune molecules, which affect the immune escape of cells. Clinical studies have shown that disordered TNF-α and IFN-γ levels have a significant impact on the stability of the body's internal environment, leading to abnormal immune function in patients, and

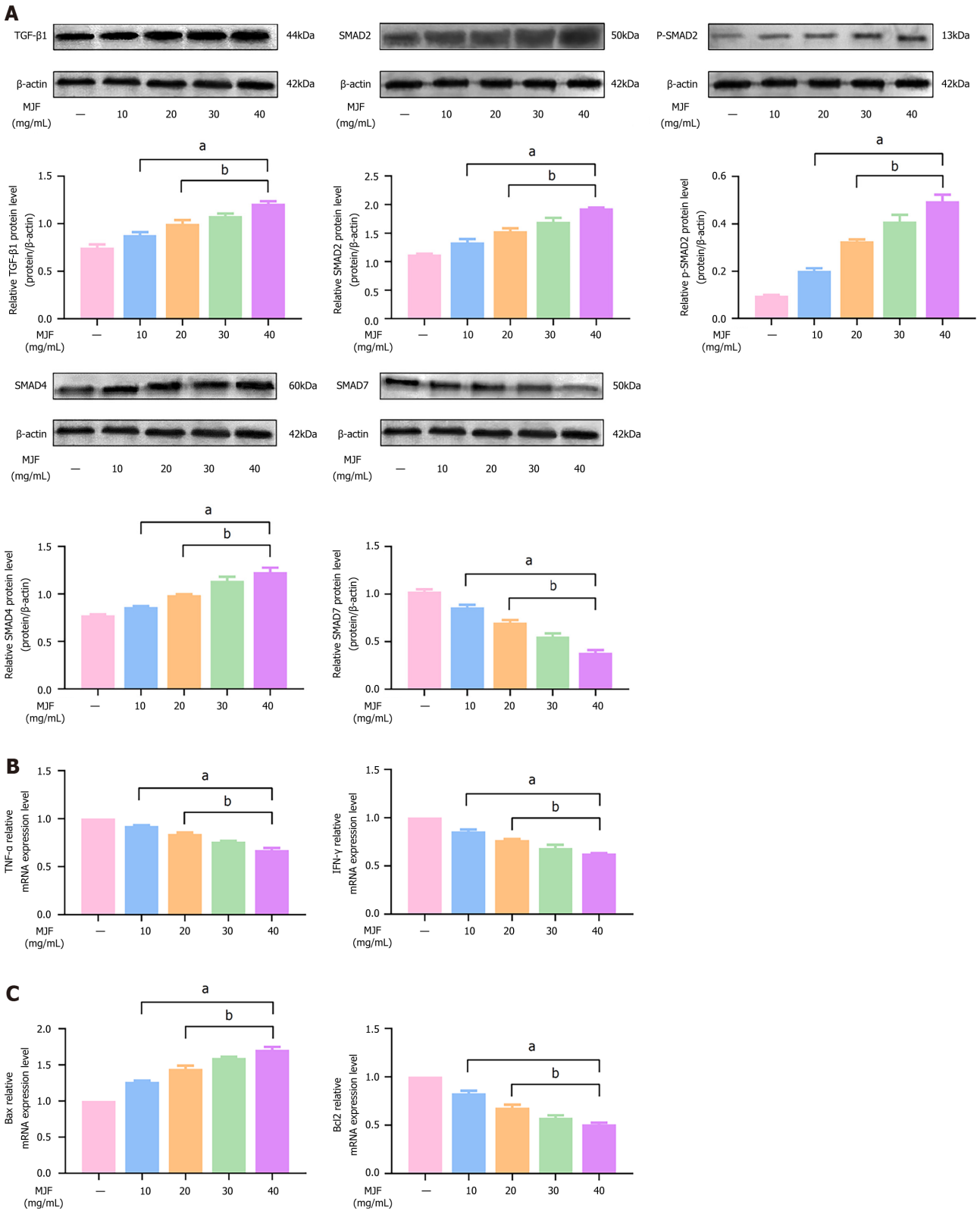

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Figure 6 Mu Ji Fang Granules modulated the Transforming growth factor β1/ Mothers against decapentaplegic homolog signaling pathway and improved the expression of immune and apoptotic cytokines in HepG2 cells. A: Relative expression of Transforming growth factor β1, Mothers against decapentaplegic homolog (SMAD) 2, p-SMAD2, SMAD4, and SMAD7 protein; B: Relative expression of Tumor necrosis factor α and Interferon gamma mRNA; C: Relative expression of *Bax*, and *Bcl2* mRNA (n = 4). Data are shown as the mean ± standard deviation. [a]P < 0.01 *vs* Control [Mu Ji Fang Granules (MJF) 0 mg/mL]. [b]P < 0.01 *vs* MJF 10 mg/mL.

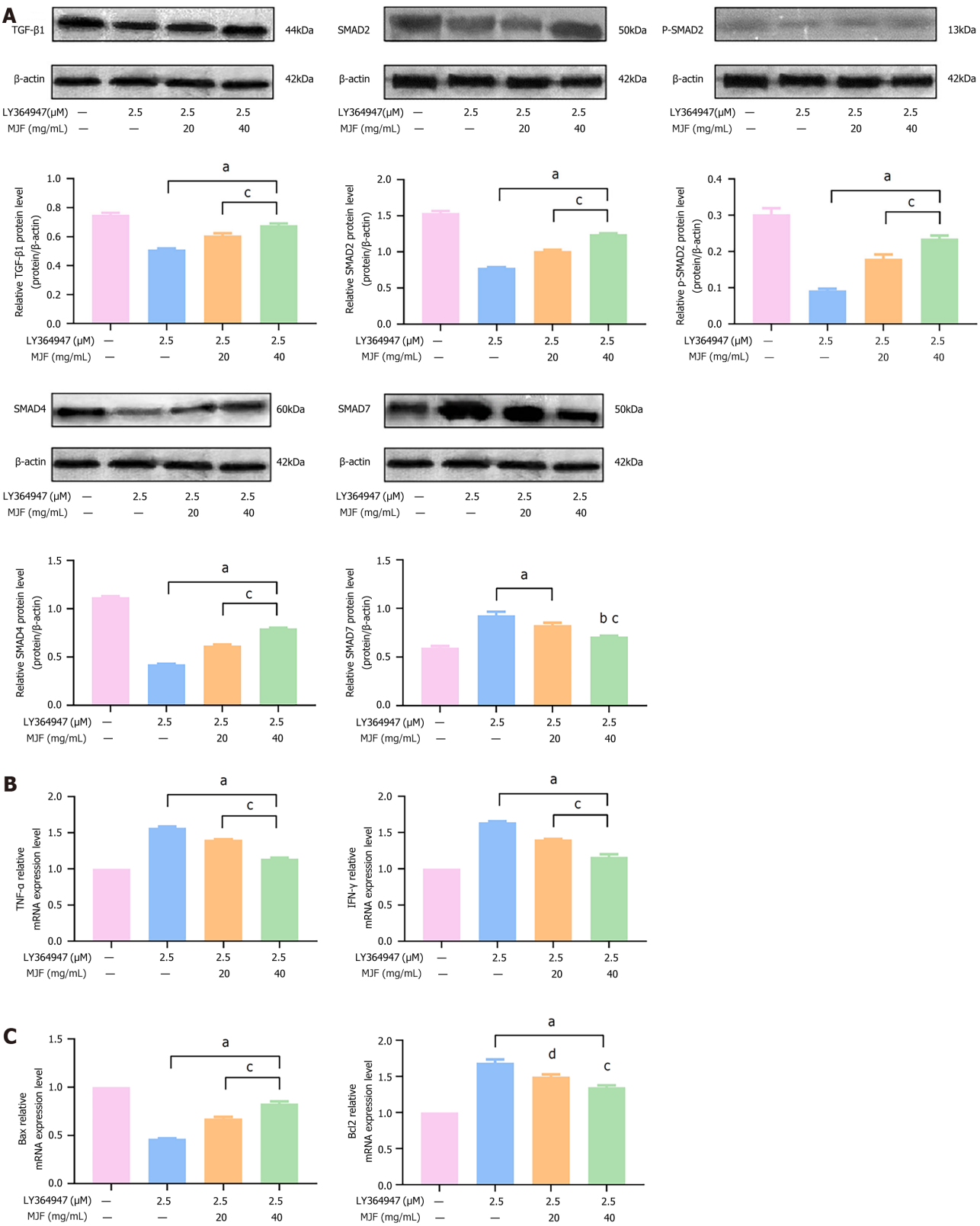

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Figure 7 Mu Ji Fang Granules alleviated the modulation of Transforming growth factor β1 inhibitor (LY364947) on the Transforming growth factor β1/ Mothers against decapentaplegic homolog signaling pathway, immune and apoptotic cytokines in HepG2 cells. A: Relative expression of Transforming growth factor β1(TGF-β1), Mothers against decapentaplegic homolog (SMAD) 2, p-SMAD2, SMAD4, and SMAD7 protein following treatment of HepG2 cells with TGF-β1 inhibitor (LY364947); B: Relative expression of Tumor necrosis factor α and Interferon gamma mRNA following treatment of HepG2 cells with TGF-β1 inhibitor (LY364947); C: Relative expression of *Bax*, and *Bcl2* mRNA following treatment of HepG2 cells with TGF-β1 inhibitor (LY364947) (n = 4). Data are shown as mean ± standard deviation. $^{a}P < 0.01$ *vs* Control [Mu Ji Fang Granules (MJF) 0 mg/mL]. $^{b}P < 0.05$ *vs* Control [Mu Ji Fang Granules (MJF) 0 mg/mL]. $^{c}P < 0.01$ *vs* Mu Ji Fang Granules 10 mg/mL. $^{d}P < 0.05$ *vs* Mu Ji Fang Granules 10 mg/mL.

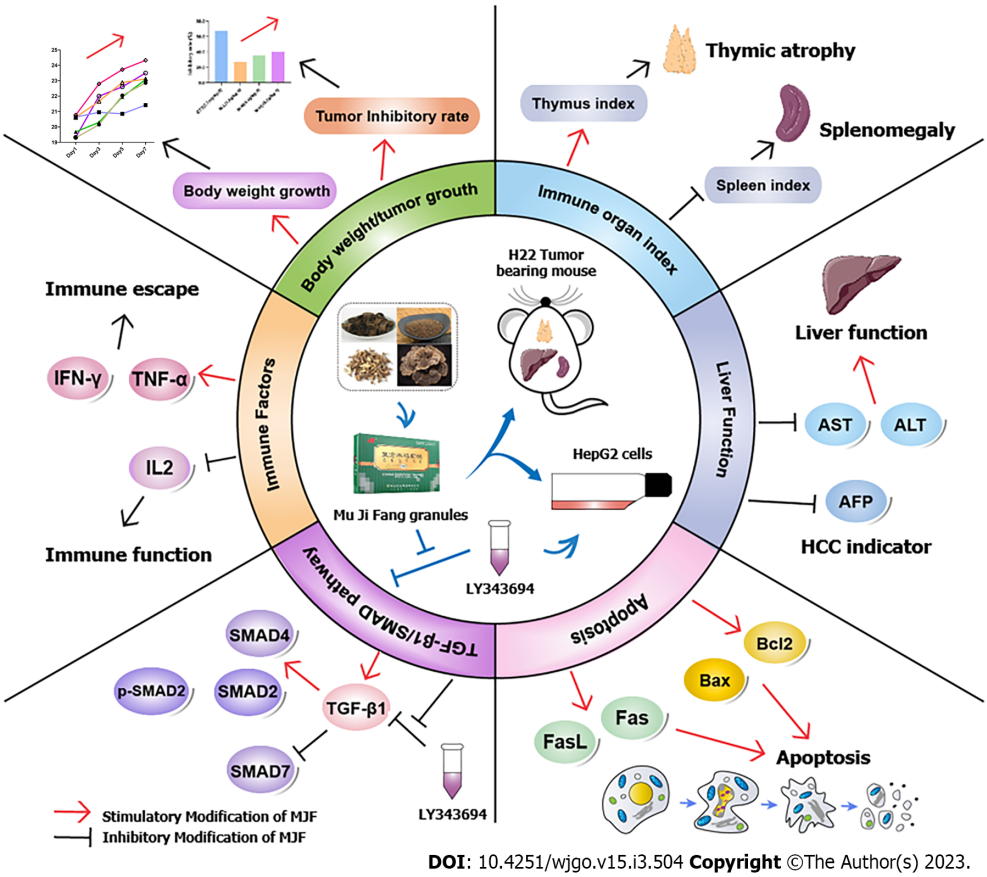

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Figure 8 Overview of the possible anti-hepatocellular carcinoma effects of Mu Ji Fang Granules. CTX: Cyclophosphamide; IFN-γ: Interferon gamma; TNF-α: Tumor necrosis factor α; IL: Interleukin; SMAD: Mothers against decapentaplegic homolog; TGF: Transforming grow; MJF: Mu Ji Fang Granules; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

inducing the occurrence and progression of tumors[32]. Tumor cells can avoid apoptosis by up-regulating *Bcl2* and down-regulating *Bax*[33]. *Bax* and *Bcl2* antagonize each other in apoptosis regulation of HCC cells. With the decrease or deletion of *Bax* expression, apoptosis of HCC cells is gradually weakened, which may be an important reason for the rapid growth and enlargement of tumors. Fas is a cell surface death receptor and an apoptosis signaling molecule. Fas which binds to FasL activates and transmits apoptosis signals, which is an important pathway in inducing tumor cell apoptosis. Previous research showed that the apoptosis of cancer cells mediated by Fas and FasL is an important part of the body's immune surveillance[34], and the abnormal expression of Fas and FasL can lead to disordered apoptosis, which results in massive cell proliferation due to escaping the killing effect of cytotoxic T cells[35,36].

In this study, we evaluated the impact of MJF on HCC using both *in vivo* (H22 tumor-bearing mice) and *in vitro* (HepG2 cells) models (Figure 8). The results indicated that MJF could prevent HCC by improving body weight gain and tumor inhibition rate, protecting immune organs and liver function, affecting immunity and apoptosis, and up-regulating the TGF-β1/SMAD signaling pathway, by increasing the relative expression of TGF-β1, SMAD3, p-SMAD2 and SMAD4 and decreasing SMAD7, reducing immune factors TNF-α and IFN-γ, and decreasing the apoptosis cytokines Fas, FasL and *Bcl2*/*Bax*. In addition, to verify these findings, we treated HepG2 cells with the TGF-β1 inhibitor LY364947, which inhibited the TGF-β1/SMAD signaling pathway, increased Laminin Subunit Alpha 4 (LAMA4), TNF-α, IFN-γ, Fas, FasL, and *Bcl2*/*Bax* expression. However, MJF significantly inhibited the effect of LY364947. This suggested that MJF has potential effects on HCC by regulating the TGF-β1/SMAD pathway, immune and apoptotic cytokines.

Based on the identification of absorbable ingredients in mouse serum, we mainly focused on the fundamental study of potential anti-HCC targets of MJF, and intensive research on the mechanism of tumor cell apoptosis and immunology are still required. Moreover, other absorbable ingredients also need to be identified.

CONCLUSION

Our results demonstrated that MJF inhibits HCC by activating the TGF-β1/SMAD signaling pathway, immune and apoptotic cytokines, which may be due to the mechanisms of MJF in adjusting immune escape and apoptosis.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is one of the most common digestive system cancers with high mortality rates worldwide.

Research motivation

Considerable effort has been expended in understanding the mechanism of Mu Ji Fang Granules (MJF) on tumor immunology in the treatment of HCC.

Research objectives

Our study explored the mechanism of MJF adjusting immune escape and apoptosis.

Research methods

We conducted Molecule Network related to High Performance Liquid Chromatography-Electron Spray Ionization-Time of Flight- Mass Spectrometry to identify the absorbable ingredients of MJF, and hub potential anti-HCC targets were screened using network pharmacology and pathway enrichment analysis. Both *in vivo* and *in vitro* experiments were demonstrated, and multiply processes including Histopathological analysis, Immunosorbent Assay, RT-qPCR, Western blotting assays were to adopted to explore the function MJF inhibits HCC.

Research results

We found that MJF improved body weight gain and tumor inhibition rate in H22 tumor-bearing mice, protected immune organs and liver function, reduced the HCC indicator alpha-fetoprotein, affected immunity and apoptosis, and up-regulated the Transforming growth factor β1(TGF-β1)/SMAD signaling pathway, by increasing the relative expression of TGF-β1, SMAD2, p-SMAD2 and SMAD4 and decreasing SMAD7, reducing immune factors Tumor necrosis factor α and Interferon gamma, decreasing apoptosis cytokines Fas, FasL and *Bcl2*/*Bax*, and inhibiting the effect of LY364947 in HepG2 cells.

Research conclusions

Our findings suggested that MJF inhibits HCC by activating the TGF-β1/SMAD signaling pathway, and affecting immune and apoptotic cytokines.

Research perspectives

Considerable effort has been expended in understanding the anti-HCC mechanism of MJF, all of which due to MJF's particular participate in adjusting immune escape and apoptosis.

FOOTNOTES

Author contributions: Zhang YB, Bao YR, Wang S, Wang BC and Meng XS contributed to conception and design of the study; Zhang YB, Wang S, Li TJ, Tai H, Leng JP, Wang BC and Yang XX conducted the experiments, organized the database, carried out the statistical analysis and drafted the manuscript; All authors contributed to manuscript revision, and approved the submitted version.

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Institutional animal care and use committee statement: All experimental procedures were performed with the approval of the animal guidelines and protocols established by the Medicine Ethics Review Committee of Animal Experiments of Liaoning University of Traditional Chinese Medicine. The study was reviewed and approved by the Animal Ethics Committee of The Affiliated Hospital of Liaoning University of TCM. [Approval No. 2019YS(DW)-033-01].

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Data are available from the corresponding authors upon request (mxsvvv@163.com).

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ORIGINAL ARTICLE

Retrospective Cohort Study

Risk of pancreatic cancer in individuals with celiac disease in the United States: A population-based matched cohort study

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Abstract

BACKGROUND

Celiac disease (CD) has been associated with gastrointestinal malignancies. However, the magnitude of the risk of pancreatic cancer (PC) associated with CD is much less clear, and risks have not been estimated from large populations.

AIM

To assess the risk of PC in CD patients.

METHODS

We conducted a population-based, multicenter, propensity score-matched cohort study with consecutive patients diagnosed with CD using the TriNeTx research network platform. We examined the incidence of PC in patients with CD compared with a matched cohort of patients without CD (non-CD, controls). Each patient in the main group (CD) was matched to a patient in the control group using 1:1 propensity score matching to reduce confounding effects. The incidence of PC was estimated using a Cox proportional hazards model with a hazard ratio (HR) and 95% confidence interval (CI).

RESULTS

A total of 389980 patients were included in this study. Among them, 155877 patients had a diagnosis of CD, and the remaining 234103 individuals without CD were considered a control cohort. The mean duration of follow-up for patients in the CD and control cohorts was 5.8 ± 1.8 and 5.9 ± 1.1 years, respectively. During the follow-up, 309 patients with CD developed PC, whereas 240 patients developed PC in the control group (HR = 1.29; 95%CI: 1.09-1.53). In the secondary

analyses in the first year after diagnosis of CD, patients with CD were at a significant increase in risk for PC; 151 patients with CD had an incidence of PC compared with 96 incidences of PC among the patients in the non-CD control group (HR = 1.56; 95%CI: 1.20-2.01) and sensitivity analysis showed similar magnitude to the one generated in the primary and secondary analysis.

CONCLUSION
Patients with CD are at increased risk of PC. Risk elevation persists beyond the first year after diagnosis to reference individuals without CD from the general population.

Key Words: Celiac disease; Cancer; Epidemiology; Pancreas; Pancreatic cancer; Malignancy; Carcinoma

Core Tip: Celiac disease (CD) is an immune-mediated disorder precipitated by the ingestion of gluten in genetically predisposed individuals. Population-based studies have shown the risk of cancer among patients with CD; however, the magnitude of the risk of pancreatic cancer (PC) in association with CD is much less clear. Therefore, In this multicenter retrospective cohort study, we analyzed the risk of PC in patients with CD. We examined the incidence of PC in patients with CD compared with a propensity-matched cohort of patients without CD. We found that patients with CD were at increased risk of PC. Risk elevation persisted beyond the first year after diagnosis to reference individuals without CD from the general population.

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INTRODUCTION

Celiac disease (CD) is an immune-mediated disorder with a worldwide prevalence approaching 1%. A higher risk of death[1], and an increased risk of cancer, particularly lymphomatous malignancies[2] and gastrointestinal malignancies, including small bowel adenocarcinoma[3], among others, have been reported in patients with CD. However, most of the reports of cancer risk in CD suffer from the small sample size and are based on data derived from the pre-serologic testing era, and thus likely selected for more severe phenotypic cases. Therefore, cancer risk in CD remains a subject of current interest.

The magnitude of the risk of pancreatic cancer (PC) in association with CD is much less clear[1,4]. Nevertheless, despite the magnitude of the risk remaining debatable, the unequivocal association between CD and PC remains. In the two most recent matched studies that included data on PC risk in CD, a higher risk of PC diagnosis was observed in CD patients; however, discordant results were obtained regarding whether PC risk persists long-term after CD diagnosis[4,5]. While Elfström *et al*[4] noted no persistent increased risk of PC after the first year of CD diagnosis, Lebwohl *et al*[5] in their recent study, have noted a persistently elevated risk of PC in patients with CD after 1 year of diagnosis. Hence, there is considerable, but not definitive, evidence that strict compliance to CD is associated with increased risk for the development of PC. Furthermore, owing to the lack of control for known risk factors of PC, any independent associations between these diseases cannot be assessed based on current data; given the high incidence of CD and the poor outcomes associated with PC, any such potential association warrants further investigation. To address the abovementioned knowledge gaps, we conducted a propensity-matched retrospective cohort study on a large multi-institutional electronic healthcare registry to assess the risk of PC in patients with CD.

MATERIALS AND METHODS

Methods

We conducted a retrospective cohort study using TriNetX multi-institutional research network (Cambridge, MA, United States). TriNetX is a federated multicenter research network that provides real-time access to an anonymized data set from participating healthcare organizations' electronic health records (EHR). Patients were included using the standard International Classification of Diseases (classi-

fication, Tenth Revision, Clinical Modification) (ICD-10-CM) and Current Procedural Terminology classification terminologies. Details of the data source, quality checks, and diagnosis codes used for patient selection are detailed in the supplementary. TriNetX has received a waiver from Western Institutional Review Board as it only provides de-identified information. At our institution West Virginia University, the Clinical and Translational Science Institute manages the TriNetX platform and provides access to the end-users.

Study participants

All patients with the diagnosis of CD from January 1, 2005, to December 31, 2021, were included. Follow-up of these patients ended on September 31, 2022. The diagnosis of CD was on the ICD-10 diagnostic code. During the same study period, patients who didn't have a diagnosis of CD were classified as non-CD comparators (control group). We excluded CD patients and controls with a history of pancreatic diseases, gallbladder diseases, procedures such as endoscopic retrograde cholangiopancreatography (ERCP) or cholecystectomy, and any malignancies, including PC preceding the index period (defined as the diagnosis of CD) or the corresponding date for controls.

Matching process

Each patient in the CD group was matched to a patient in the control (non-CD comparators) group using 1:1 propensity score matching (PSM) to reduce confounding effects. Covariates in the propensity score model were adjusted for apriori–identified potential confounders: Age, sex, race/ethnicity (Hispanic, non-Hispanic white, non-Hispanic black, or non-Hispanic other), nicotine dependence, alcohol-related disease, body mass index (BMI), type 2 diabetes mellitus (T2DM), hypertension, hyperlipidemia, type 1 diabetes, autoimmune thyroid disease, rheumatoid arthritis, inflammatory bowel disease, disease of gallbladder, diseases of the pancreas, hypercholesterolemia, hypercalcemia, familial hypercholesterolemia, family history of primary malignant neoplasm, ERCP, cholecystectomy, cholesterol level, low-density lipoprotein, and serum triglyceride level, hemoglobin A1C (HbA1C) and genomics (Table 1). Logistic regression on these input matrices was used to obtain propensity scores for each patient in both cohorts. Logistic regression was performed in Python 3.6.5 (Python Software Foundation) using standard libraries NumPy and sklearn. The same analyses were also performed in R 3.4.4 software (R Foundation for Statistical Computing, Vienna, Austria) to ensure outputs match. After calculating propensity scores, matching was performed using a greedy nearest-neighbor matching algorithm with a caliper of 0.1 pooled standard deviations. The balancing of potential confounding variables between the CD and the control group after PSM was evaluated using standardized differences (SMD) with a threshold set a priori at 0.10. SMD was used to measure the magnitude of difference between the groups rather than the *P*-value because of their insensitivity to sample size. The order of the rows in the covariate matrix can affect the nearest neighbor matching; therefore, the order of the rows in the matrix was randomized to eliminate this bias.

Outcomes

Our primary outcome was the incidence of PC. We defined PC according to relevant ICD-10 codes (details depicted in the Supplementary material).

Statistical analysis

All statistical analyses were performed in real-time using TriNetX. Categorical variables were compared using the Pearson chi-square test, and continuous variables using an independent-sample *t*-test. Continuous variables are expressed as mean ± SD. Categorical variables were presented as frequency and percentage. The balancing of potential confounding variables between the CD and the control group after PSM was evaluated using SMD with a threshold set a priori at 0.10. SMD was used to measure the magnitude of difference between the groups rather than the *P*-value because of their insensitivity to sample size. For each outcome, Cox proportional hazards models were used to estimate the hazard ratio (HR). HR and confidence interval (CI), along with tests for proportionality, were calculated using R's Survival package v3.2-3. Numbers were then validated by comparing them with the output from SAS version 9.4. Kaplan-Meier survival analyses were also used to estimate the risk of PC at the end of 7 years after the index event. Patients were censored when the time window ended or the day after the last fact in their record. Hypothesis testing for Kaplan-Meier survival curves was conducted by using the log-rank test. A priori-defined 2-sided alpha of < 0.05 was used for statistical significance.

Secondary analyses

We performed a prespecified secondary analysis. To assess the possible duration of the disease and risk of PC, we performed a follow-up time-specific risk of PC. For these time-dependent analyses, we estimated time-specific HRs and HRs in which follow-up time commences 1-year after CD diagnosis.

Table 1 Baseline characteristics of the study cohort, *n* (%)

Variables	Before the propensity score match			After the propensity score match		
	Celiac disease (*n* = 155877)	Non-CD controls (*n* = 234103)	SMD	Celiac disease (*n* = 134680)	Non-CD controls (*n* = 134680)	SMD
Age in years, mean ± SD	38.8 ± 22.0	47.2 ± 19.0	0.4069	42.5 ± 21.0	43.7 ± 20.3	0.0577
Age by categories						
< 18 yr	34169 (21.9)	18120 (7.7)	0.4072	18105 (13.4)	16935 (12.5)	0.0258
18 to < 40 yr	47471 (30.4)	63011 (26.9)	0.0783	42960 (31.8)	42268 (31.3)	0.0110
40 to < 60 yr	41430 (26.5)	85840 (36.6)	0.2183	41031 (30.4)	41526 (30.8)	0.0080
≥ 60 yr	32807 (21.0)	67132 (28.6)	0.1772	32584 (24.1)	33951 (25.2)	0.0235
Sex, female	109848 (70.4)	170658 (72.8)	0.0539	98469 (73.1)	98950 (73.4)	0.0081
Ethnicity						
Hispanic or Latino	7520 (4.8)	20344 (8.6)	0.1545	7175 (5.3)	6132 (4.5)	0.0357
Race						
White	128024 (82.1)	159285 (68.0)	0.3302	108441 (80.5)	108517 (80.5)	0.0014
Black or African Americans	6276 (4.0)	37239 (15.9)	0.4047	6259 (4.6)	6008 (4.4)	0.0089
Others	19302 (12.3)	31595 (13.4)	0.0332	17799 (13.2)	18092 (13.4)	0.0064
Nicotine dependence	9998 (6.4)	25637 (10.9)	0.1617	9609 (7.1)	8495 (6.3)	0.0330
Alcohol-related disorders	3065 (1.9)	8176 (3.4)	0.0938	2977 (2.2)	2849 (2.1)	0.0065
BMI (kg/m^2), mean ± SD	25.7 ± 7.5	28.7 ± 7.3	0.0574	26.6 ± 7.34	27.9 ± 7.4	0.0221
Comorbidities						
Type 2 diabetes	14086 (9.0)	30411 (12.9)	0.1265	12304 (9.1)	11461 (8.5)	0.0221
Hyperlipidemia	15800 (10.1)	45916 (19.6)	0.2687	15660 (11.6)	15919 (11.8)	0.0060
Hypercholesterolemia	7290 (4.6)	17282 (7.3)	0.1138	7084 (5.2)	6971 (5.1)	0.0038
Hypercalcemia	1086 (0.6)	3149 (1.3)	0.0645	1030 (0.7)	1300 (0.9)	0.0217
Other autoimmune diseases						
Type 1 diabetes	6750 (4.3)	6561 (2.8)	0.0824	3825 (2.8)	3626 (2.6)	0.0090
Rheumatoid arthritis	2669 (1.7)	16810 (7.1)	0.2677	2533 (1.8)	8052 (5.9)	0.2121[1]
Autoimmune thyroiditis	2994 (1.9)	3868 (1.6)	0.0203	2479 (1.8)	2582 (1.9)	0.0056
Crohn's disease	2938 (1.8)	2513 (1.0)	0.0672	2165 (1.6)	2081 (1.5)	0.0050
Ulcerative colitis	2107 (1.3)	2133 (0.9)	0.0417	1605 (1.1)	1544 (1.1)	0.0042
Gallbladder diseases						
Cholecystitis	1407 (0.9)	2402 (1.0)	0.0126	1335 (0.9)	1089 (0.8)	0.0193
Cholelithiasis	3311 (2.1)	8088 (3.4)	0.0809	3184 (2.3)	3225 (2.3)	0.0020
Other diseases of gallbladder	1635 (1.0)	3164 (1.3)	0.0278	1554 (1.1)	1483 (1.1)	0.0050
Other diseases of biliary tract	1574 (1.0)	3008 (1.2)	0.0258	1453 (1.1)	1465 (1.1)	0.0009
Pancreatic diseases						
Acute pancreatitis	1560 (1.0)	2637 (1.1)	0.0122	1372 (1.0)	1334 (0.9)	0.0028
chronic pancreatitis	823 (0.5)	1661 (0.7)	0.0232	763 (0.5)	745 (0.5)	0.0018
Alcohol-induced acute pancreatitis	56 (0.03)	106 (0.04)	0.0046	52 (0.03)	46 (0.03)	0.0023
Alcohol-induced chronic pancreatitis	42 (0.02)	109 (0.04)	0.0102	42 (0.03)	31 (0.02)	0.0050
Idiopathic acute pancreatitis	101 (0.06)	167 (0.07)	0.0025	90 (0.06)	82 (0.06)	0.0024

Other diseases of pancreas	572 (0.3)	903 (0.3)	0.0031	519 (0.3)	494 (0.3)	0.0030
Cyst of pancreas	478 (0.3)	966 (0.4)	0.0177	465 (0.3)	427 (0.3)	0.0049
Disease of pancreas, unspecified	383 (0.2)	778 (0.3)	0.0161	363 (0.2)	345 (0.2)	0.0026
Pseudocyst of pancreas	296 (0.2)	703 (0.3)	0.0223	290 (0.2)	253 (0.1)	0.0061
Family history						
Familial hypercholesterolemia	106 (0.06)	280 (0.1)	0.0169	102 (0.07)	113 (0.08)	0.0029
Family history of primary malignant neoplasm	10498 (6.7)	15773 (6.7)	0.0001	9265 (6.8)	9417 (6.9)	0.0044
Procedures						
ERCP	424 (0.272)	867 (0.37)	0.0174	398 (0.296)	378 (0.281)	0.0028
Cholecystectomy	1158 (0.74)	2217 (0.94)	0.0223	1078 (0.80)	1116 (0.82)	0.0031
Genomics						
KRAS	21 (0.013)	16 (0.007)	0.0066	14 (0.01)	12 (0.009)	0.0015
TP53	16 (0.01)	15 (0.006)	0.0042	14 (0.01)	12 (0.009)	0.0015

[1]$P > 0.1$.

SD: Standard deviation; BMI: Body mass index; CD: Celiac disease; ERCP: Endoscopic retrograde cholangiopancreatography; KRAS: Kirsten rat sarcoma viral oncogene homolog; TP: Tumor protein.

Sensitivity analysis

Due to the heterogeneous nature of cancer and to assess the robustness of our findings. We performed a sensitivity analysis by estimating risk after excluding patients with outcomes within 1-year after the index event. In this analysis, the outcome was the first and new diagnosis of PC that developed after the start of follow-up, as some cases of PC have been reported to occur shortly after diagnosis of CD.

RESULTS

Baseline characteristics

A total of 389980 patients were included in this study. Among them, 155877 patients had a diagnosis of CD and were thus placed in the main cohort (CD) (Figure 1). The remaining 234103 individuals did not have CD and were considered as a control cohort (non-CD comparators). The mean duration of follow-up for patients in the CD and control cohorts was 5.8 ± 1.8 and 5.9 ± 1.1 years, respectively. The baseline characteristics of the study cohorts are depicted in Table 1. Patients with CD were generally younger (38.8 ± 22.0 *vs* 47.2 ± 19.0) than the control cohort group. In both groups, the majority of the patients were females and white race. There were differences in comorbidities between the groups at baseline before PSM (Table 1). Type 1 diabetes, autoimmune thyroiditis, Crohn's disease, and ulcerative colitis were slightly more common in patients with CD than in controls. However, compared with the CD cohort, the non-CD cohort had a higher prevalence of comorbidities, including T2DM, hyperlipidemia, and hypercholesterolemia. In addition, 76% of the CD cohort had positive for tissue transglutaminase immunoglobulin A serology. After PSM, both cohorts (134680 patients each) were well-matched and balanced (Supplementary Figure 1). In contrast, compared with the CD cohort, the control cohort had a higher prevalence of rheumatoid arthritis (1.8% *vs* 5.9%). No significant difference was observed between other comorbidities, procedures, genomic profile, family history of primary malignant neoplasm, or hypercholesterolemia between both cohorts after propensity matching.

Study outcome

During the follow-up, 309 patients with CD developed PC, whereas 240 patients developed PC in the control group. Patients with CD had a significantly higher risk of PC (HR = 1.29; 95%CI: 1.09-1.53) than the non-CD controls (Figure 2). In the secondary analyses for the incidence of PC, when starting follow-up at 1-year after diagnosis of CD, 151 patients with CD had an incidence of PC compared with 96 incidences of PC among the patients in the non-CD control group. CD was associated with a significantly higher risk of PC (HR = 1.56; 95%CI: 1.20-2.01). Kaplan-Meier survival analysis showed a cumulative incidence of PC at 7 years among patients with CD compared to non-CD controls (log-rank test $P < 0.001$, Figure 3).

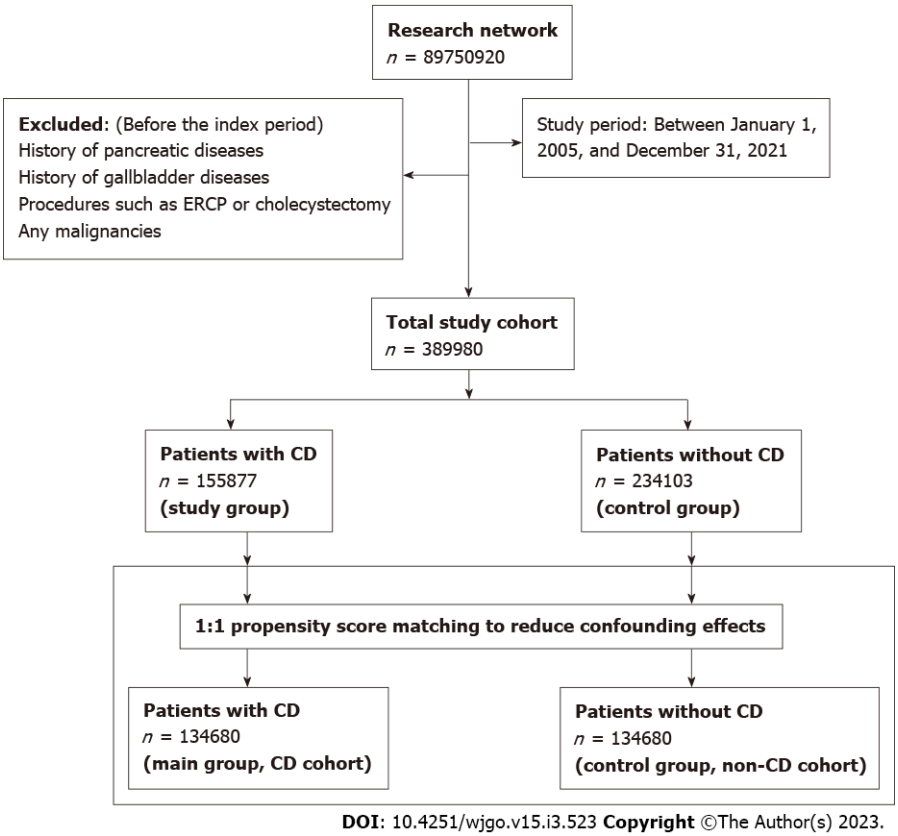


Figure 1 Flow chart showing patient selection for study cohorts. CD: Celiac disease; ERCP: Endoscopic retrograde cholangiopancreatography.

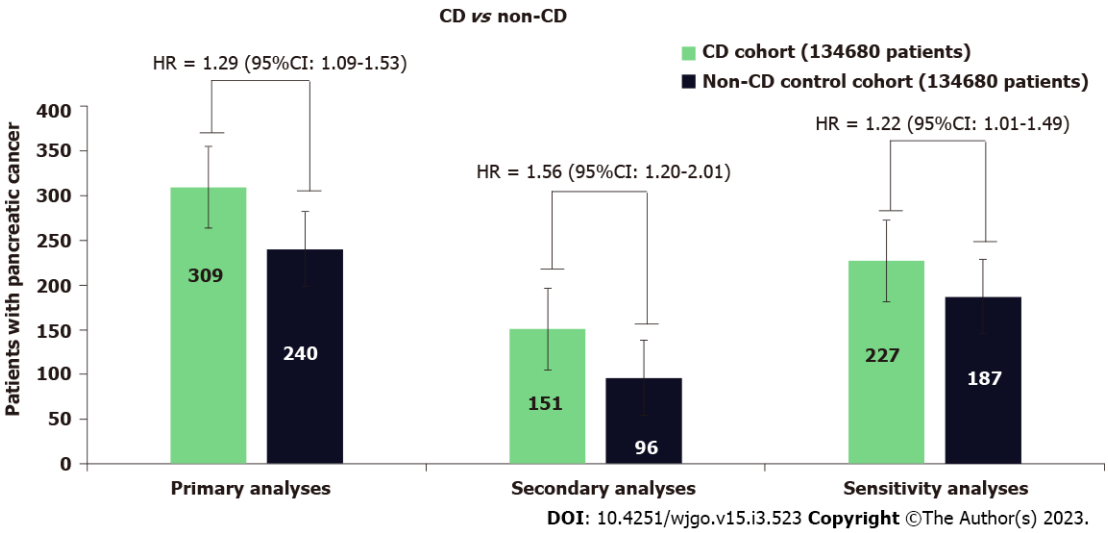
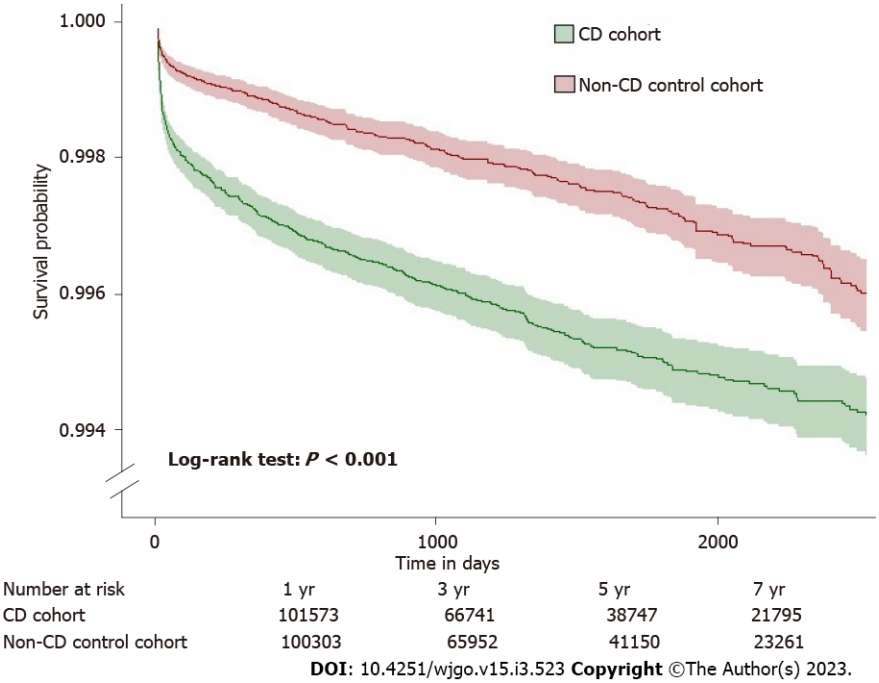


Figure 2 Risk of pancreatic cancer for patients with celiac disease compared to patients without celiac disease (non-celiac controls). The numbers inside the bars represent the number of patients who developed pancreatic cancer during follow-up. CD: Celiac disease; HR: Hazard ratio; CI: Confidence interval.

Sensitivity analyses

The sensitivity analyses led to consistent results when starting follow-up at 1 year after diagnosis of CD by excluding the outcomes within 1 year after the index event. CD generated a hazard ratio of similar magnitude to the one generated in the primary analysis (HR = 1.22; 95%CI: 1.01-1.49) (Figure 2).



Number at risk	1 yr	3 yr	5 yr	7 yr
CD cohort	101573	66741	38747	21795
Non-CD control cohort	100303	65952	41150	23261

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Figure 3 Kaplan-Meier curve shows the risk of pancreatic cancer in patients with celiac disease and matched non-celiac controls.

DISCUSSION

This was a population-based, multicenter retrospective study analysis that used a large sample size from a nationally representative database and statistical adjustments with apriori-identified potential confounders to balance the critical variables at the baseline by PSM. Patients with CD were found to have a modest excess risk of PC. The association of CD with PC remained unchanged in the secondary and sensitivity analyses, where a lag period of 1 year was applied. Our study confirmed that the risk for PC is significantly greater in patients with CD than in the unaffected population.

Previous retrospective cohort studies have noted an increase in overall cancer risk and gastrointestinal cancer risk in patients with CD[4,6,7]. However, as most of the reported data on cancer risk in CD is derived from patients diagnosed in the 20th century, concerns have been raised about its current validity as these CD cases likely represent a more severe disease phenotype[4]. Some recent reports have noted that the overall excess risk of malignancy diagnosis in CD may be elevated only in the short term following CD diagnosis (about 1 year), and the risk elevation was noted to subside after the first year of CD diagnosis[4,5,8]. Two hypothetical explanations have been proposed in the literature to explain this finding: (1) The risk elevation may be mitigated over time due to the introduction of a gluten-free diet after diagnosis[9,10]; or (2) The elevated risk of malignancy in the short term may be a reflection of the increased surveillance and diagnostic testing that these patients may be undergoing in the peridiagnostic period due to presentation with weight loss and other mutual symptoms that may result in the discovery of CD in malignancy cases and vice versa, and thus not be a true elevation in risk [10,11,12]. Thus any excess overall malignancy risk in CD is still unclear. However, an excess risk of certain individual malignancies in CD, *e.g.*, small bowel adenocarcinoma, lymphoproliferative malignancies, and liver cancer, have been strongly noted in CD and have been reported to persist in the longer term beyond 1 year[3]. More recently, in their retrospective cohort analysis, Lebwohl *et al*[5] noted an increase in the incidence of PC in a large Swedish CD cohort; the risk elevation persisted at a longer duration of follow-up, contrary to the earlier report by Elfström *et al*[4]. Nevertheless, data on PC risk in CD patients is limited, and these prior studies were not designed to study PC specifically; thus, control for PC-specific risk factors was not adequately performed.

Our results are largely consistent with prior observations of elevated PC risk in CD[5,9]. We were able to confirm and strengthen this prior observed elevated risk of PC due to robust PSM and control for PC risk factors. This study has one major advantage over those that have preceded it: Our propensity score-matched analysis with covariables that are directly correlated with the risk of PC. The present study includes an expansion of clinical outcomes addressed in previous studies and an adjustment for confounders such as BMI, race, ethnicity, disease of gallbladder, diseases of the pancreas, hypercholesterolemia, hypercalcemia, familial hypercholesterolemia, family history of primary malignant neoplasm, ERCP, cholecystectomy, HbA1C, and genomics. Further experimental data and large multicenter cohort studies are required to ensure better comprehension of the association between CD and PC.

Most importantly, we conducted a sensitivity analysis where only PC diagnosed after 1 year of lag time following CD diagnosis was included as the outcome, and the study conclusion remained unchanged. When taken in totality, these data point towards a persistently elevated risk of PC in CD patients. One important observation to note is that the absolute number of events (PC cases) captured in our cohort was much higher than in previous studies due to the large population included in our analysis, as well as the long duration of follow-up. We believe that the high number of events coupled with available follow-up time increased the study's power and allowed us to deduce the persistence of risk elevation. In the reports by Lebwohl *et al*[5] and Landgren *et al*[13], an adjusted HR of 2.29 (1.84-2.84) and 2.27 (1.22-4.23) was described; the risk noted by Lebwohl *et al*[5] beyond the first year was 1.66 (1.32-2.10), and we noted HRs of 1.29 and 1.56 for PC in our cohort of CD patients. The overall risk elevation that we noted was thus slightly lower than these earlier reports.

Previously, some authors have hypothesized that an increased incidence of pancreatitis, impaired exocrine pancreatic function, as well as malnutrition, and resulting pancreatic atrophy may be the cause for the elevation in PC risk in patients with CD[9]. The mechanism for developing malignancies in patients with CD is still unknown. Autoimmunity has also been proposed as the etiopathologic culprit, as autoantibodies targeting the pancreas have been noted in CD patients[14]. In addition, chronic inflammation, chronic antigenic stimulation, the release of proinflammatory cytokines, increased intestinal permeability of environmental carcinogens, immune surveillance problems, and the gluten-free diet or nutritional deficiencies due to the disease have been suggested to be contributory[15,16]. Owing to the design of our study, we cannot deduce the pathophysiological basis of our observation of increased PC risk in CD.

Strengths and limitations

The major strengths of this study include the robust control and comprehensive organ-specific assessment focused on the pancreas. In addition, we adjusted for various lifestyle risk factors and baseline and potential confounders that may have confounded the association of CD and PC risk. The large sample in the propensity-matched analyses resulted in narrow confidence intervals. It allowed us to capture a significant number of outcomes, which lends strength to the conclusions that we have derived. Lastly, including a sensitivity analysis lends strength to our study and its findings.

However, our study is limited by its retrospective design and its analysis of EHR-based data, which can be subject to documentation and coding errors, resulting in some selection bias. Some residual confounding can exist, and we cannot exclude any possible confounding factors not controlled for in our study that may have an effect on the risk of PC. Due to the retrospective cohort nature of this study, any potential underlying pathophysiological mechanisms explaining the observed association of CD with PC cannot be explored. Furthermore, histological data on villous atrophy and compliance with a gluten-free diet was not studied. Whether villous atrophy and its persistence over time pose any elevation of risk for PC needs to be explored. Also, whether mitigation of the excess risk is observed with the normalization of villous architecture or in patients who strictly adhere to a gluten-free diet should be explored in further studies.

CONCLUSION

This nationwide study with large propensity-matched analysis found an increased risk of PC in patients with CD; this risk elevation persisted beyond the first year after diagnosis.

ARTICLE HIGHLIGHTS

Research background

Studies have shown an increased risk for various malignancies, particularly lymphomas, small intestinal adenocarcinoma, and other gastrointestinal malignancies, in celiac disease (CD) patients. However, the magnitude of the risk of pancreatic cancer (PC) in association with CD is much less clear. Nevertheless, despite the magnitude of the risk remaining debatable, the unequivocal association between CD and PC remains.

Research motivation

Although malignancy occurring in the setting of CD has been well recognized; however, there is considerable, but not definitive, evidence that strict compliance to CD is associated with increased risk for the development of PC. Furthermore, owing to the lack of control for known risk factors of PC, any independent associations between these diseases cannot be assessed based on previous studies; given the high incidence of CD and the poor outcomes associated with PC, any such potential association warrants further investigation.

Research objectives
This study aimed to assess the risk of PC in patients with CD.

Research methods
A population-based, multicenter, propensity score-matched cohort study included 155877 patients with CD and 234103 patients without CD (non-CD, controls). To reduce confounding effects, we performed a 1:1 propensity score matching with each patient in the main group to a patient in the control group. The incidence of PC was estimated using a Cox proportional hazards model with a hazard ratio (HR) and 95% confidence interval (CI).

Research results
During the follow-up, 309 patients with CD developed PC, whereas 240 patients developed PC in the control group (HR = 1.29; 95%CI: 1.09-1.53). In the secondary analyses in the first year after diagnosis of CD, patients with CD were at a significant increase in risk for PC; 151 patients with CD had an incidence of PC compared with 96 incidences of PC among the patients in the non-CD control group (HR = 1.56; 95%CI: 1.20-2.01) and sensitivity analysis showed similar magnitude to the one generated in the primary and secondary analysis.

Research conclusions
This multicenter, propensity score-matched cohort study reveals that patients with CD are at increased risk of PC. Risk elevation persists beyond the first year after diagnosis to reference individuals without CD from the general population.

Research perspectives
We still know little about the risk factors and mechanisms contributing to developing malignancies among individuals. Further experimental data and long-term follow-up studies are required to elucidate the pathogenic mechanisms and to ensure better comprehension of the association between PC and CD.

FOOTNOTES

Author contributions: Krishnan A conceptualized and designed the research and performed the formal analysis and interpretation of the data; Krishnan A and Hadi YB wrote the original draft and performed the review and editing of the draft; Krishnan A performed a critical revision of the manuscript; and all authors revised the manuscript for important intellectual content and approved the article's final version, including the authorship list.

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ORIGINAL ARTICLE

Retrospective Study

Nomogram model predicting the overall survival for patients with primary gastric mucosa-associated lymphoid tissue lymphoma

Dan Wang, Xin-Lin Shi, Wei Xu, Rui-Hua Shi

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Abstract

BACKGROUND

Increasingly extranodal marginal B-cell lymphoma of mucosa-associated lymphoid tissue, known as mucosa-associated lymphoid tissue (MALT) lymphoma, is a type of non-Hodgkin's lymphoma. The prognosis of primary gastric MALT (GML) patients can be affected by many factors. Clinical risk factors, including age, type of therapy, sex, stage and family hematologic malignancy history, also have significant effects on the development of the disease. The available data are mainly focused on epidemiology; in contrast, few studies have investigated the prognostic variables for overall survival (OS) in patients with primary GML. Based on the realities above, we searched a large amount of data on patients diagnosed with primary GML in the Surveillance, Epidemiology and End Results (SEER) database. The aim was to develop and verify a survival nomogram model that can predict the overall survival prognosis of primary GML by com-bining prognostic and determinant variables.

AIM

To create an effective survival nomogram for patients with primary gastric GML.

METHODS

All data of patients with primary GML from 2004 to 2015 were collected from the SEER database. The primary endpoint was OS. Based on the LASSO and COX regression, we created and further verified the accuracy and effectiveness of the

survival nomogram model by the concordance index (C-index), calibration curve and time-dependent receiver operating characteristic (td-ROC) curves.

RESULTS

A total of 2604 patients diagnosed with primary GML were selected for this study. A total of 1823 and 781 people were randomly distributed into the training and testing sets at a ratio of 7:3. The median follow-up of all patients was 71 mo, and the 3- and 5-year OS rates were 87.2% and 79.8%, respectively. Age, sex, race, Ann Arbor stage and radiation were independent risk factors for OS of primary GML (all $P < 0.05$). The C-index values of the nomogram were 0.751 (95%CI: 0.729-0.773) and 0.718 (95%CI: 0.680-0.757) in the training and testing cohorts, respectively, showing the good discrimination ability of the nomogram model. Td-ROC curves and calibration plots also indicated satisfactory predictive power and good agreement of the model. Overall, the nomogram shows favorable performance in discriminating and predicting the OS of patients with primary GML.

CONCLUSION

A nomogram was developed and validated to have good survival predictive performance based on five clinical independent risk factors for OS for patients with primary GML. Nomograms are a low-cost and convenient clinical tool in assessing individualized prognosis and treatment for patients with primary GML.

Key Words: Primary gastric mucosa-associated lymphoid tissue lymphoma; Nomogram; Prognosis; Overall survival; SEER database

Core Tip: Increasingly extranodal marginal B-cell lymphoma of mucosa-associated lymphoid tissue, known as mucosa-associated lymphoid tissue (MALT) lymphoma, is a type of non-Hodgkin's lymphoma. The prognosis of primary gastric MALT (GML) patients can be affected by many factors. The available data are mainly focused on epidemiology; in contrast, few studies have investigated the prognostic variables for overall survival (OS) in patients with primary GML. Based on the realities above, we searched a large amount of data on patients diagnosed with primary GML in the Surveillance, Epidemiology and End Results database. The aim was to develop and verify a survival nomogram model that can predict the OS prognosis of primary GML by combining prognostic and determinant variables. In this manuscript, our results demonstrated that Nomogram is developed and validated to have a good survival predictive performance, basing on five clinical independent risk factors of OS for primary GML patients. Nomogram will be a low cost and convenient clinical tool in assessing individualized prognosis and treatment for primary GML patients.

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INTRODUCTION

Increasingly extranodal marginal B-cell lymphoma of mucosa-associated lymphoid tissue, known as mucosa-associated lymphoid tissue (MALT) lymphoma, is a type of non-Hodgkin's lymphoma[1,2]. Approximately one-third of cases of MALT lymphoma and 85% of gastrointestinal MALT lymphomas present in the stomach as the affected site[3,4]. Primary gastric MALT lymphoma (GML) has a rare incidence and only accounts for approximately 5% of all primary gastric neoplasms[5].

This disease demands high attention. Researchers have observed that GML patients had significant risk of atrophic gastritis, intestinal metaplasia[6,7] and secondary tumors[8]. Compared to the healthy population, the incidence of gastric adenocarcinoma in GML patients was 6 times higher in the GML population[9,10].

The prognosis of primary GML patients can be affected by many factors. Clinical risk factors, including age, type of therapy, sex, stage and family hematologic malignancy history, also have significant effects on the development of the disease[11-14]. Meanwhile, previous studies demonstrated a good prognosis of the disease, with 5-year survival rates of up to 99%. However, more than 95% of studies were only based on stage I/II patients with a small sample size, and few of them even used

different staging standards[15-19]. Matysiak-Budnik *et al*[11] conducted a multicenter study in France, and 416 GML patients were retrospectively enrolled. They surprisingly found that 25% of subjects diagnosed at stage III/IV and 11% of patients obtained missed or false diagnoses, which was similar to other studies[20-22].

Furthermore, the available data are mainly focused on epidemiology; in contrast, few studies have investigated the prognostic variables for overall survival (OS) in patients with primary GML. Based on the realities above, we searched a large amount of data on patients diagnosed with primary GML in the Surveillance, Epidemiology and End Results (SEER) database. The aim was to develop and verify a survival nomogram model that can predict the OS prognosis of primary GML by combining prognostic and determinant variables.

MATERIALS AND METHODS

Patient selection and data extraction from the SEER database

The data for our study were extracted from the SEER database (username: 12262-November 2019, software version: SEER * Stat 8.3.6). Due to the openness of the database, our research was exempted from the need for approval by the Ethics Committee of Zhongda Hospital, the Affiliated Hospital of Southeast University.

All patients diagnosed with primary GML from 2004 to 2015 were ultimately included in this study. Subjects meeting the following conditions were excluded: (1) Hospitalized death and autopsy source patients; (2) patients with tumor history; (3) patients who were not followed up or who were lost follow-up; (4) age at diagnosis < 20; (5) unknown data (race, stage, and cause of death); and (6) survival months < 1.

A total of 2604 selected GML patients were randomly assigned to the training and validation sets with a ratio of 7:3. There were 1823 and 781 people in the two groups, respectively.

The clinical covariates included sex, age, race, primary site, Ann Abor stage, surgery, chemotherapy, and radiation. Data on the survival month and vital status of patients were also analyzed. The primary endpoint was OS.

Establishment and verification of the survival nomogram

In the training set, the least absolute shrinkage and selection operator (LASSO) and multivariate Cox proportional hazard regression were combined to identify the significantly correlated prognostic factors that influenced OS. The nomogram model was established based on the above results. Meanwhile, primary GML patients were divided into low- and high-risk groups at the cutoff point of the risk score. Scatter plots, forest plots and Kaplan-Meier curves were generated to visually compare the OS times of patients in the two different risk groups.

Internal validation was performed on the patients in the validation set. The discriminatory performance of the model was measured by the concordance index (C-index) value[23]. We also adopted time-dependent receiver operating characteristic (td-ROC) curves to assess the 3- and 5-year OS predictive power of the nomogram. Additionally, calibration plots were applied to compare the agreement between actual and predicted probability.

Data analysis

Statistical analysis was performed by IBM SPSS version 22.0 (Chicago, IL, United States) and R version 4.0.2. Continuous variables were grouped and transformed into categorical variables and are expressed as frequency and percentage values, *n* (%). The chi-square test was used to compare categorical variables between groups. Two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

Clinical characteristics of subjects

The specific clinical and pathological characteristics of all enrolled patients and the training and testing groups are presented in Table 1.

Initially, 4475 GML patients were searched in the SEER database. The following populations were excluded: One primary tumor = NO (*n* = 1408), age < 20 (*n* = 9), unknown race (*n* = 74), unknown stage (*n* = 317), unknown COD (*n* = 16), and survival month < 1 (*n* = 47). In total, 2604 primary GML patients were included in our study. The median survival time was 71 mo.

All patients (median age: 65 years old) were divided into three subgroups (age < 45, 45-65, and > 65; Table 1). Compared to patients < 45 years old (8.98%), the 45-65 years (39.94%) group had a higher proportion (*P* = 0.001, HR 4.29, 95%CI: 1.881-9.815). The proportion of patients > 65 years old was 51.08%. In total, 1250 (48.00%) were male, and 2059 (79.07%) were white. In addition, primary GML could be located in all parts of the stomach and was mostly diagnosed at stage I (2088/80.18%)

Table 1 Clinical pathology characteristics of patients with primary gastric mucosa-associated lymphoid tissue in all subjects, the training and validation sets, *n* (%)

Variable	All cohort	Training cohort	Validation cohort	*P* value[a]
Total	**2604**	**1823**	**781**	
Age				0.423
<45	234(8.98)	168 (71.79)	66(28.21)	
45-65	1040(39.94)	739 (71.06)	301(28.94)	
≥65	1330(51.08)	916 (68.87)	414(31.13)	
Sex				0.262
Male	1250(48.00)	862 (68.96)	388(31.04)	
Female	1354(52.00)	961 (70.97)	393(29.03)	
Race				0.699
White	2059(79.07)	1434 (69.65)	625(30.35)	
Black	284(10.91)	201 (70.77)	83 (29.23)	
Other	261(10.02)	188 (72.03)	73 (27.97)	
Location				0.734
Upper third	314(12.06)	212 (67.52)	102(32.48)	
Middle third	642(24.65)	455 (70.87)	187(29.13)	
Low third	425(16.32)	291 (68.47)	134(31.53)	
Stomach, NOS	1020(39.17)	723 (70.88)	297(29.12)	
Overlapping lesion	203(7.80)	142 (69.95)	61 (30.05)	
of stomach				0.516
Stage (Ann Arbor)		1452 (69.54)	636(30.46)	
I	2088(80.18)	187 (73.91)	66 (26.09)	
II	253(9.71)	47 (72.31)	18 (27.69)	
III	65(2.50)	137 (69.19)	61 (30.81)	
IV	198(7.60)			0.332
Surgery		1700 (70.25)	720(29.75)	
No/unknown	2420(92.93)	123 (66.85)	61 (33.15)	
Yes	184(7.07)			0.82
Chemotherapy		1440 (69.90)	620(30.10)	
No/unknown	2060(79.11)	383 (70.40)	161(29.60)	
Yes	544(20.89)			0.36
Radiation		1231 (70.58)	513(29.42)	
No/unknown	1744(66.97)	592 (68.84)	268(31.16)	
Yes	860(33.03)			

[a]χ^2 test.

Categorical variables were represented by n(%).The *P* value result is the comparison result between the training and validation groups.

according to the Ann Arbor staging system[24]. The numbers of patients who received surgery, chemotherapy and radiation were 184 (7.07%), 544 (20.89%) and 860 (33.03%), respectively. The 3- and 5-year OS rates of primary GML were 87.2% and 79.8%, respectively.

To establish the nomogram model, 1823 subjects were randomly assigned to the training cohort, while 781 were assigned to the validation cohort. No significant difference in variables was observed between the two groups (all $P > 0.05$).

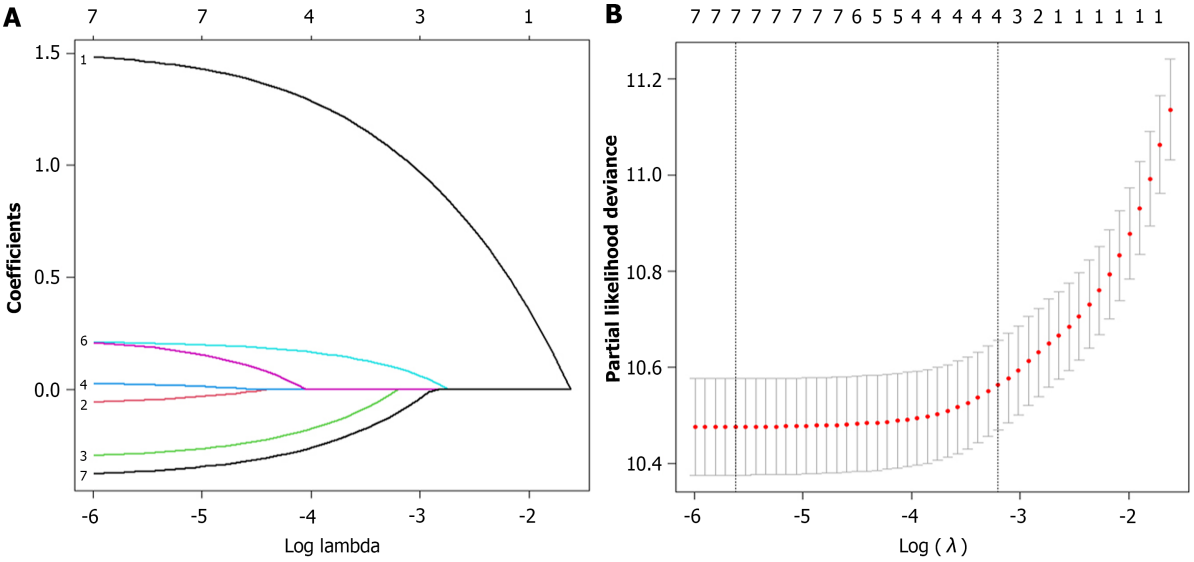

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Figure 1 Identify the correlated prognostic factors by least absolute shrinkage and selection operator regression. A: Least absolute shrinkage and selection operator coefficient profiles of the 8 primary gastric mucosa-associated lymphoid tissue lymphoma-associated variables; B: The selection of optimal prognostic factors by cross-validation. GML: Gastric mucosa-associated lymphoid tissue lymphoma.

Multivariate risk factor analysis and establishment of the nomogram. In the training set, the LASSO Cox regression model was adopted to filter risk factors for OS. The results revealed that 5 out of 8 factors were significantly associated with 3- and 5-year OS, and the specific information is listed in Figure 1 and Table 2. Increased age, male sex, black race and higher disease stage were inversely correlated with survival, while radiation treatment showed a positive correlation with survival (all $P <$ 0.05). Based on the five variables above, we established an efficient survival nomogram to precisely calculate the probability of 3- and 5-year OS in primary GML patients (Figure 2). In the nomogram, the C-index value was 0.751 (95%CI: 0.729-0.773) and demonstrated satisfactory discrimination ability. Patients were separated into high- and low-risk groups according to the median risk score (cutoff: 0.28; Figure 3A). A scatter plot (Figure 3B) visually showed a shorter survival time and a higher mortality rate of high-risk patients. The Kaplan-Meier curve (Figure 4A and B) clearly revealed that, compared to the high-risk population, more low-risk patients had a better OS (all $P < 0.0001$, HR 0.190, 95%CI: 0.153-0.236), which is consistent with the results shown in Figure 5 (all HR > 2). Td-ROC curves also showed good predictive power of the nomogram assessing the 3- and 5-year OS. The areas under the curve (AUCs) were 0.727 (Figure 6A) and 0.734 (Figure 6B), respectively. Moreover, another analysis, the calibration curves of the model, shown in Figure 7A and B, confirmed a high agreement between actual and predictive survival proportions. All the above results indicate the good discrimination and predictive capacities of the model.

Internal validation in the validation set

To better validate the nomogram model, we carried out the relevant analysis in the validation set. The C-index of validation patients was 0.718 (95%CI: 0.680-0.757), indicating good predictive accuracy of the nomogram model. There were significant survival differences between the low- and high-risk groups on Kaplan-Meier curves (HR 0.233, 95%CI: 0.174-0.312, $P < 0.0001$; Figure 4C). As shown in Figure 6C and D, the AUC values of the 3- and 5-year OS td-ROC curves were 0.689 and 0.715, respectively, which were similar to the results in the training set, further confirming the reliable predictive ability of the model. The same phenomenon was also observed between the calibration plots in the training and validation groups (Figure 7C and D). In conclusion, the survival nomogram model displayed a favorable performance to discriminate and predict the OS of primary GML patients in 2 sets.

DISCUSSION

Primary GML is confirmed as a low-grade, rare incidence rate lesion, and the main risk for the disease is a histological transformation to diffuse large B-cell lymphoma[19,25]. Studies mostly focus on epidemiology[26,27] and the prognosis affected by different treatment methods of the disease[14,22,28,29]. Few large studies have reported the relationship between clinical variables and prognostic survival in primary GML. Until now, no available survival model has been established for predicting the prognosis of patients with primary GML. Our study successfully constructed and validated an effective

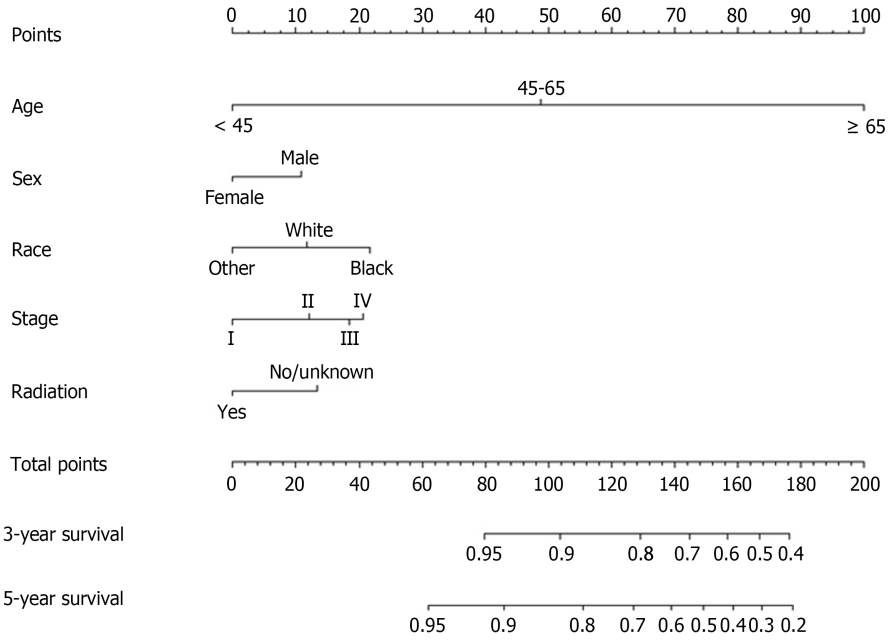

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Figure 2 Survival nomogram for primary gastric mucosa-associated lymphoid tissue lymphoma in 3- and 5-year overall survival.

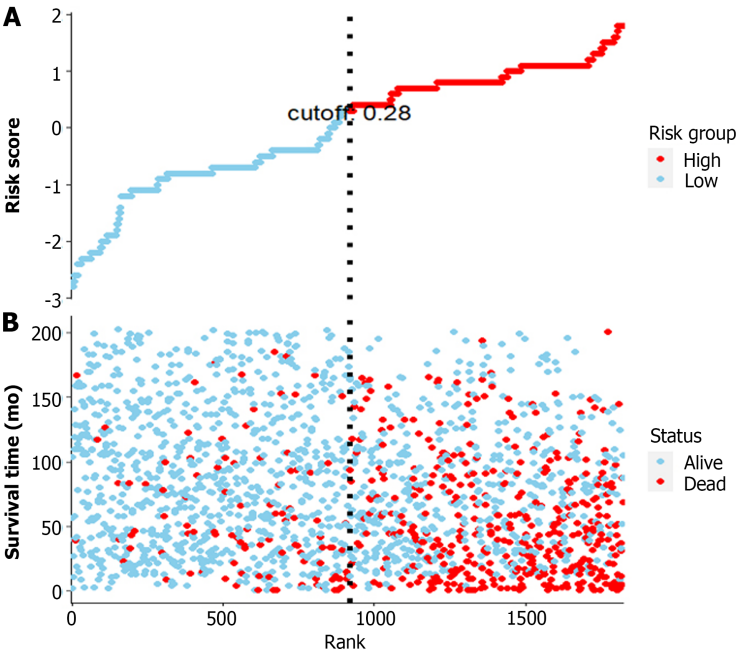

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Figure 3 Patients in the training set were separated into high- and low-risk groups. A: The Cox regression risk score (cutoff point: 0.28); B: Scatter plot showing that patient survival time was sorted by risk score; high-risk patients had shorter survival times and higher mortality rates.

nomogram model to predict the overall survival of the disease based on clinical and pathological risk factor analysis. We demonstrated that age, gender, race, Ann Arbor stage and radiation therapy (RT) were independent risk factors for OS of primary GML. The nomogram was proven to have good predictive ability for disease prognosis.

Considering the indolent natural development of the disease, long-term and very large follow-up clinical datasets are needed. In our study, all of the data were obtained from the SEER database. This is a national cancer database gathering a large amount of data from different hospitals in the United States, and patient information is strictly managed and reliable[30]. Meanwhile, we developed and validated a survival nomogram. In recent years, nomograms have been widely used as an effective tool for cancer prognosis[31-33]. Tailored to the specific information of every patient, the nomogram can visually

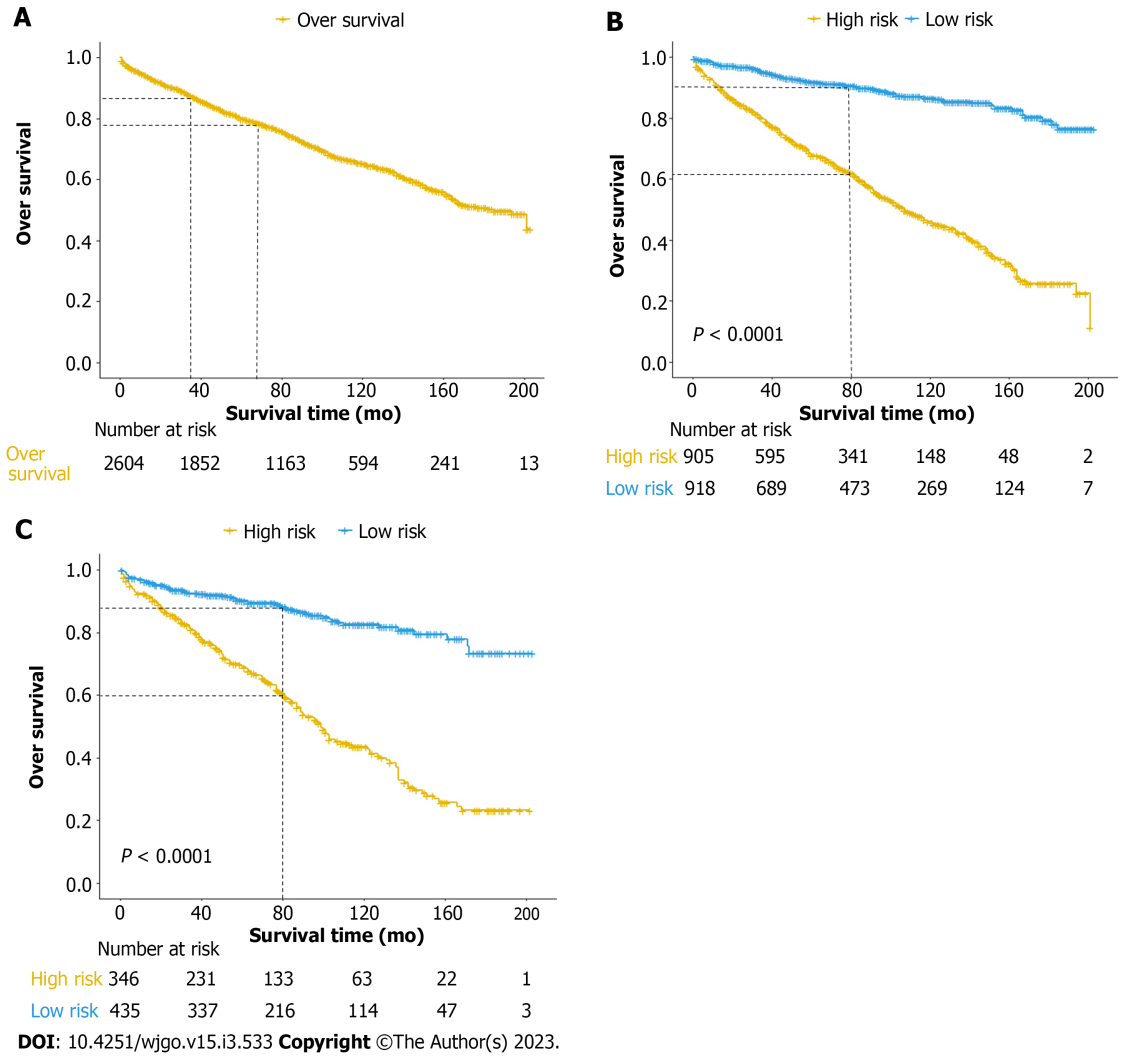

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Figure 4 Kaplan–Meier curves of overall survival for patients in the high- and low-risk groups. A-C: Analysis results of all patients (A), the training set (B), and the testing set (C). OS: Overall survival.

analyze and present the disease event (such as OS) with a single numerical probability[34]. In summary, on the basis of high-quality data and validation analysis methods, our model has good clinical prediction ability and can be applied to clinical work.

The 3- and 5-year OS rates calculated in our study were 87.2% and 79.8%, respectively. In 2019, the first national study was conducted on the general population in France. They confirmed that the 5-year OS of all populations was 79% (95%CI: 75-83)[11]. This rate is similar to published studies and may reflect a better prognostic outcome of GML disease than other gastric malignancies.

In 2017, Thieblemont *et al*[35] generated a novel MALT lymphoma prognostic index (MALT-IPI), including age ≥ 70 years, elevated LDH levels and Ann Arbor stage III or IV. They concluded that this index would be an effective method to predict poor outcome for MALT lymphoma. A similar conclusion was found in other studies[11,14,19,29]. Matysiak-Budnik *et al*[11] conducted a multiple retrospective study in French, including 416 cases of GML. They found that 5-year OS was better for patients < 67 years old (93.6%) than for those with an older age (93.6% *vs* 68.5%, $P < 0.0001$). Another multicenter cohort follow-up study of 420 patients found that age (each incremental year) was an independent prognostic factor for OS ($P = 0.024$)[14]. In our study, age had the highest risk and showed a significant correlation with the prognosis of primary GML ($P < 0.0001$, HR 19.843). The nomogram obviously indicated that increased age, especially > 65 years old, had a negative impact on the OS of the disease. Although several researchers found no association between them[20,29], an insufficient number of subjects in these studies need to be considered. Therefore, we still insist that age and prognosis are closely related. Regardless of the specific cutoff point of age, it is recognized that advanced age means an increased risk of primary GML[13]. Multiple analyses also indicated that Ann Arbor stage was a significant independent prognostic factor for the disease. In our study, most patients presented with stage I-II disease (89.9%), and as the severity of the disease increased, the prognosis of the disease worsened (all $P < 0.05$). The proportion of patients with localized and advanced disease at diagnosis varies among reported series[11,14,36], and we have a consensus that the prognosis of these patients is

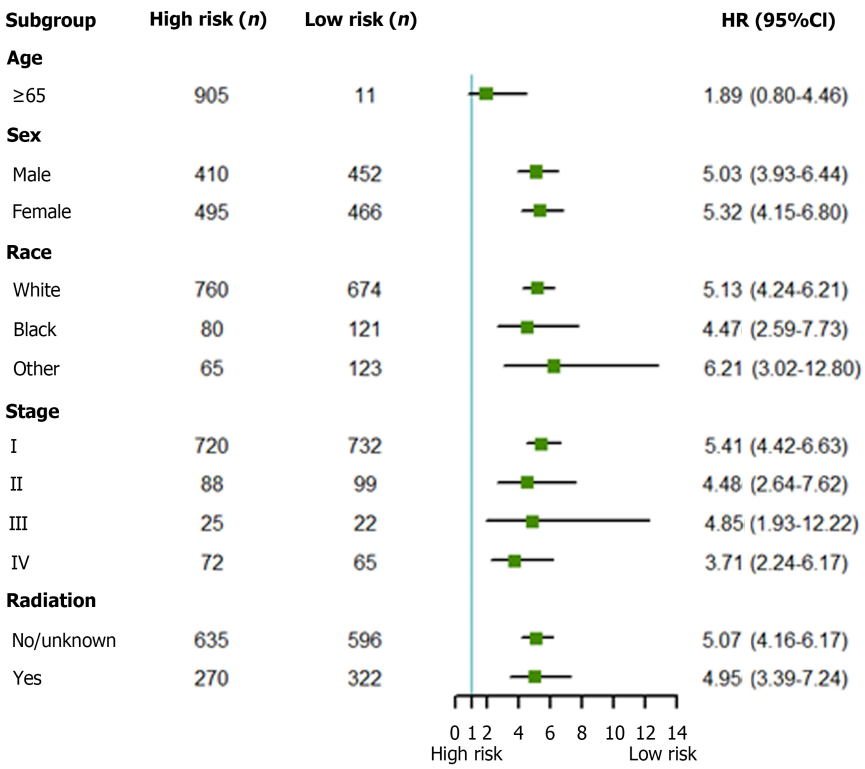

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Figure 5 Subgroup analysis results of associations between independent factors and overall survival time between high- and low-risk patients in the training set. 95%CI: 95% confidence interval; HR: Hazard ratio.

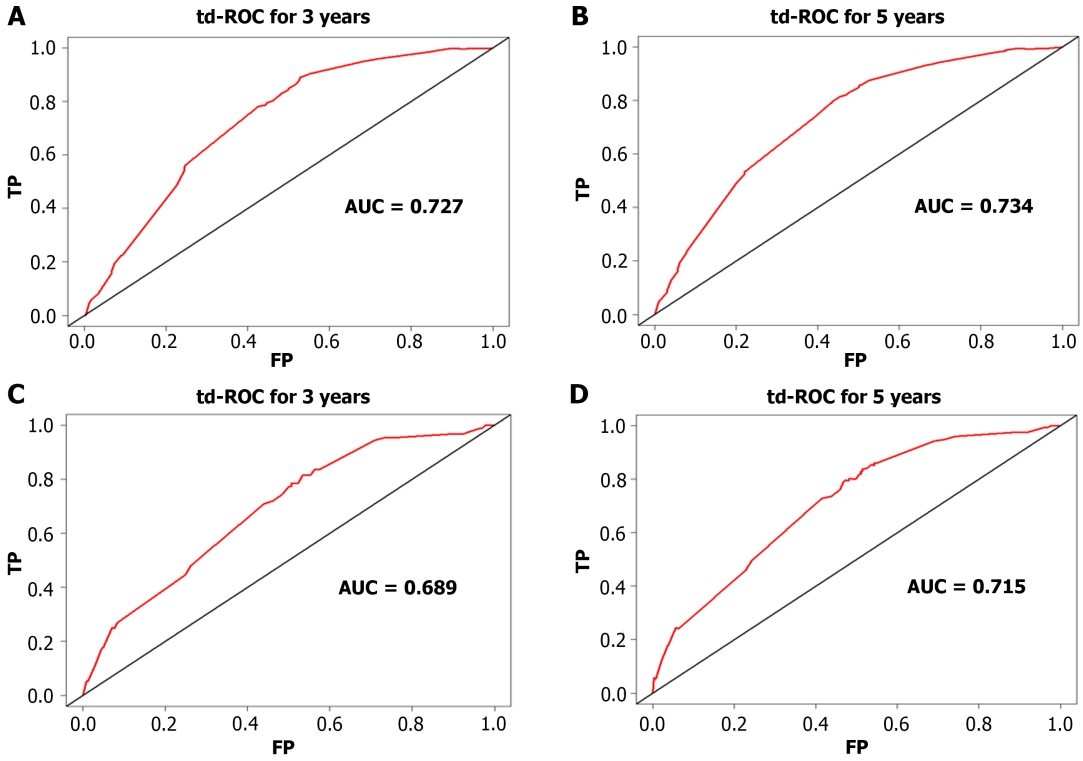

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Figure 6 Time-dependent receiver operating characteristic curves to assess the predictive power of the nomogram. A-D: Curves of 3- and 5-year overall survival in the training set (A and B) and in the validation set (C and D). td-ROC: Time-dependent receiver operating characteristic; OS: Overall survival; AUC: Areas under the curve.

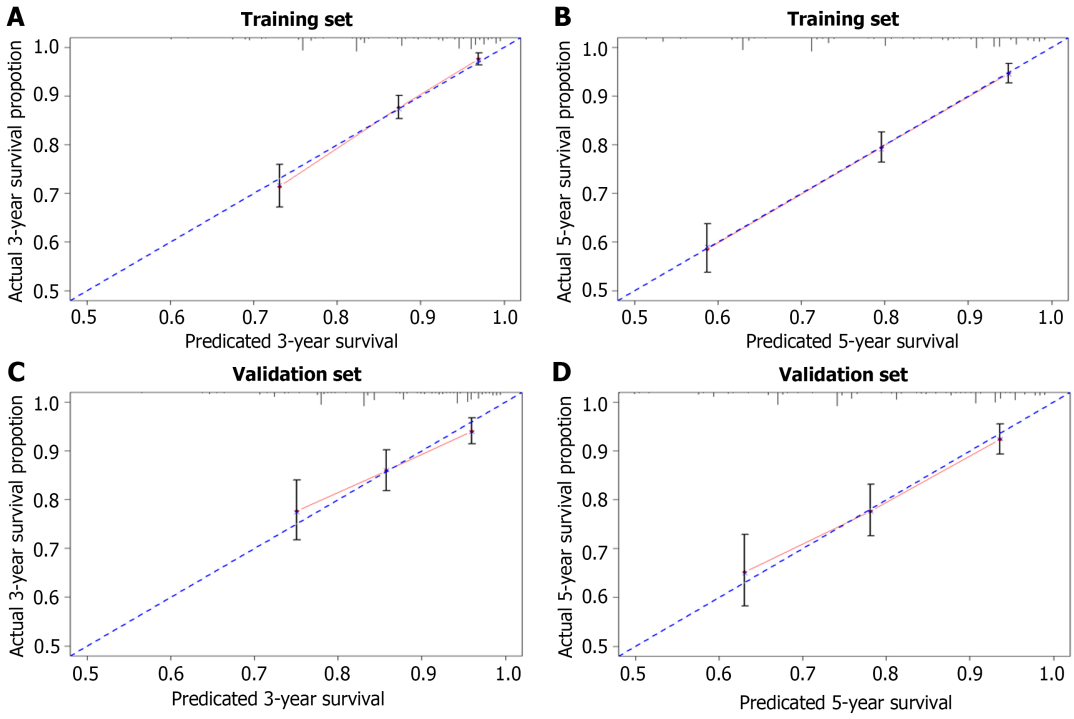

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Figure 7 The calibration plot. A-D: The calibration plot was applied to compare the agreement between actual and predicted probability of 3, 5 years overall survival in training (A and B) and testing set (C and D), respectively. OS: Overall survival.

different[13].

The percentage of female patients was higher than that of male patients (52.0% *vs* 48.0%), and more people were white (79.07%). Statistically significant correlations were found between male sex ($P < 0.001$), black race ($P = 0.036$) and primary GML overall survival. Whether sex is related to the disease remains controversial. Some studies have reported that males have a 2-3 times higher incidence rate of development and a worse prognosis than females[37,38], while other studies have not[11,35]. Until now, no study has focused on the relationship between race and primary GML. SEER provided us with detailed race data and indicated that black individuals are more likely to develop primary GML (HR: 1.374, 95%CI: 1.020-1.779). We still need more studies to further investigate prognostic factors in primary GML.

Management and treatment guidelines for MALT lymphomas have been extremely heterogeneous until the last few years. Over the past 2-3 decades, eradiation of *Helicobacter pylori* (*H. pylori*) has been the preferred choice for GML regardless of the histological status of *H. pylori*[21,39]. Chemotherapy and RT were only suggested to be second-line therapies for nonresponders or advanced patients[39]. In contrast, ESMO guidelines suggested that RT might be the first option for GML patients with localized stages, and chemotherapy was an effective method in patients with all stages[40]. RT alone was also reported to have excellent treatment effects on GML with a total dose of 24-30 Gy[29,41]. Compared with other therapy methods, surgery showed no advantage over treatments in other trials[42]. In our study, no survival difference was found between patients with medication and surgical treatment. Multivariate analysis showed that only RT was significantly associated with better disease prognosis ($P < 0.001$). These data are consistent with previous studies of RT for gastric MALT lymphoma. In total, radiotherapy is a good choice for primary GML disease.

The limitations of this study are very obvious. First, this is a retrospective analysis. All information is from 2004-2015, and part of the data was recorded before the publication of guidelines for primary GML. This may cause heterogeneity in the clinical management of patients, resulting in data bias. Second, SEER cannot provide us with specific details of helicobacter pylori treatment and this is very important for primary GML. Some data even show unknown labels. Third, we have no external verification data. We collected a few cases of primary GML, but they are not worth analyzing considering analysis errors. More multiple, prospective datasets of primary GML are still necessary for further investigation.

CONCLUSION

In conclusion, a nomogram was developed and validated to have good survival predictive performance based on five clinical independent risk factors for OS for primary GML patients. Nomograms are a low-cost and convenient clinical tool in assessing individualized prognosis and treatment for patients with primary GML.

Table 2 Multivariate analysis of overall survival for patients with primary gastric mucosa-associated lymphoid based on least absolute shrinkage and selection operator regression in the training set (*n* = 1823)

Variable		Multivariate		Analysis
		HR	95%CI of HR	*P* value
Age				
< 45	Reference			
45-65		4.290	1.881-9.815	0.001[b]
≥ 65		19.843	8.843-44.528	< 0.001[c]
Sex				
Male				
Female		0.722	0.606-0.860	< 0.001[c]
Race				
White	Reference			
Black		1.374	1.020-1.779	0.036[a]
Other		0.704	0.493-1.003	0.052
Stage (Ann Arbor)				
I	Reference	1.437	1.084-1.904	0.012[a]
II		1.732	1.076-2.786	0.024[a]
III		1.844	1.401-2.428	< 0.001[c]
IV				
Radiation	Reference			
No/unknown				
Yes		0.670	0.542-0.829	

[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
OS: Overall survival; HR: Hazard ratio; CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Extranodal marginal B-cell lymphoma of mucosa-associated lymphoid tissue, known as MALT lymphoma, is a type of non-Hodgkin's lymphoma. The prognosis of primary gastric MALT (GML) patients can be affected by many factors. Few studies have investigated the prognostic variables for overall survival (OS) in patients with primary GML. We searched a large amount of data on patients diagnosed with primary GML in the Surveillance, Epidemiology and End Results (SEER) database.

Research motivation

In this study, we investigated the significant risk factors of primary GML and build an effective survival nomogram model for primary GML patients.

Research objectives

To develop and verify a survival nomogram model that can predict the OS prognosis of primary GML by combining prognostic and determinant variables.

Research methods

All data of patients with primary GML were collected from the SEER database. Based on the LASSO and COX regression, we created and further verified the accuracy and effectiveness of the survival nomogram model by the concordance index (C-index), calibration curve and time-dependent receiver operating characteristic (td-ROC) curves.

Research results

A total of 2604 patients diagnosed with primary GML were selected for this study. A total of 1823 and 781 people were randomly distributed into the training and testing sets at a ratio of 7:3. The median follow-up of all patients was 71 mo, and the 3- and 5-year OS rates were 87.2% and 79.8%, respectively. Age, sex, race, Ann Arbor stage and radiation were independent risk factors for OS of primary GML (all $P < 0.05$). The C-index values of the nomogram were 0.751 (95%CI: 0.729-0.773) and 0.718 (95%CI: 0.680-0.757) in the training and testing cohorts, respectively, showing the good discrimination ability of the nomogram model. Td-ROC curves and calibration plots also indicated satisfactory predictive power and good agreement of the model. Overall, the nomogram shows favorable performance in discriminating and predicting the OS of patients with primary GML.

Research conclusions

A nomogram was developed and validated to have good survival predictive performance based on five clinical independent risk factors for OS for primary GML patients.

Research perspectives

Nomograms are a low-cost and convenient clinical tool in assessing individualized prognosis and treatment for patients with primary GML.

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FOOTNOTES

Author contributions: Shi RH contributed to conceptualization and supervision; Wang D contributed to conceptualization, data curation, formal analysis, and writing-original draft preparation; Shi XL contributed to data curation and formal analysis; Xu W contributed to supervision and editing; all authors approved the manuscript.

Institutional review board statement: The study was reviewed and approved by the Zhongda Hospital, Affiliated Hospital of Southeast University, all our data comes from public database (SEER database) and need no ethical approval.

Informed consent statement: The informed consent was waived because all of data were downloaded from open database "SEER Database".

Conflict-of-interest statement: All authors of this paper have no conflict of interests to disclose.

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Retrospective Study

Mitophagy-related gene signature predicts prognosis, immune infiltration and chemotherapy sensitivity in colorectal cancer

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Abstract

BACKGROUND

Mitophagy plays essential role in the development and progression of colorectal cancer (CRC). However, the effect of mitophagy-related genes in CRC remains largely unknown.

AIM

To develop a mitophagy-related gene signature to predict the survival, immune infiltration and chemotherapy response of CRC patients.

METHODS

Non-negative matrix factorization was used to cluster CRC patients from Gene Expression Omnibus database (GSE39582, GSE17536, and GSE37892) based on mitophagy-related gene expression. The CIBERSORT method was applied for the evaluation of the relative infiltration levels of immune cell types. The performance signature in predicting chemotherapeutic sensitivity was generated using data from the Genomics of Drug Sensitivity in Cancer database.

RESULTS

Three clusters with different clinicopathological features and prognosis were

identified. Higher enrichment of activated B cells and $CD4^+$ T cells were observed in cluster III patients with the most favorable prognosis. Next, a risk model based on mitophagy-related genes was developed. Patients in training and validation sets were categorized into low-risk and high-risk subgroups. Low risk patients showed significantly better prognosis, higher enrichment of immune activating cells and greater response to chemotherapy (oxaliplatin, irinotecan, and 5-fluorouracil) compared to high-risk patients. Further experiments identified CXCL3 as novel regulator of cell proliferation and mitophagy.

CONCLUSION
We revealed the biological roles of mitophagy-related genes in the immune infiltration, and its ability to predict patients' prognosis and response to chemotherapy in CRC. These interesting findings would provide new insight into the therapeutic management of CRC patients.

Key Words: Colorectal cancer; Mitophagy; Tumor microenvironment; Immunotherapy; Prognosis

Core Tip: We for the first time revealed the biological roles of mitophagy-related genes in the immune infiltration, and its prediction of patients' prognosis and chemotherapy response in colorectal cancer. These novel findings would provide new insight into the therapeutic management of colorectal cancer patients.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant diseases in the world[1]. Though the past decades have witnessed a great improvement in treatment programs, the 5-year survival for patients with CRC after tumor resection remains poor and approximately 50% of patients will develop distant metastasis[2]. Thus, effective prognostic markers and novel potential therapeutic targets are necessary to help individualize therapy for CRC patients in clinical practice.

AS a specific type of autophagy, mitophagy is essential in eliminating damaged or excessive mitochondria through autophagolysosomes[3,4]. The process of mitophagy is frequently activated under oxidative and energetic stress, which occur frequently during cancer initiation and progression[5-7]. Previous studies have reported the correlation between mitophagy dysregulation and colorectal cancer formation and invasion. Yin *et al*[8] revealed that the mitophagy protein PTEN induced putative kinase 1 suppresses CRC cell proliferation by rewiring metabolism *via* activating p53 and reducing acetyl-CoA production. In addition, induction of mitophagy was shown to contribute to drug resistance in CRC stem cells[9]. A recent study showed that in intestinal epithelial cells, adaptive immunity could be activated by mitophagy during CRC initiation, confirming the correlation between mitophagy and anti-tumor immunity in colorectal cancer[10]. Therefore, targeting mitophagy in CRC cells represents a promising anticancer strategy. Therefore, we aimed to reveal the underlying association between mitophagy and prognosis, immune infiltration and the response to chemotherapy in CRC patients by analyzing the transcriptomics of mitophagy-associated genes in CRC comprehensively.

In this study, we analyzed three datasets (GSE39582, GSE17536, and GSE37892) from the GEO database to elucidate the pattern of mitophagy in CRC. Three mitophagy-related phenotypes in CRC were identified. A prognostic signature was established to quantify the death risk, immune infiltrating characteristics and therapeutic response. Our findings may provide new insights for treatment strategies in CRC based on the mitophagy-related gene signatures.

MATERIALS AND METHODS

Data preparation

GSE39582, GSE17538, and GSE37892 microarray datasets were retrieved from the GEO database, whose website is https://www.ncbi.nlm.nih.gov/geo/. The three abovementioned datasets were generated using the Affymetrix HG-U133 plus 2.0 platform and the Robust Multichip Average method was used to perform data normalization. When multiple datasets were combined as a whole cohort, the ComBat method was used to remove the batch effects. This method was executed in the SVA R package (version 3.2.1).

Dataset selection

The inclusion criteria for dataset selection were: (1) All datasets were generated using the Affymetrix platform (Affymetrix HG-U133 plus 2.0); (2) sample size > 100; (3) patients were pathologically diagnosed with colorectal cancer; (4) basic clinical information for patients was available for analysis; and (5) patients had complete follow-up information. Based on the inclusion criteria, the CRC datasets GSE39582, GSE17538 and GSE37892 were included in this study. The three datasets were combined together and randomly separated into training sets and validation sets at a ratio of 1:1.

Mitophagy-related genes

the Gene Set Enrichment Analysis (http://www.gsea-msigdb.org/gsea/index.jsp) and h GeneCards (https://www.genecards.org/) were utilized to generate a total of 51 genes related to mitophagy. The gene list is shown in Supplementary Table 1.

Clustering by non-negative matrix factorization

We performed consensus clustering "Nonnegative Matrix Factorization (NMF)" with the nrun parameter being set to 50 for CRC patients classification based on the expression levels of mitophagy-related genes[11,12]. This procedure was performed using the "NMF" package in R (version 0.24.0).

Gene set variation analysis

Based on gene expression data, Gene set variation analysis (GSVA) has been widely used to evaluate the activity of signaling pathways *via* the unsupervised method. The "GSVA" R package (version 1.42.0) was used to quantify the activity of the signaling pathways in each patient. For GSVA processing, the MSigDB database (https://www.gsea-msigdb.org/gsea/index.jsp) was used to download gene set information.

Immune infiltration analysis

We performed a CIBERSORT deconvolution approach to evaluate the relative abundance of 22 tumor-infiltrating immune cells, which included naïve B cells, naïve T cells, memory B cells, activated $CD4^+$ memory T cells, plasma cells, resting $CD4^+$ memory T cells, $CD8^+$ T cells, regulatory T cells (Tregs), $CD4^+$ T cells, activated NK cells, follicular helper T cells, resting NK cells, gamma delta T cells, activated mast cells, activated dendritic cells, monocytes, M2 macrophages, M0 macrophages, resting mast cells, M1 macrophages, resting dendritic cells, neutrophils, and eosinophils in each CRC sample[13].

Construction of a mitophagy-related prognostic gene signature

To develop the mitophagy-related prognostic gene signature, we overlapped differentially expressed genes (DEGs) of each cluster identified by "NMF" function to explore the mitophagy related gene expression pattern. The DEGs were identified by lusing LIMMA package (version 3.26.9). Then univariate Cox regression analysis was adopted to identify those significantly associated with overall survival. Next, the least absolute shrinkage and selection operator method (LASSO) Cox regression model was carried out at 10-fold cross-validation to identify the most meaningful mitophagy related genes[14]. The LASSO Cox model was perfomed by lusing glmnet package (version 2.0.5). Finally, the gene signature was established based on selected 36 target genes to calculate risk_score, which was calculated as follows: Risk_score = Σ (Expi × coefi).

Based on the median value of individual risk score, the patients in the training and validation sets were categorized into low and high-risk groups and a Kaplan-Meier survival analysis was conducted to analyze survival difference. The subsequent survival receiver operator characteristic (ROC) as utilized for the evaluation of the prognostic accuracy of the gene signature.

Drug susceptibility analysis

We trained our prognostic signature in pharmacogenomics database (Genomics of Drug Sensitivity in Cancer, https://www.cancerrxgene.org/) to compare the difference of chemotherapeutic sensitivities between low and high-risk patients. The R package "pRRophetic" (version 0.5) was used to conduct the prediction procedure and a ridge regression model was used to estimate the IC_{50} value of each chemotherapeutic drug.

Statistical analysis

All statistical analysis was performed based on R (version 4.1.2). A Wilcoxon rank-sum test was carried out for data displaying a skewed distribution. A Student's *t*-test was performed for data with a normal distribution to compare the differences between groups. A Kruskal-Wallis test was adopted to compare the differences between the three groups. A P value < 0.05 was considered to be significant statistically.

RESULTS

Identification of mitophagy-related subtypes in CRC

A total of 836 CRC patients from the three GEO datasets were identified in this study. The expression correlation among mitophagy-related genes was shown in Figure 1A and survival analysis found that 18 of them were prognostic for CRC (Supplementary Table 2). To further characterize the expression profiling patterns mediated by mitophagy in CRC, we used the "NMF" method and stratified patients into three clusters based on the optimal clustering number (Figure 1B and C). 435 samples were grouped in cluster I, 172 samples in cluster II, and 229 samples in cluster III. The three clusters could be notably discriminated according to PCA analysis (Figure 1D). Survival analysis showed that patients in cluster III exhibited the best prognosis (Figure 1E; P = 0.0057). Further correlation analysis did not observe significant connection between Cluster III and early stage of CRC, suggesting that this identified molecular cluster may be an independent prognostic factor (Figure 1F).

Pathway enrichment and immune infiltration analysis among mitophagy-related clusters

Pathway enrichment analysis based on GSVA showed that the metastasis related pathways, such as epithelial mesenchymal transition, KRAS signaling, myogenesis, coagulation, and angiogenesis were mainly enriched in cluster I and cluster II compared to cluster III (Figure 2A and B). To further characterize the immune infiltration among the three clusters, we employed CIBERSORT[15] analyses and found that activated dendritic cells, memory B cells, and activated memory $CD4^+$ T cells (Figure 2C) were predominantly enriched in cluster III.

Construction and validation of the mitophagy-related gene signature

To investigate the prognostic prediction of mitophagy-related genes, we overlapped the DEGs of three clusters and obtained a total of 3112 genes (Figure 3A). Further GO enrichment indicated that these genes were associated with mitochondrial biological function, including mitochondrial gene expression (Figure 3B). Next, univariate Cox analysis was performed and we found 347 prognostic genes with a P value < 0.001 (Supplementary Table 3). The least absolute shrinkage and selection operator (LASSO) regression model was applied to the training set and the minimize λ method resulted in 36 mitophagy-related DEGs (Figure 3C and D). The risk score of each patient was then calculated for the training set based on the established formula. The coefficients of the selected 36 mitophagy-related DEGs were shown in Table S4. Basing on the median risk score, we separated the patients in the training set into low-risk (n = 209) or high-risk (n = 209) groups. The distribution patterns of risk scores and patients' survival status were shown in Figure 3E and it was noticed that the risk of death increased gradually with the incremental risk scores. The same analyses were then conducted in the validation set and a similar distribution was found (Figure 3F).

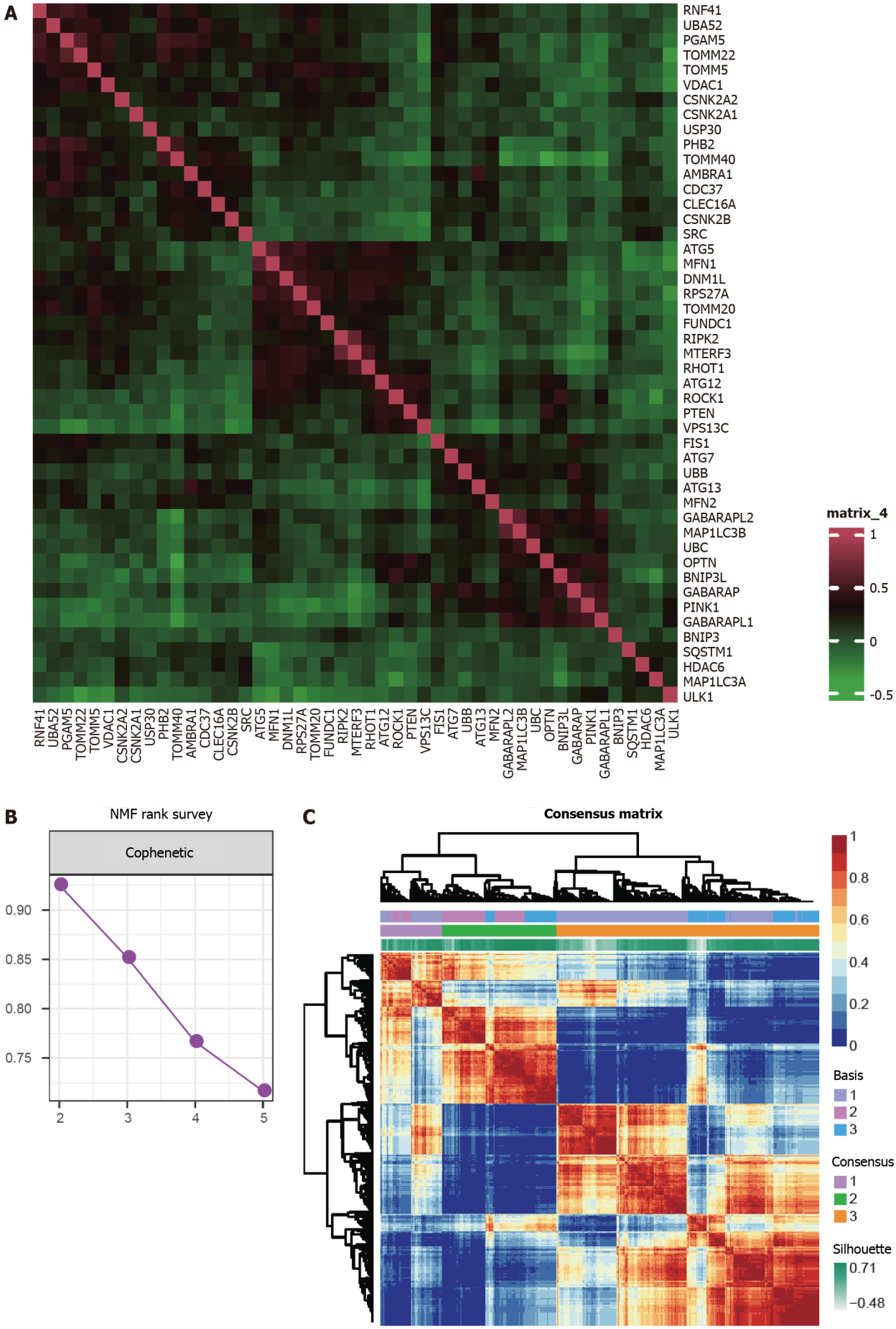
Predictive capacity of the mitophagy-related gene signature

Based on the median mitophagy risk score, we explored patients' survival rates between the low- and high-risk groups. We observed that high-risk patients showed a significantly worse survival rate than low risk patients in both training (Figure 4A) and validation sets (Figure 4B). Next, we calculated the area under the curve (AUC) values of survival rates in two cohorts. The results showed that the 5-year AUC values all reached 0.7 in the training (Figure 4C) and validation sets (Figure 4D).

After other clinicopathological parameters were adjusted, the mitophagy-related gene signature remained an independent prognostic factor in the whole cohort (Figure 5A). To further facilitate the clinical use of prognostic signatures, a nomogram was developed based on the multivariate result (Figure 5B). The mitophagy-related gene signature, TNM stage and tumor location were integrated into nomogram. The developed nomogram exhibited outstanding prognostic accuracy and displayed superior performance compared to any other factors alone in predicting survival (Figure 5C and D).

External validation of mitophagy-related gene signature in TCGA cohort

To further externally validate the prognostic and predictive value of the establised mitophagy-related gene signature, CRC patients were identified from TCGA database. Consistent with the result in training and internal validation sets, the risk of death increased gradually with the incremental risk scores in TCGA cohort (Supplementary Figure 1A). By applying the prognostic signature in TCGA cohort, it was found that patients were successfully separated into two groups with significantly different overall survival (Supplementary Figure 1B). In addition, survival ROC showed that the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CDC37
CLEC16A
CSNK2B
SRC
ATG5
MFN1
DNM1L
RPS27A
TOMM20
FUNDC1
RIPK2
MTERF3
RHOT1
ATG12
ROCK1
PTEN
VPS13C
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ATG7
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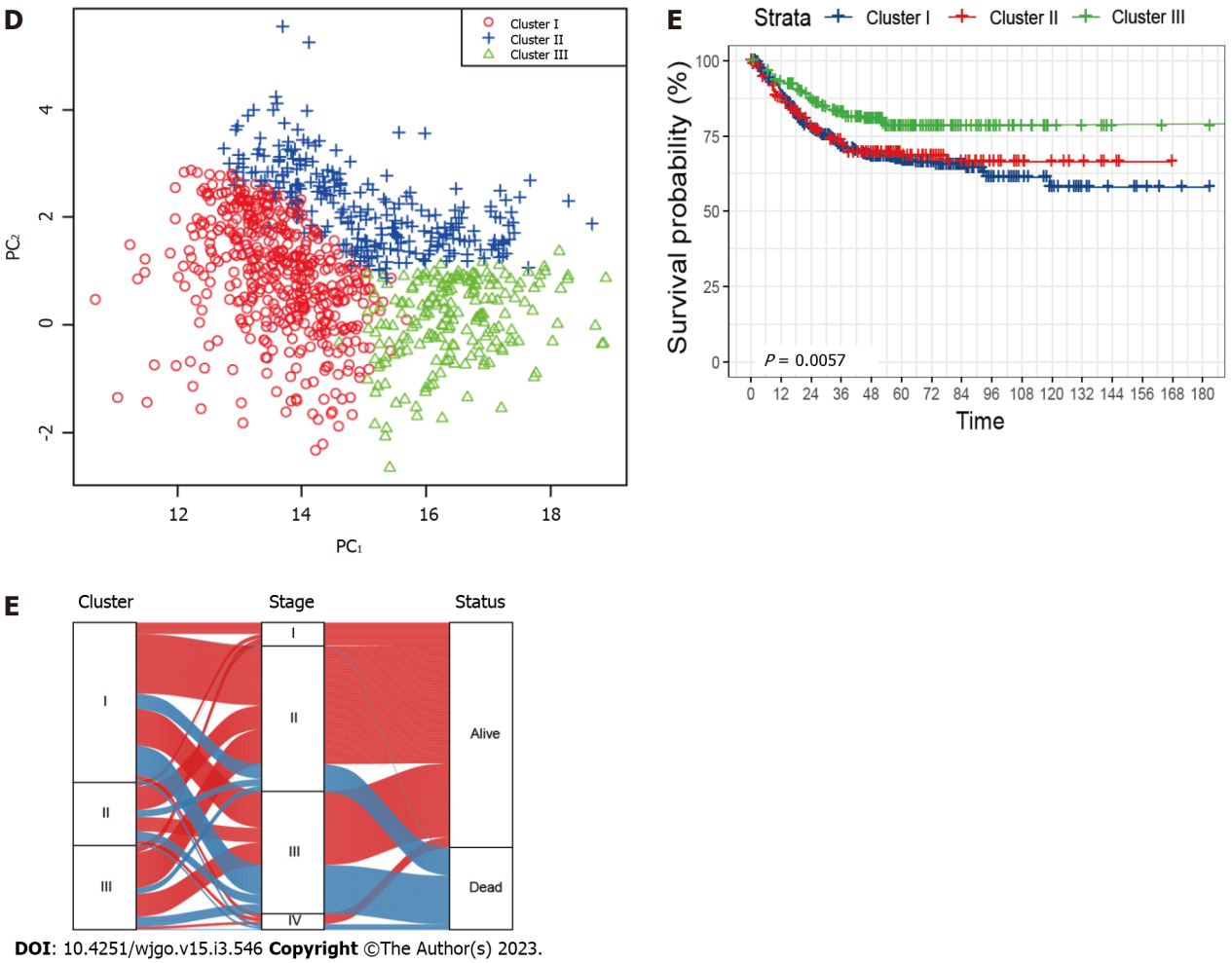

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Figure 1 Grouping of mitophagy-related clusters in colorectal cancer. A: Expression correlation among mitophagy-related genes; B: The cophenetic plot of NMF clustering analyses; C: Heatmap showing three clusters were identified, along with the optimal value for consensus clustering; D: Principal component analysis of three mitophagy-related clusters; E: Kaplan-Meier curves for survival rate of three clusters showing patients in cluster III have the best prognosis (P = 0.0057). The P value was calculated using the log-rank test; F: Alluvial diagram of clusters shows patients in cluster III tend to be early-stage. NMF: Nonnegative Matrix Factorization.

developed mitophagy-related gene signature performed good in predicting prognosis (5-year AUC = 0.72, Supplementary Figure 1C).

Characterization of Immune infiltration between high- and low-risk groups

Since mitophagy-related gene signatures exhibited good predictive value in predicting survival, we next evaluated the pathway enrichment and immune infiltration patterns in patients from the high- and low-risk groups. The GSVA analysis showed that several important metastasis-related pathways, including epithelial mesenchymal transition, myogenesis, coagulation and angiogenesis were mainly enriched in high-risk group (Figure 6A). By using CIBERSORT analyses, we observed that memory B cells, activated dendritic cells, activated mast cells, plasma cells, and activated memory CD4$^+$ T cells were most notably enriched in low-risk patient group (Figure 6B and C).

Prediction of chemotherapy drug sensitivity

Chemotherapy drugs including oxaliplatin, 5-Fluorouracil and irinotecan are the mainstay treatment for CRC. Considering the distinct long-term survival and signaling pathway enrichment between the risk groups, the GSDC database was used to compare the sensitivity to chemotherapy drugs and several targeted inhibitors between low and high-risk patients. It demonstrated the estimated IC_{50} of oxaliplatin, 5-Fluorouracil and irinotecan were dramatically higher in the high-risk group. In addition, the IC_{50} of several other inhibitors including the Wnt inhibitor, KRAS inhibitor, Erlotinib, and Afatinib were notably higher in high-risk group (Figure 7).

The effect of CXCL3 on mitophagy in CRC cells

Paired transcriptome profiling data of cancer and normal tissues were obtained from GSE32323 dataset. We focused on the upregulated mitophagy related gene CXCL3, which showed the highest fold change

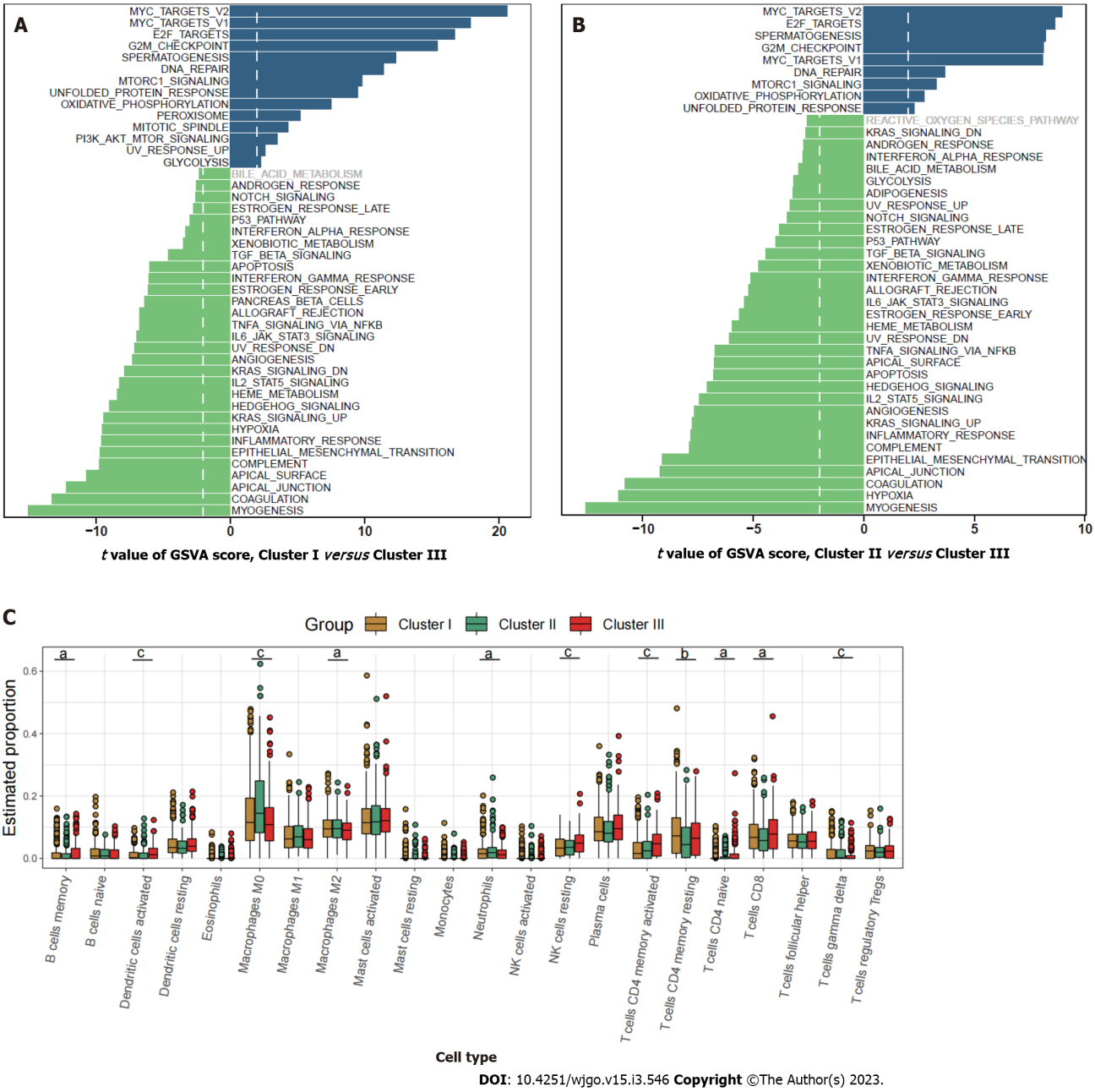

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Figure 2 Characterization of the three clusters. A: Differences in metastasis-related pathways scored by gene set variation analysis (GSVA) between cluster I and cluster III; B: Differences in pathway activities scored by GSVA between cluster II and cluster III; C: Relative abundance of 22 tumor-infiltrating immune cells in each of the three clusters. The statistical differences between the three clusters as determined through the Kruskal-Wallis H test. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

in cancer tissues (Figure 8A). We then enforced CXCL3 expression in CRC cells (Figure 8B). The result showed that CXCL3 over-expression increased the proliferative ability of cells (Figure 8C) and weakened their sensitivity to oxaliplatin treatment (Figure 8D). We next examined mitochondrial DNA (mtDNA) to observe how ULK1 modulated mitophagy. The decrease in mtDNA was attenuated in the groups with CXCL3 overexpressed under hypoxic conditions (Figure 8E and F).

DISCUSSION

In our study, we integrated and analyzed transcriptomic patterns mediated by mitophagy-related genes. Using the "NMF" method, we grouped patients in the GEO datasets into three clusters with distinct clinical features, among which cluster III showed the most favorable prognosis and the most enriched immune cell infiltration. Here, we demonstrated that cluster III exhibited the enrichment of memory B cells, activated dendritic cells and activated memory CD4^{+} T cells. As previously reported, dendritic cells are a diverse group of specialized antigen-presenting cells with key roles in the initiation and regulation of innate and adaptive immune responses[16,17]. With their potent antigen presenting

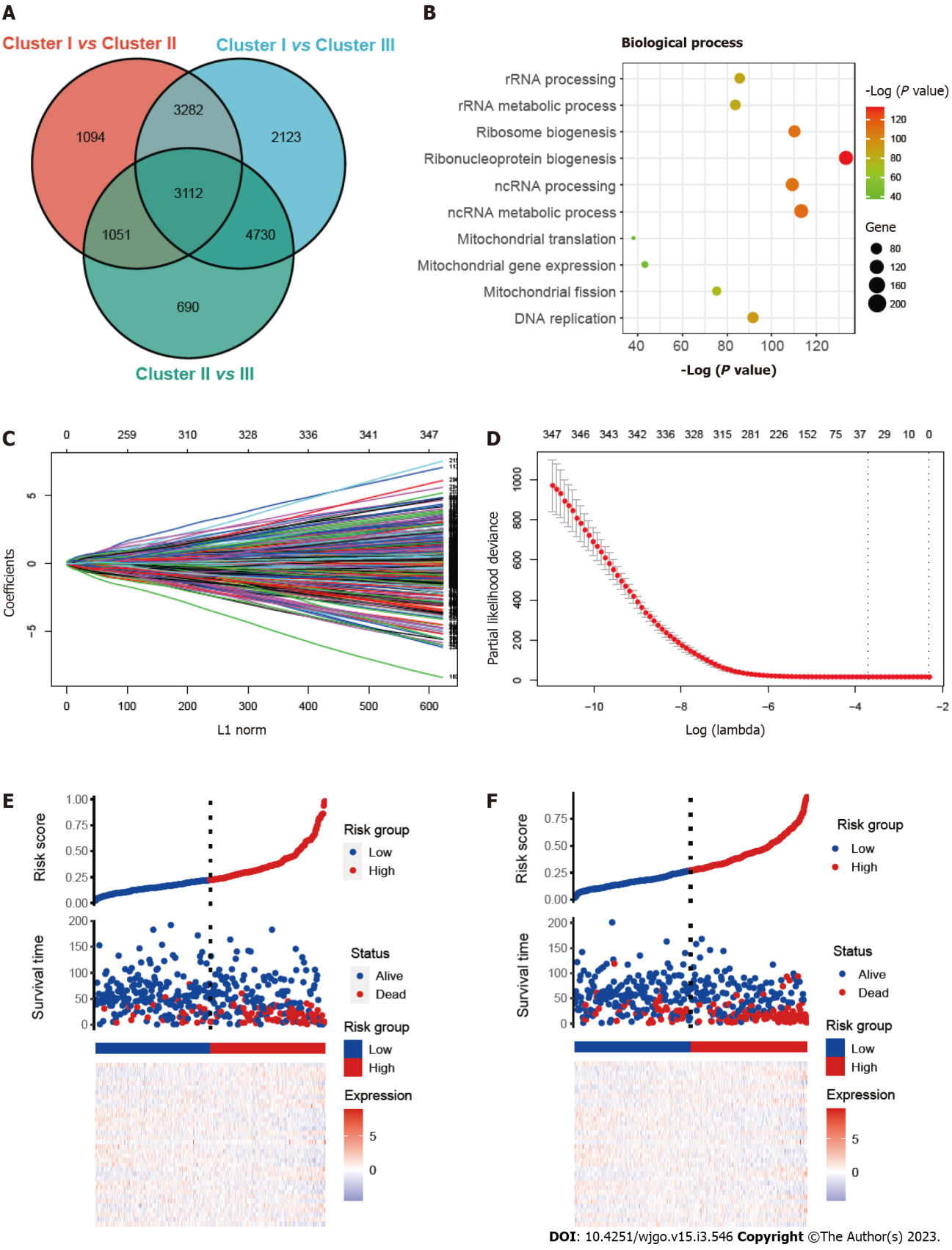

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Figure 3 Construction and validation of the prognostic risk_score. A: Venn diagram showing overlapped genes between the three clusters; B: Gene ontology enrichment (GO) analysis of mitophagy-related genes; C and D: Thirty-six candidate genes screened out by least absolute shrinkage and selection operator method analysis with minimal lambda; E and F: Ranked dot and scatter plots showing risk_score distribution, patient survival status, survival time and expression of 36 mitophagy-related genes in training set and validation set.

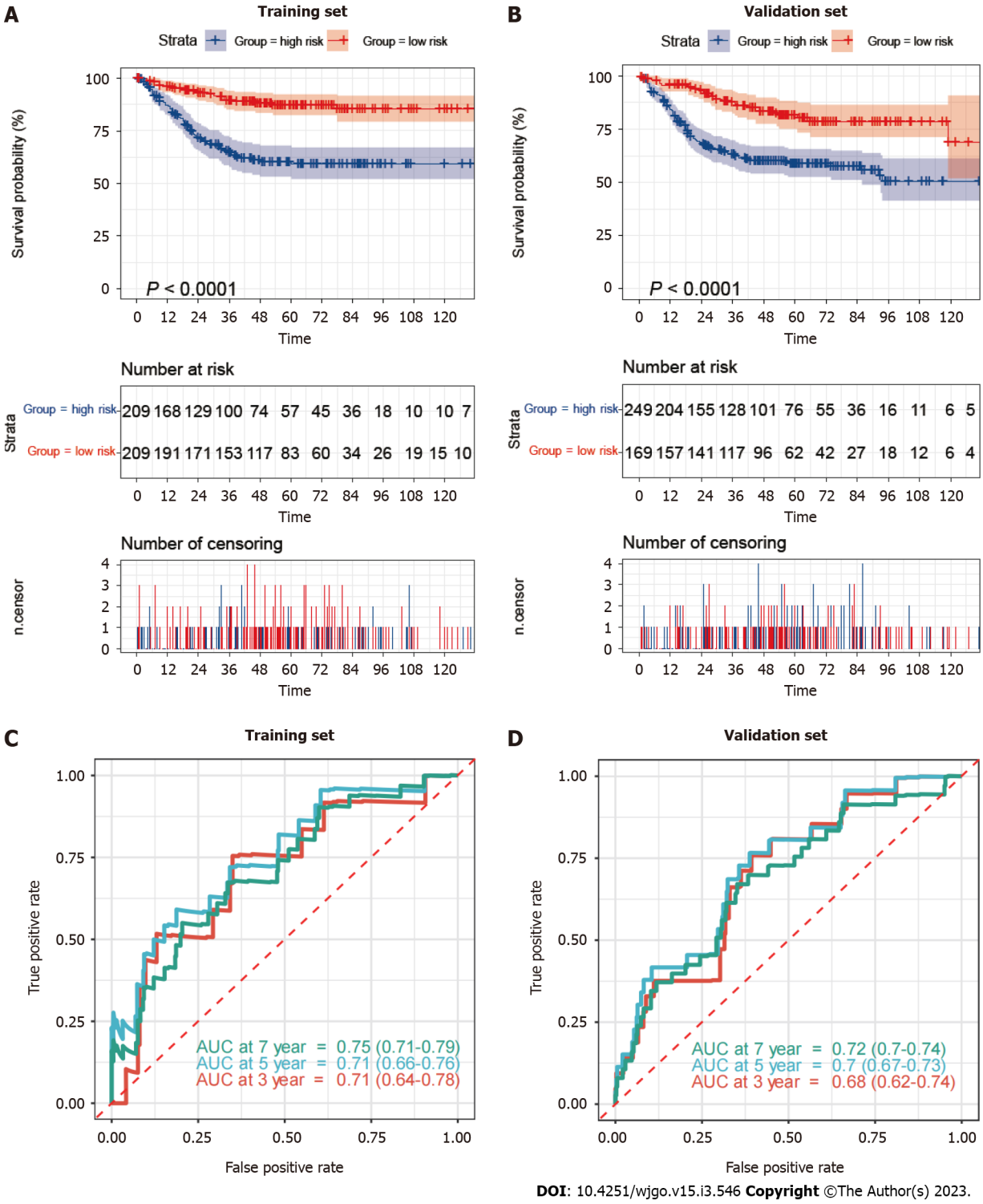

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Figure 4 Mitophagy-related gene signatures in training and validation sets. A and B: Kaplan–Meier survival analysis between high-risk and low-risk patients; C and D: Receiver operator characteristic curves and area under the curve to predict the sensitivity and specificity of 3-, 5-, and 7-year survival according to the risk_score. AUC: Area under the curve.

ability, dendritic cells have been long considered as critical factor in antitumor immunity[18]. $CD4^+$ T cells, as a major population of cells in the tumor microenvironment (TME), are required for efficacious anti-tumor immunity[19-21]. $CD4^+$ T cell are important in fully equipping cytotoxic T lymphocyte cells to target and eliminate cancer cells and facilitate the improvement in outcomes of all cancer immunotherapy strategies[22-25]. In this part, our research delineated intra-tumor heterogeneity of transcriptomic classifications mediated by mitophagy-related genes, and characterized their TME infiltration characteristics. We believe that the molecular classification mediated by mitophagy-related genes could provide new insight for risk stratification and therapeutic management in patients with CRC.

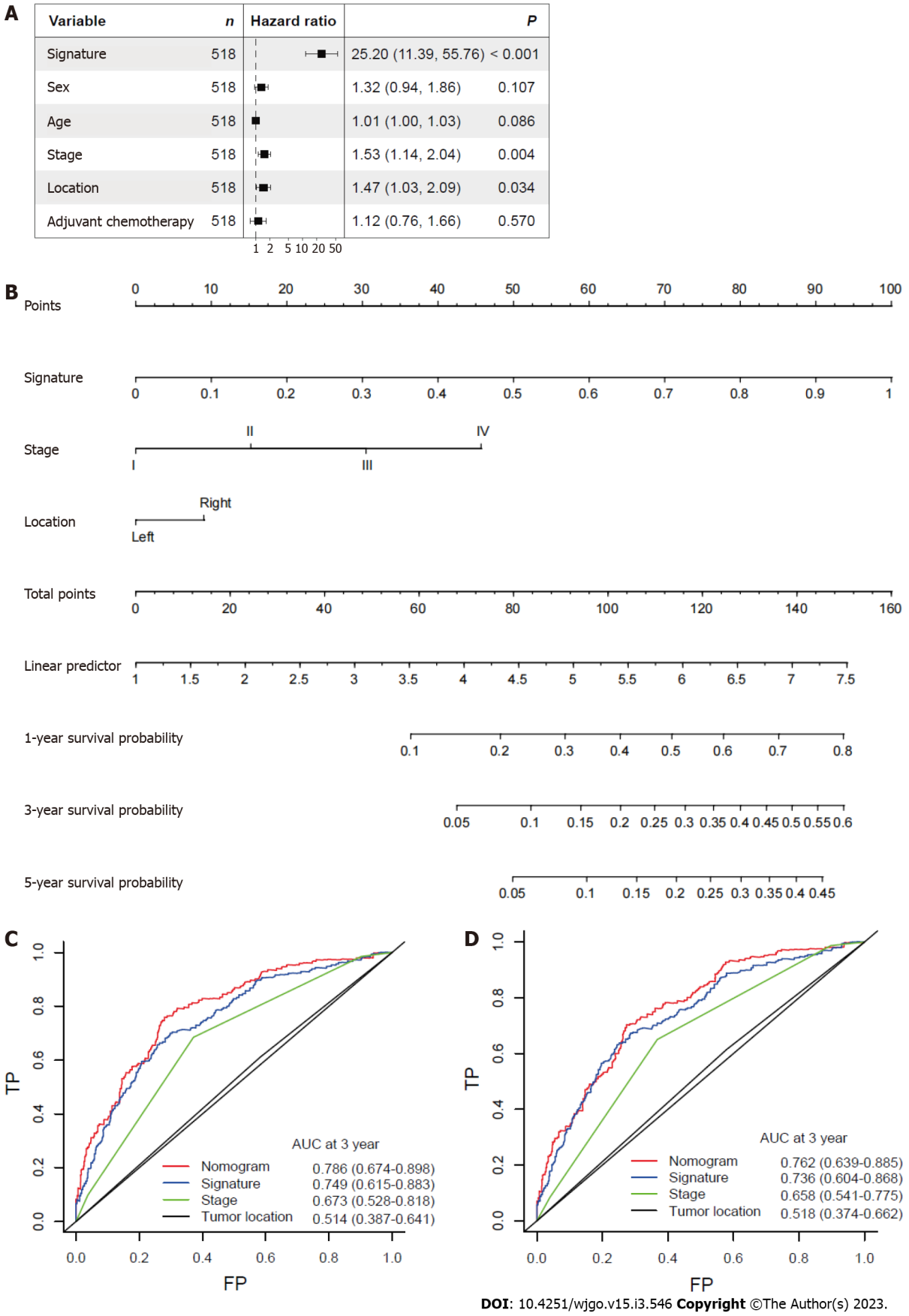

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Figure 5 Nomogram based on gene signature. A: Hazard ratios of the mitophagy-related gene signature after adjusting for other clinicopathological variables by multivariate Cox regression analysis; B: Nomogram integrating risk_score and clinical characteristics; C: Receiver operator characteristic curves of the nomogram, gene signature, stage and tumor location at 3 years; D: Receiver operator characteristic curves of the nomogram, gene signature, stage and tumor location at 5 years. AUC: Area under the curve.

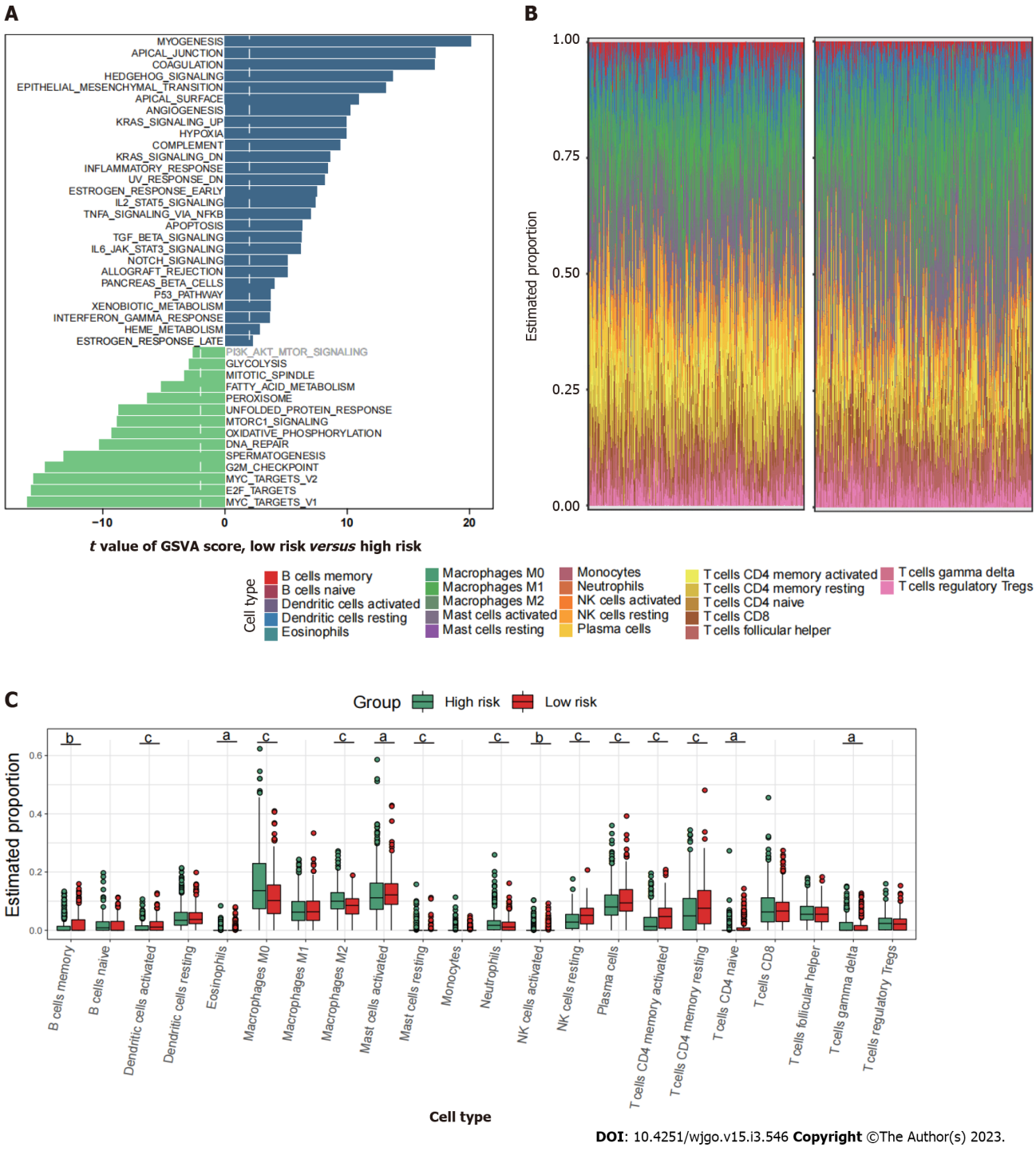

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Figure 6 Immune characteristics of the high- and low-risk groups. A: Differences in metastasis-related pathways scored by gene set variation analysis; B and C: Comparison of the TIICs between the two risk groups. [a]*P* < 0.05; [b]*P* < 0.01; [c]*P* < 0.001.

To further quantify the survival risk of CRC patients and facilitate prognostic predictions, we next identified mitophagy subtype related genes and determined the most useful genes to develop the final prognostic signature. Based on the LASSO regression model, a total of 36 mitophagy-related DEGs were identified. In training and validation sets, patients were successfully divided into two groups with significantly different prognoses. Here, we demonstrated that a high risk score was consistently related to a worse prognosis, and that this model exhibited excellent prognostic prediction abilities. In addition, we developed a nomogram based on mitophagy-related gene signatures to facilitate clinical follow-up and management in colorectal cancer. In both the training and validation sets, we confirmed the strong ability of prediction in our nomogram.

Most of the mitophagy-related genes have been experimentally investigated in colorectal cancer and 18 of them were prognostic for CRC[26-31]. We found that PTEN-induced kinase 1 (PINK1) was one of the most significant risk factors. Previous studies have revealed the restrained role of PINK1 mediated

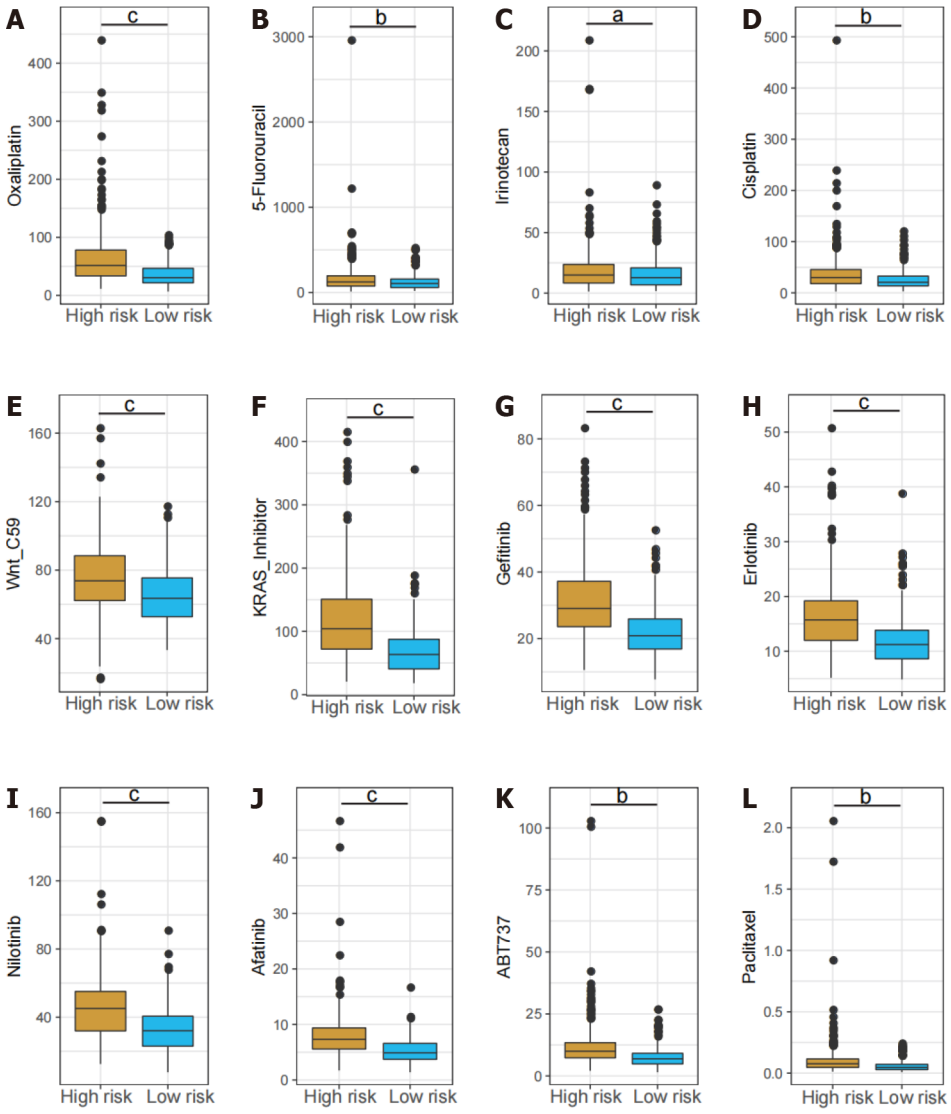

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Figure 7 Estimated IC_{50} of different chemotherapy drugs between the high- and low-risk groups. A: Oxaliplatin; B: 5-Fluorouracil; C: Irinotecan; D: Cisplatin; E: Wnt_C59; F: KRAS_Inhibitor; G: Gefitinib; H: Erlotinib; I: Nilotinib; J: Afatinib; K: ABT737; L: Paclitaxel. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

mitophagy in innate immunity[32]. In addition, a pan-cancer analysis showed a correlation between PINK1 expression and immune infiltration, including infiltration of B cells, $CD8^{+}$ T cells, $CD4^{+}$ T cells, macrophages, neutrophils, and dendritic cells, suggesting that PINK1 can work as a biomarker for prognosis and immune responses. However, this finding requires further experimental validation[33].

Though a novel mitophagy-related-gene-based signature has been developed in this study, our study mainly focused on the bioinformatic analyses, which still requires a more robust confirmation. Consequently, more in-depth experimental validation is needed to validate these findings.

CONCLUSION

In this study, we have shown for the first time the biological function of mitophagy-related genes in immune infiltration in CRC, and its correlations with patients' prognosis and chemotherapy response in CRC. These interesting findings would offer novel insights for therapeutic management of CRC patients.

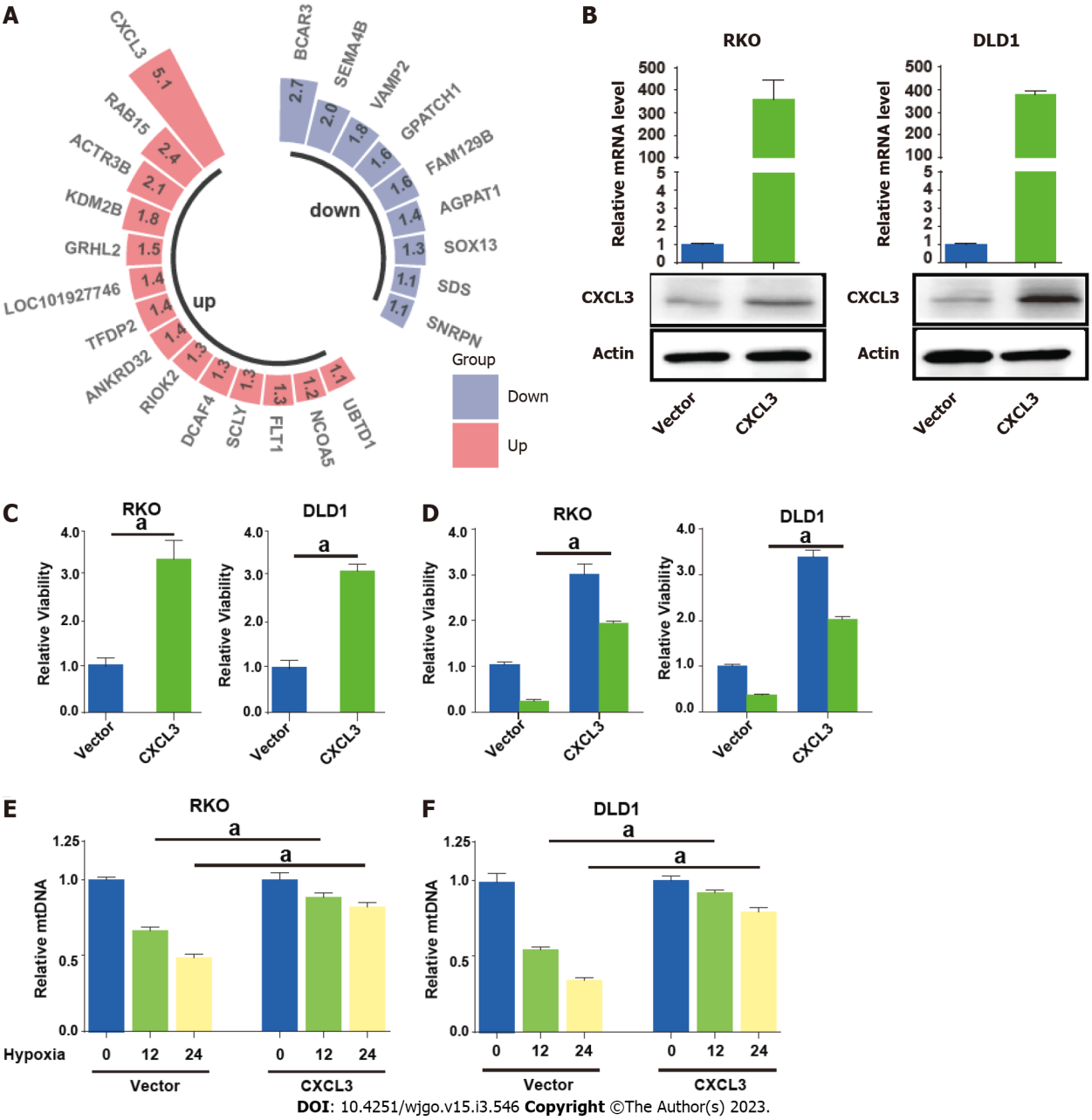

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Figure 8 CXCL3 attenuates mitophagy in colorectal cancer cells. A: Differentially expressed mitophagy related genes included in the signature; B: quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and Western blot showing enforced expression of CXCL3 in colorectal cancer (CRC) cells; C: CXCL3 overexpression significantly enhanced cell proliferation and cell viability; D: CXCL3 overexpression weakened the sensitivity of CRC cells to oxaliplatin treatment; E and F: The mtDNA of CRC cells with or with CXCL3 overexpression was detected using qRT-PCR. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

ARTICLE HIGHLIGHTS

Research background

Mitophagy plays essential role in the initiation and progression of colorectal cancer (CRC). Mitophagy plays essential role in the initiation and progression of CRC.

Research motivation

The effect of mitophagy-related genes in CRC remains largely unknown.

Research objectives

To develop a mitophagy-related gene signature to predict the survival, immune infiltration and chemotherapy response of CRC patients.

Research methods

Non-negative matrix factorization was used to cluster CRC patients from Gene Expression Omnibus database (GSE39582, GSE17536, and GSE37892) based on mitophagy-related gene expression. The CIBERSORT method was applied for the evaluation of the relative infiltration levels of immune cell types. The performance signature in predicting chemotherapeutic sensitivity was evaluated based on the Genomics of Drug Sensitivity in Cancer database.

Research results

Three clusters with different clinicopathological features and prognosis were identified. Higher enrichment of activated B cells and $CD4^+$ T cells were observed in cluster III patients with the most favorable prognosis. Next, a gene risk model related to mitophagy was produced and patients in training and validation sets were categorized into low-risk and high-risk groups. Low risk patients showed significantly better prognosis, higher enrichment of immune activating cells and greater response to chemotherapy (oxaliplatin, irinotecan and 5-fluorouracil) compared to high risk patients. Further experiments identified CXCL3 as novel regulator of cell proliferation and mitophagy.

Research conclusions

We revealed the biological roles of mitophagy-related genes in the immune infiltration, and its ability to predict patients' prognosis and response to chemotherapy in CRC. These new findings would offer meaningful insights for the therapeutic management of CRC patients.

Research perspectives

The developed signature in this study will aid in individualizing treatment and follow-up scheme in CRC patients.

FOOTNOTES

Author contributions: Weng JS, Huang JP, and Yu W contributed equally to this work; Lin JP and Ye Y had the idea for this study; Weng JS, Huang JP, and Yu W supervised the acquisition of the data; Xiao J and Zang WD undertook the statistical analysis; Lin F, Lin KN, and Zang WD provided statistical advice; all authors contributed to interpretation of the results; Weng JS, Huang JP, and Yu W wrote the article and other authors contributed to the content; all authors approved the final version of the manuscript, including the authorship list.

Institutional review board statement: The study was reviewed and approved by the Fujian Cancer Ethics Committee (Approval No. K2023-030-01).

Informed consent statement: All the data were downloaded from GEO and TCGA database, thus the informed consent statement is not applicable in this study.

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

Data sharing statement: Source data and reagents are available from the corresponding author upon reasonable request.

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CASE REPORT

Carcinosarcoma of common bile duct: A case report

Yao Yao, Hong-Gang Xiang, Lei Jin, Ming Xu, Shu-Yan Mao

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Abstract

BACKGROUND

Carcinosarcomas of the common bile duct (CBD) are an extremely rare finding in the clinical setting. Based on a review of 12 literatures, 3 cases had the imaging features of ossification. Carcinosarcomas are prone to distant metastasis, as they possess clinical features of both carcinoma and sarcoma, and generally have with a poor prognosis. Due to the small number of cases reported, clinical experience in the diagnosis and treatment of the disease is lacking.

CASE SUMMARY

The patient was a 75-year-old woman who had experienced recurrent chills with nausea and vomiting for 3 mo. Computed tomography, magnetic resonance imaging, endoscopic ultrasonography and endoscopic retrograde cholangiopancreatography led to the diagnosis of malignant tumor of the CBD. The patient ultimately underwent cholecystectomy, CBD resection, and choledochojejunostomy. Postoperative pathological examination revealed carcinosarcoma of the CBD, and the latest follow-up showed that the patient is recovering well. Based on previous case reports, some carcinosarcoma has ossification characteristics in imaging. If it is misdiagnosed as biliary calculi, the use of laser lithotripsy in surgery may lead to tumor diffusion. Choledochoscopy and narrow band staining of mucosa are very important for diagnosis.

CONCLUSION

We herein present a rare case of carcinosarcomas of the CBD, we found the tumours may have imaging features of polypoid growth and ossification only when the sarcomal components are bone differentiation, while show soft tissue

shadow when non bone differentiation. Confirmation of diagnosis depends greatly upon postoperative pathological examination and the adjuvant treatment has not been established, which leads to the poor prognosis.

Key Words: Carcinosarcoma; Sarcocarcinoma; Sarcomatoid carcinoma; Extrahepatic bile duct tumor; Common bile duct tumor; Case report

Core Tip: Carcinosarcomas of the common bile duct are extremely rare. Polypoid growth and ossification in the tumor could be representative features in cases of the sarcomal components being differentiated from bone. If misdiagnosed as biliary calculi, the use of laser lithotripsy may lead to tumor diffusion. Our case showed soft tissue exclusively on imaging and was initially misdiagnosed as acute cholangitis, highlighting the critical dependence of diagnosis confirmation on postoperative pathological examination, which itself is important for establishment of adjuvant treatment and the consequent prognosis. Other critical investigations to avoid misdiagnosis are choledochoscopy and narrow band staining of mucosa.

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INTRODUCTION

Carcinosarcomas of the common bile duct (CBD) are an extremely rare finding in the clinical setting, as they are mixture of carcinoma and sarcoma. Polypoid growth and ossification in the tumor could be representative features of carcinosarcomas of the extrahepatic bile duct[1]. For the case discussed herein, the patient's clinical presentation suggested recurrent cholangitis, and the follow-up examination showed no obvious ossification. Confirmation of diagnosis almost always depends on postoperative pathological examination so, currently, the main treatment is surgery. However, these tumors are prone to recurrence and distant metastasis, as they possess clinical features of both carcinoma and sarcoma.

CASE PRESENTATION

Chief complaints

A 75-year-old woman was admitted to our practice for recurrent chills with nausea and vomiting.

History of present illness

The patient's symptoms had started 3 mo prior.

History of past illness

The patient was healthy in the past and did not disclose any long-term drug use, smoking or alcohol abuse, or any history of operations.

Personal and family history

The patient did not disclose any family genetic or aggregation diseases.

Physical examination

Slight yellowing of skin and sclera, the abdomen is soft, without tenderness or obvious mass.

Laboratory examinations

The patient's liver function profile showed the following: Total bilirubin, 40.0 μmol/L (normal range: 0-17.1 μmol/L); direct bilirubin, 31.4 μmol/L (normal range: 0-5.1 μmol/L); aspartate aminotransferase, 99 IU/L (normal range: 0-40 IU/L); alanine aminotransferase, 85 IU/L (normal range: 0-40 IU/L); gamma-glutamyl transferase, 377 U/L (normal range: 6-71 U/L); and carbohydrate antigen 19-9 (CA19-9), 25.4 IU/mL (normal range: 0-37 IU/mL). The patient's white blood cell count was 13.67 × 10^9/L [normal range: (3.5-9.5) × 10^9/L]; her neutrophils were 89.8% and her C-reactive protein level was 80.25

mg/L (normal range: 0-10 mg/L).

Imaging examinations

Computed tomography (CT) showed localized thickening with iso-low signal nodules in the middle part of the CBD, approximately 12 mm × 13 mm in size. The nodules were significantly enhanced heterogeneously. Magnetic resonance imaging (MRI) revealed dilation of intrahepatic and CBDs, soft tissue mass signal at the lower end of CBD, with rough edge, limited diffusion of dispersion weighting, low signal of apparent dispersion coefficient (Figure 1).

FINAL DIAGNOSIS

Endoscopic ultrasound (Figure 2) showed a solid occupancy in the middle part of the CBD with medium elasticity and imaging quality, partial compression of the portal vein, and an enlarged lymph node next to the CBD, approximately 12.9 mm in size. The intrahepatic bile duct was widened, to approximately 6 mm in diameter. We found a cystic occupancy in the neck of the pancreas, approximately 15.9 mm in size, with a clear border, no communication with the main pancreatic duct, and no significant dilatation of the pancreatic duct. Endoscopic retrograde cholangiopancreatography (ERCP) was recommended before reaching the final diagnosis (Figure 3).

Bile duct biopsy was performed, and a 7.5 Fr × 7 cm bile duct plastic stent was implanted during ERCP. Postoperative bile duct biopsy revealed small fibrous tissue with inflammatory cell infiltration and individual glands, which was considered tumor marginal tissue. Immunohistochemistry (IHC) revealed the following findings: Ki-67 (+ 20%); P53 (+), CD68 (+); ALK (-); Des (-); CD34 (vascular +); vimentin (+); and S-100 (-). Therefore, intraoperative frozen sectioning was recommended.

TREATMENT

The patient was referred for general surgery. After general anesthesia, in the supine position, intraoperative exploration revealed a tumor located in the middle and lower part of the CBD (approximately 2 cm × 1.5 cm in size) with a hard texture and relatively limited, so cholecystectomy, CBD resection, and choledochojejunostomy were performed. The lesser omentum was cut at the lower edge of the liver, and the hepatoduodenal ligament and the lymph nodes near the common hepatic artery were cleaned. The gallbladder was removed and the hilar lymph nodes were cleaned. The common hepatic duct above the cystic duct was cut off and the lymph nodes around the CBD and behind the pancreatic head were cleaned. The CBD was cut 1 cm away from the edge of the tumor and the distal bile duct was ligated and sutured. The jejunum was cut 20 cm away from the flexor ligament, and end-to-side intermittent anastomosis was performed between the distal jejunum and the common hepatic duct. The jejunal stump was then closed. The distal jejunum (50 cm away from the anastomosis) was anastomosed side-to-side with the severed proximal jejunum (Roux-en-Y anastomosis with Ankang linear cutting stapler, Changzhou Ankang Medical Instrument Co., Ltd). Intraoperative frozen section revealed CBD mesenchymal tumor inclined to sarcoma with mild dysplasia of the CBD epithelium, negative upper and lower margins of CBD (Figure 4).

Postoperative pathological examination revealed carcinosarcoma of the CBD with a tumor volume of 1.0 cm × 0.6 cm × 0.6 cm. The tumor had invaded the muscular layer and did not involve the outer membrane. A small amount of pancreatic tissue was observed at the edge of the CBD with no tumor involvement. The upper and lower margins of the CBD were negative (Figure 5). IHC examination revealed the following: CK7 (+); CK19 (+); CK18 (+); Ki-67 (approximately 40%); carcinoembryonic antigen (+); CA19-9 (+); ventralis intermedius (+); s-100p (+); diethylstilbestrol (-); Calponin (+); spinal muscular atrophy (SMA) (-); and CKpan (+). Seven lymph nodes were detected in the twelfth and eighth groups, and no tumor metastasis was observed in any of the nodes (0/7) (Figure 6).

OUTCOME AND FOLLOW-UP

In this case, the postoperative pathology showed a negative margin and no clear lymph node metastasis. After consulting the literature, the patient was not given additional radiotherapy or chemotherapy. In the outpatient follow-up of 6 mo and telephone follow-up of 8 mo after the operation, the patient was in good condition.

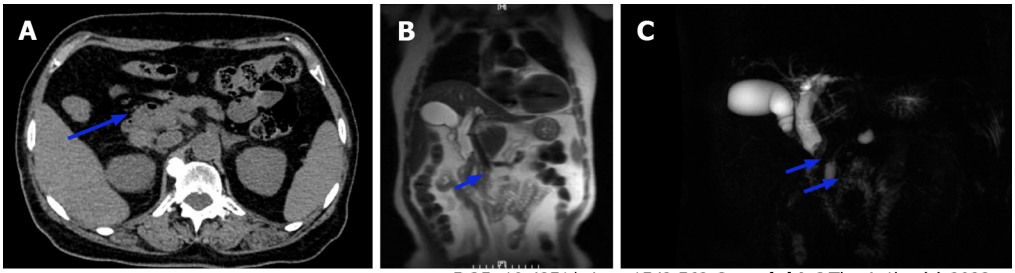

Figure 1 Imaging revealed an occupying lesion of the middle segment of the common bile duct. A: Computed tomography showed localized thickening with iso-low signal nodules in the middle part of the common bile duct (CBD), approximately 12 mm × 13 mm in size. The nodules were significantly enhanced heterogeneously; B and C: Magnetic resonance imaging by the (B) coronal plane FIESTA sequence and (C) magnetic resonance cholangiopancreatography revealed dilation of the intrahepatic duct and CBD, a soft tissue mass signal at the lower end of the CBD (with rough edge), limited diffusion on weighted imaging, and low signal intensity on apparent diffusion coefficient mapping.

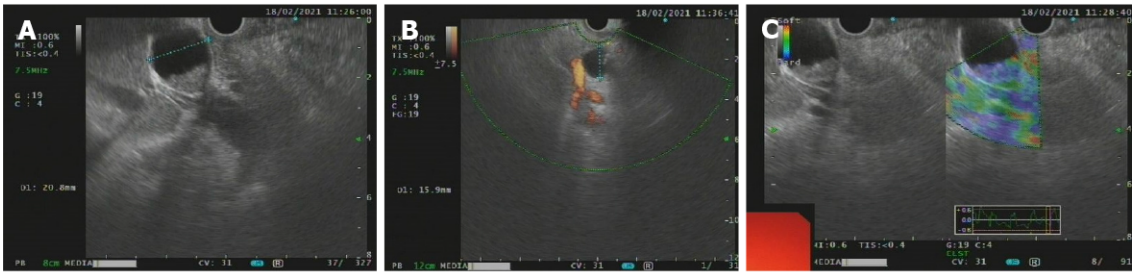

Figure 2 Endoscopic ultrasound showed solid occupancy of the middle segment of the common bile duct, partial compression of the portal vein, The intrahepatic bile duct was widened, to approximately 6 mm in diameter. A: Solid mass of common bile duct, upper common bile duct dilatation; B: An enlarged lymph node next to the common bile duct, approximately 12.9 mm in size; C: Occupancy elastic imaging shows medium texture.

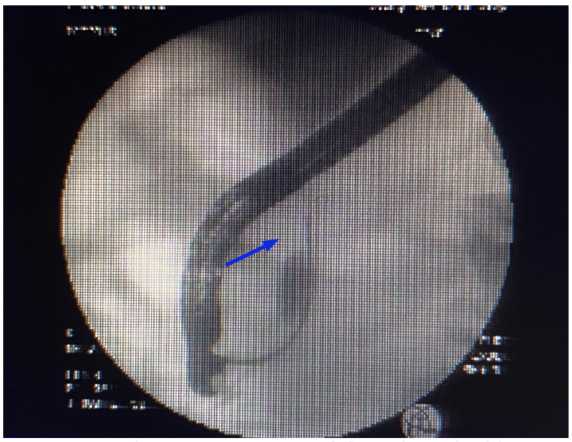

Figure 3 Endoscopic retrograde cholangiopancreatography was recommended for bile duct biopsy and a 7.5 Fr × 7 cm bile duct plastic stent was implanted.

DISCUSSION

Carcinosarcomas are rare malignant tumors consisting of a mixture of carcinoma and sarcoma, first named by Virchow (1864). According to the 1990 World Health Organization (WHO) histologic classification of tumors[2], a carcinosarcoma is defined as a neoplastic organism containing both carcinoma and sarcoma components with no detectable epithelial markers on the sarcoma component by IHC and a sarcoma component with a clear mesenchymal tissue cell origin. According to the 2006 WHO classification of tumors[3], carcinosarcoma is classified as a subset of sarcomatoid carcinoma and defined as a mixed tumor with cancer and differentiated sarcomatous components (*e.g.*, malignant cartilage, bone, or rhabdomyosarcoma). The mechanism of its development is currently discussed in several ways: (1)

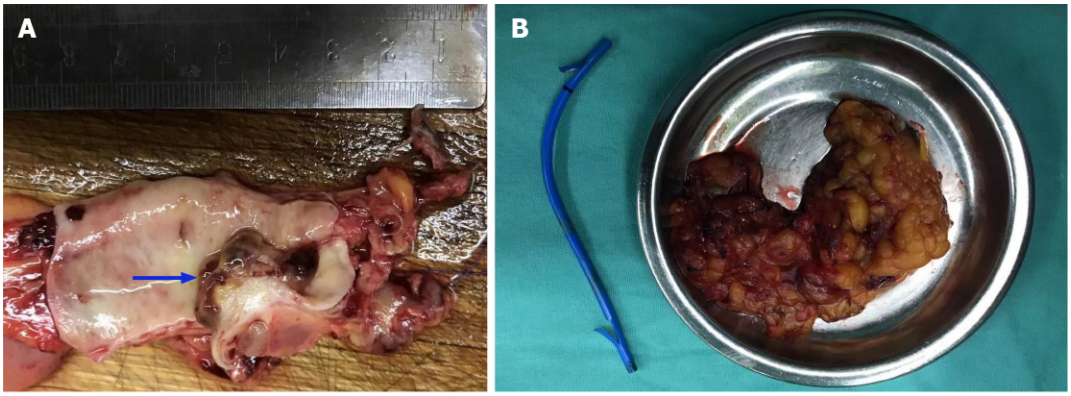

Figure 4 Intraoperative exploration revealed a tumor located in the middle and lower part of the common bile duct. A: Cauliflower-like new organisms approximately 2 cm × 1.5 cm with a hard texture; B: Cholecystectomy, common bile duct resection, and choledochojejunostomy were performed.

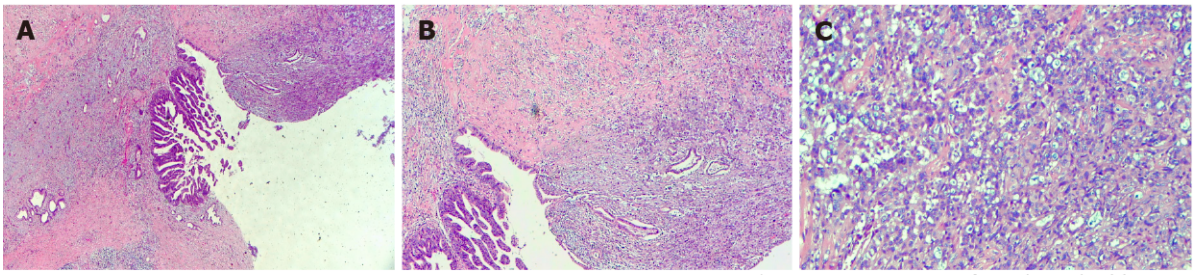

Figure 5 Postoperative pathological examination revealed carcinosarcoma of the common bile duct with a tumor volume of 1.0 cm × 0.6 cm × 0.6 cm. Cancerous tissue accounted for 40% and sarcoma for 60% of the tissue. A: Heterotypic glands can be seen in the mucosa of the bile duct, and infiltrating growth can be seen. Adenocarcinoma can be seen in the muscle wall of the bile duct; B: The muscle wall stroma of bile duct can be composed of heterotypic large cells, epithelial cancer nests and sarcoma-like components; C: Sarcoma-like area, large nuclear heterotypic cells in various forms and pathological mitotic image can be seen.

Collision theory: Two independent malignancies colliding; (2) Combination theory: The tumor is thought to have a dual origin[4], wherein the cancer is derived from epithelial cells and the sarcoma from mesenchymal tissue, and that the stem cells develop into both cancer and sarcoma and infiltrate each other at the same location in the same organ to form a carcinosarcoma; (3) Conversion theory: The sarcomatous part is derived from the carcinomatous part *via* metaplastic transformation; and (4) Composition theory: The carcinoma drives a pseudosarcomatous stromal reaction[5]. The current study proposes an alternative hypothesis[6]: That carcinosarcomas are monoclonal tumors with heterologous differentiation, in which cancer and sarcoma components are derived from a subpopulation of epithelial stem cells that de-differentiate and express mesenchymal markers. Evidence supporting this hypothesis is the migratory transition between the carcinoma and heterologous mesenchymal components of carcinosarcoma, the maintenance of consistent genetic alterations, and the positivity of both epithelial markers. Molecular biology studies have shown that the cumulative effect of multiple oncogenic events, including genetic alterations and genetic instability, leads to tumor formation followed by epithelial-to-mesenchymal transition (EMT), resulting in a sarcomatoid transformation. The EMT pathway activation mechanism is currently considered to be closely associated with the development and progression of sarcomatoid carcinoma of the lung[7].

The pathological presentation of this case was carcinosarcoma, which is extremely rare in solid hepatic cholangiocarcinoma. Histologically, carcinosarcoma is characterized by the presence of a mixture of malignant epithelial and mesenchymal components, with no transition between carcinoma and sarcoma. The epithelial component is mainly adenocarcinoma, squamous carcinoma, or both; the mesenchymal components of the tumor include fibrosarcoma, smooth muscle sarcoma, rhabdomyosarcoma, angiosarcoma, osteosarcoma, and chondrosarcoma. The immunohistochemical markers such as CD34, CD68, SMA, *etc.* can be used to identify the components of sarcoma. Studies have shown that the most common malignant epithelial component is adenocarcinoma, followed by a mixed state of adenocarcinoma and squamous carcinoma, which is less common; the malignant mesenchymal component most commonly shows spindle cells, while osteoid cells are less common. The mesenchymal component can have both spindle cells and chondrocytes. In such cases, the differential diagnosis should be sarcomatoid carcinoma, which belongs to the epithelial-derived tumor and is essentially a

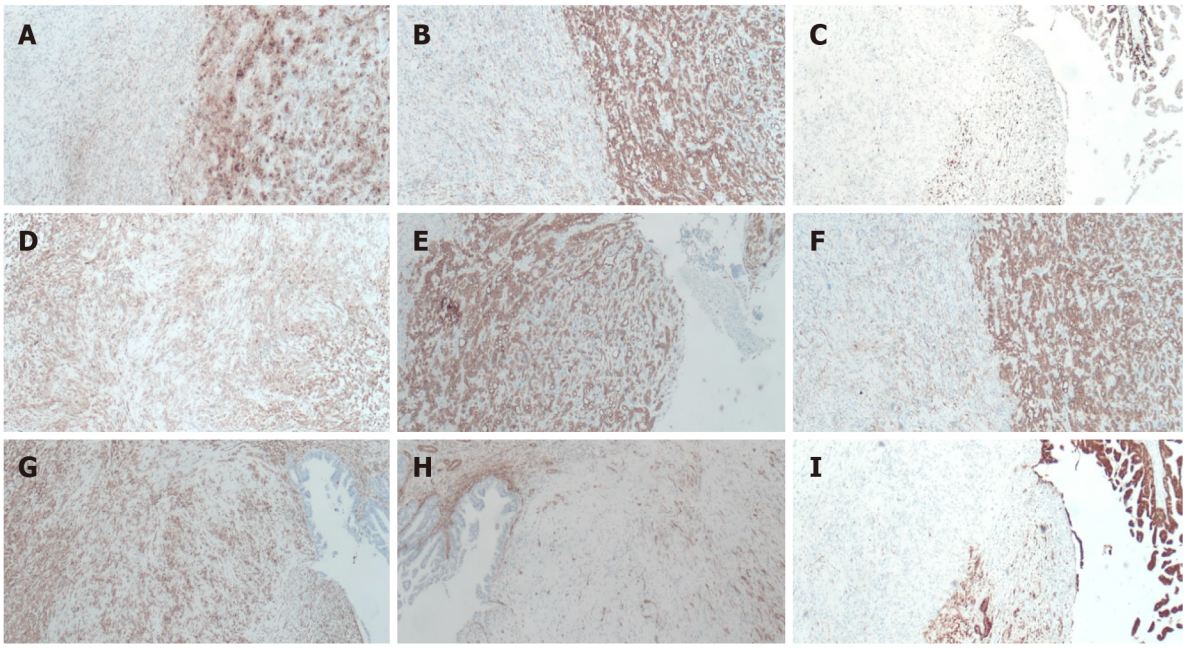

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Figure 6 Immunohistochemical examination findings. A: The tissue was positive for carbohydrate antigen 19-9; B: The tissue was positive for CK7; C: Ki-67 was approximately 40%; D: The tissue was positive for Calponin; E: The tissue was positive for CK19; F: The tissue was positive for CKpan; G: The tissue was positive for vimentin; H: The tissue was positive for S-100p; I: The tissue was negative for spinal muscular atrophy.

poorly differentiated carcinoma. The morphology may be all single sarcomatoid spindle or pleomorphic cells, or it may have both carcinoma and sarcomatoid morphology, with a predominant sarcomatoid component. In typical cases, the carcinoma and sarcomatoid forms have a migratory transition; IHC staining of both carcinoma and sarcomatoid components reveals epithelial markers such as creatine kinase, and the sarcomatoid component also expresses mesenchymal markers such as vimentin, which indicates the migratory pattern between malignant epithelial cells and sarcomatoid cells typical of sarcomatoid carcinoma[8].

Carcinosarcomas occurring in the CBD are extremely rare. Through literature review, we found only 12 case reports (Table 1). Through these 12 cases[1,5,9-18], we found that the prevalence is consistent between male and female. Among them, 9 patients were more than 70 years old. The chief complaint of 8 patients was jaundice, and others were abdominal pain and liver dysfunction. Six cases were diagnosed as tumor before operation, three were diagnosed as CBD stone, and two were cholangitis. All 11 patients received surgical treatment, of which 6 patients died, with an average survival time of 8.3 mo. 2 patients survived, but the follow-up time was short, only 7 mo. 4 patients lost follow-up. In general, these cases indicate that the tumor can occur throughout the bile duct and has a poor prognosis; even after surgery, they are prone to local recurrence and liver or lung metastasis (the clinical features of both carcinomas and sarcomas).

A recent report from China described the case of cholangiocarcinoma sarcoma who had been initially diagnosed with bile duct stones by B-ultrasound and had undergone a biliary stent implantation after failed ERCP lithotripsy[1]. During the subsequent laparoscopic choledochotomy, blood vessels were observed at the root of the stone, which prompted further investigation by narrow band imaging (NBI); histological staining confirmed the presence of blood vessels, supporting the possibility that the stone-like mass could actually be a tumor. The clinical care team, therefore, performed an open pancreaticoduodenectomy; the postoperative pathological and IHC findings confirmed a CBD carcinosarcoma. The authors concluded that polypoid growth and ossification are likely typical features of extrahepatic cholangiocarcinoma sarcomas. Such tumors can be easily misdiagnosed as a bile duct stone. Unfortunately, the well-established treatment of laser lithotripsy for stones can lead to undesirable events if targeting tumorous tissue (*i.e.*, tumor spread). Thus, it is critical to understand this uncommon differential diagnosis between bile duct stones and carcinosarcomas, since their treatments are not interchangeable. Other critical investigations to avoid misdiagnosis are choledochoscopy and NBI.

The other two cases which were misdiagnosed by CT or B-ultrasound as CBD stone before operation were also diagnosed by CT or B-ultrasound. But our case and other cases with correct preoperative diagnosis showed only a soft tissue shadow on imaging. Therefore, we propose the possibility that carcinosarcoma only shows ossification when the sarcomal components involve malignant cartilage and/or are differentiated from bone.

Table 1 Twelve reported cases of carcinosarcoma of the biliary tract

Ref.	Age/sex	Main complaints	Location	Preoperative diagnosis	Surgery	Outcome at time of last follow-up
Sasamoto *et al*[5], 2021	79/male	General malaise	Lower bile duct	Bile duct cancer	PD	Death, 26 mo
Moreno Moraleda *et al*[9], 2020	39/male	Jaundice	CBD	CBD cancer	NA	Death, 2 mo
Xie *et al*[1], 2020	76/male	Abdominal pain	Distal	CBD stone	Lap-C	NA
Usui *et al*[10], 2019	74/male	High γ-glutamyl transferase level	Right intrahepatic and CBD	Bile duct cancer	Right hepatectomy	NA
Lee *et al*[11], 2016	91/female	Liver dysfunction	Hilar	Bile duct cancer	Resection of the hilar cholangiocarcinoma	Death, 2 mo
Kumei *et al*[12] 2015	73/female	Abdominal pain, jaundice	Middle	Bile duct cancer	SSPPD	Death, 6 mo
Hoshino *et al*[13], 2013	73/male	Abdominal pain, jaundice	NA	Cholangitis	PD	Death, 4 mo
Tanaka *et al*[14], 2012	71/male	Appetite loss, jaundice	Distal	Repeated cholangitis	PD	Death, 10 mo
Aurello *et al*[15], 2008	73/female	Jaundice	Distal	Intrapancreatic	PD	Alive, 7 mo
Xia and Xu[16], 2006	57/female	Abdominal pain, jaundice	Distal	CBD cancer	BDR	NA
Kadono *et al*[17] 2005	75/female	Jaundice	Middle-distal	CBD stone	PD	Alive, 7 mo
Loud *et al*[18], 1997	35/female	Jaundice	Hilar	CBD stone	BDR	NA

BDR: Biliary duct resection; Lap-C: Laparoscopic cholecystectomy; NA: Not available; PD: Pancreatoduodenectomy; PPPD: Pylorus-pre-serving pancreatoduodenectomy; SSPPD: Subtotal stomach-preserving pancreatoduodenectomy.

Early diagnosis of cholangiocarcinoma sarcoma and sarcomatoid carcinoma is challenging due to few early clinical manifestations and unspecific imaging features. Confirmation of diagnosis largely depends on postoperative pathological examination. Regardless of whether the cancer is in an early-stage or has progressed to the stages of invasion and metastasis, pathological examination of carcinosarcomas should be performed using adequate and extensive sampling of the biopsy samples and should be supplemented with IHC staining to avoid misdiagnosis. If choledochoscopy is available, observation through NBI and performance of a biopsy under direct vision will be beneficial for clinical decision-making.

CONCLUSION

Carcinosarcomas of the CBD may have imaging features of polypoid growth and ossification only when the sarcomal components are bone differentiation, while show soft tissue shadow when non bone differentiation. Confirmation of diagnosis depends greatly upon postoperative pathological examination and the adjuvant treatment has not been established, which leads to the poor prognosis.

FOOTNOTES

Author contributions: Yao Y authored the manuscript; Xu M co-authored the manuscript and contributed to the formulation of the diagnosis and treatment plan; Xiang HG performed the surgical treatment; Jin L and Mao SY provided the diagnosis of pathology.

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