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Mechanistic study of hepatocellular carcinoma cell lines promoted by *Clonorchis sinensis* infection

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Abstract. Clonorchiasis is an important food-borne parasitic disease. The International Agency for Research on Cancer has classified *Clonorchis sinensis* as a Class I carcinogen for cholangiocarcinoma. *Clonorchis sinensis* infection is closely associated with the occurrence and progression of hepatocellular carcinoma, but the specific molecular mechanism remains unclear. This article systematically reviews the three main mechanisms by which *Clonorchis sinensis* infection promotes the occurrence and development of hepatocellular carcinoma. First of all, insect excretory and secretory products induce genome instability by activating oncogenic signaling pathways such as MAPK, PI3K-AKT, and NOTCH, and by inhibiting cell apoptosis and promoting cell migration through functional proteins such as Severin and GRN, thereby directly exerting carcinogenic effects. Secondly, in the infection microenvironment, liver cancer cells acquire enhanced proliferation, anti-apoptosis, invasion, and metastasis abilities, as well as treatment resistance, and the malignant phenotype is significantly intensified. Finally, in the tumor microenvironment reshaped by infection, the infection-remodeled immunosuppressive microenvironment, thereby indirectly promoting the survival and progression of liver cancer cells. This article concludes that *Clonorchis sinensis* infection synergistically promotes the development and progression of hepatocellular carcinoma through direct and indirect mechanisms, and that key molecules targeting these mechanisms are expected to become new targets for the diagnosis and treatment of hepatocellular carcinoma.

Keywords: *Clonorchis sinensis*, hepatocellular carcinoma, excretory secretory products, tumor microenvironment, immunosuppression

1. Introduction

Clonorchiasis is an important food-borne parasitic disease. More than 15 million people worldwide are infected with this parasite [1]. *Clonorchis sinensis* has a complex life cycle, and humans can become infected by eating raw or semi-raw freshwater fish containing metacercariae. Adult worms can survive in the host body for more than 20 years. Based on sufficient epidemiological evidence, the World Health Organization's International Agency for Research on Cancer classified *Clonorchis sinensis* as a Class I carcinogen for cholangiocarcinoma in 2009. However, recent clinical observations indicate that in areas where *Clonorchis sinensis* is endemic, the incidence of hepatocellular carcinoma remains high, and a considerable proportion of patients with hepatocellular carcinoma are complicated by *Clonorchis sinensis* infection. More and more

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studies have shown that *Clonorchis sinensis* infection may be closely associated not only with cholangiocarcinoma but also with hepatocellular carcinoma [2].

Currently, research on the molecular mechanisms by which *Clonorchis sinensis* infection promotes hepatocellular carcinoma is still in its infancy, and many key issues remain unclarified. Existing research mainly focuses on three aspects: the direct carcinogenic effects of parasite excretory and secretory products, changes in the malignant behavior of liver cancer cells in the infection microenvironment, and the indirect promotion of liver cancer cells by the infection-remodeled tumor microenvironment. The article reviews recent research on *Clonorchis sinensis* infection and hepatocellular carcinoma, summarizing progress in these three areas to provide a theoretical framework and potential therapeutic targets.

2. Direct carcinogenic effects of *Clonorchis sinensis* infection on liver cells

2.1. Cestode Secretions (CsESPs) activate oncogenic signaling pathways in liver cells

During the long-term parasitism of *Clonorchis sinensis* adults in the host body, they continue to release large amounts of Excretory Secretory Products (CsESPs) into the surrounding environment. These secretions contain a variety of proteases, growth factor-like proteins, and other bioactive molecules that can directly contact and act on adjacent hepatocytes and bile duct epithelial cells. Studies have shown that after CsESPs enter host cells, they can activate multiple classic oncogenic signaling pathways, thereby driving cells to transform into malignant phenotypes. Using single-cell transcriptome sequencing and spatial transcriptome technology to analyze hepatocellular carcinoma samples combined with *Clonorchis sinensis* infection and hepatitis B virus infection, the results showed that p53, NOTCH, hypoxia-inducible factor, and PI3K-AKT-mTOR pathways were significantly activated in the hepatocellular carcinoma cells in the dual infection group. Immunofluorescence staining further confirmed that the expression levels of p53 and p21 in tumor tissues from the dual infection group were significantly higher [1].

The *Clonorchis sinensis* Granulin (CsGRN) protein isolated from the secretions of *Clonorchis sinensis* can activate the RAS/MAPK/ERK and PI3K/AKT pathways through the epidermal growth factor receptor, thereby promoting the malignant transformation of hepatocytes and bile duct epithelial cells. In vitro experiments show that the proliferation rate of hepatocytes treated with CsGRN is significantly accelerated, and early characteristics of epithelial-mesenchymal transition appear, providing direct molecular evidence for elucidating the activation of oncogenic pathways by secretions [3].

2.2. Parasitic secretions make liver cell genes unstable

Genomic instability is one of the important signs of cellular carcinogenesis, manifested as chromosome copy-number variation, gene-mutation accumulation, chromosome structural abnormalities, and other phenomena. Increasing evidence suggests that *Clonorchis sinensis* infection can induce genomic instability in host cells, thereby increasing the risk of cancer.

Copy number variation analysis of liver cancer tissues with different infection states revealed that the degree of variation in chromosome regions 9, 10, and 22 in the double-infection group with *Clonorchis sinensis* and hepatitis B virus was significantly higher than that in the single-infection group and the control group, suggesting that the two infection factors can synergistically aggravate the instability of the genome of hepatocytes. At the same time, liver cancer cells in the dual infection group showed higher expression levels of malignant markers, and the overall prognosis of the patients was worse [1].

2.3. Specific proteins in parasite secretions directly regulate liver cell function

The secretion of *Clonorchis sinensis* is not a mixture of homogeneous components, but a complex system containing dozens or even hundreds of different proteins. In recent years, researchers have identified multiple proteins with specific biological functions in secretions and have initially revealed their mechanisms of action in promoting cancer.

The *Clonorchis sinensis* Severin protein can significantly inhibit the apoptosis of human liver cancer cells [4]. This protein exerts anti-apoptotic effects via the mitochondria-mediated endogenous apoptosis pathway [5].

The CsGRN protein mainly promotes cell migration and invasion. Recombinant CsGRN significantly promotes the migration and invasion of liver cancer cells. CsGRN enhances cell invasion by activating the EGFR downstream MAPK/ERK and PI3K/AKT pathways [3].

When *Clonorchis sinensis* Severin protein and hepatitis B virus X protein work together, the inhibitory effect on cell apoptosis and cell cycle is significantly stronger than a single treatment, suggesting that in the context of dual infection, the two pathogen proteins can synergistically exert a cancer-promoting effect, which is consistent with the clinical phenomenon of higher malignancy in tumors in patients with dual infection in endemic areas [6].

3. Changes in the malignant behavior of liver cancer cell lines in the infection microenvironment

3.1. Infection makes liver cancer cells proliferate faster and makes them less likely to die

Upon direct stimulation by parasite secretions, the regulatory network governing hepatocyte proliferation and apoptosis undergoes significant changes, particularly in established liver cancer cell lines. In vitro cell experiments provide direct experimental evidence for understanding how parasite secretions change the malignant phenotype of liver cancer cells.

Studies have confirmed that CsESPs treatment can significantly upregulate CD24 expression in liver cancer cells. CD24 is highly expressed in a variety of malignant tumors and is closely associated with cell proliferation and anti-apoptotic capabilities. After treatment with CsESPs for 48 hours, the viability and colony-forming ability of liver cancer cells were significantly enhanced, while the apoptosis rate was significantly decreased. Further mechanisms show that CsESPs can directly activate CD24 transcription via the transcription factor E2F1, thereby promoting proliferation and inhibiting apoptosis; silencing CD24 can significantly reverse this cancer-promoting effect [7].

Single-cell transcriptomic data show that liver cancer cells derived from patients infected with *Clonorchis sinensis* exhibit high expression of cell-cycle-related genes, including TOP2A, CDC20, and MKI67. This feature fully demonstrates that liver cancer cells in the infection microenvironment are in a highly proliferative state [1].

3.2. Infection makes liver cancer cells more likely to invade and metastasize

Invasion and metastasis are two of the core characteristics of malignant tumors and are also the main causes of treatment failure and death in patients with hepatocellular carcinoma. Whether and how *Clonorchis sinensis* infection enhances the invasion and metastasis ability of liver cancer cells is an important scientific issue in assessing the clinical harm of infection.

Cell scratch experiments showed that 10 and 20 μg/mL CsESP significantly improved HepG2 cell migration. Quantitative fluorescence PCR results showed that CsESP treatment can up-regulate the expression of genes related to migration, invasion, and epithelial-mesenchymal transition, such as MMP2, N-cadherin, Vimentin, and α-catenin, thereby enabling cells to exhibit stronger motility and invasion [8]. The study found that liver cancer tissues infected with simple *Clonorchis sinensis* and simple hepatitis B virus infection were significantly enriched in epithelial-mesenchymal transition-related gene sets; at the same time, liver cancer cells in the dual infection group highly expressed CD44 molecules, and their high expression was closely related to the enhanced ability of tumor invasion and metastasis and poor prognosis [1].

In vitro experiments further confirmed that CsESPs can enhance the migration, invasion, and angiogenesis of liver cancer cells. Multi-omics analysis showed that infection can significantly activate extracellular matrix remodeling and adhesion pathways, while inhibiting T cell-related pathways, suggesting that *Clonorchis sinensis* infection can synergistically promote tumor invasion and metastasis through matrix remodeling and immune suppression [9].

3.3. Infection makes liver cancer cells resistant to treatment

Treatment resistance is a major challenge in the clinical management of hepatocellular carcinoma. Increasing clinical evidence suggests that *Clonorchis sinensis* infection status may affect the response to chemotherapy and immunotherapy in patients with hepatocellular carcinoma and cholangiocarcinoma.

Studies have shown that CsESPs treatment can upregulate CTLA-4 and LAG-3 expression in co-culture systems, and this effect can be reversed after CD24 knockdown. CTLA-4 and LAG-3 are key immune checkpoint molecules, and their high expression is closely related to T cell exhaustion and immunotherapy resistance. This result reveals the molecular mechanism of secretion-induced immunotherapy resistance [7].

From the perspective of the tumor microenvironment, regulatory T cells and CD8-positive exhausted T cells were significantly enriched in samples from the dual infection group. Regulatory T cells can exert immunosuppressive effects through inhibitory cytokines and immune checkpoint molecules; exhausted T cells highly express PD-1, LAG3, TIM-3, and other molecules, and their proliferation and killing functions are significantly reduced, leading to tolerance to immunotherapy [1].

4. The mechanism by which the tumor microenvironment is reshaped by infection "helps" liver cancer cells

4.1. Bug infection recruits "bad" macrophages (SPP1+ macrophages)

Macrophages in the tumor microenvironment play a dual role in the occurrence and development of tumors: under normal physiological conditions, macrophages are an important part of the body's immune defense and have the ability to phagocytose and eliminate abnormal cells; however, in the tumor microenvironment, macrophages can be "tamed" by tumor cells and stromal cells and transform into Tumor-Associated Macrophages (TAM), which in turn promote tumor growth, angiogenesis, invasion and metastasis.

In liver cancer tissues co-infected with *Clonorchis sinensis* and hepatitis B virus, a well-characterized subpopulation of macrophages with high expression of SPP1 and IL1B is present. SPP1 is involved in cell adhesion, migration, angiogenesis, and immune regulation, and IL1B can promote angiogenesis and suppress immune function. Survival analysis showed that patients with high infiltration levels of this macrophage subpopulation had a significantly increased risk of overall death and had important prognostic predictive value [1].

In dual-infection samples, the proportion of SPP1-positive macrophages was significantly higher; at the same time, CsESP treatment can promote the differentiation of M0 macrophages toward the M2 pro-tumor phenotype, further confirming that infection can promote tumor progression by reshaping macrophage phenotype [7].

4.2. Insect infection makes T cells "tired" (T cell exhaustion)

Cellular immunity, mediated by T cells, is the main mechanism by which the body eliminates tumor cells. However, in chronic infections and tumor microenvironments, continued antigen stimulation can cause T cells to lose function and enter a state called "exhaustion" gradually. Exhausted T cells express multiple immune checkpoint molecules, reduce their ability to produce cytokines, weaken their proliferation ability, and ultimately cannot effectively control tumor growth.

There is an obvious CD8-positive exhausted T cell subpopulation in dual-infected liver cancer tissues. This subpopulation also highly expresses multiple immune checkpoint molecules, such as LAG3, PD-1, and TIM-3, and has a significantly higher exhaustion score. Pseudo-time trajectory analysis showed that CD8+ T cells derived from dual infection tended to differentiate toward immunosuppression, suggesting that *Clonorchis sinensis* infection can accelerate and intensify T cell exhaustion [1].

In vitro experiments confirmed that CsESP treatment inhibited T cell activation and promoted T cell exhaustion, consistent with single-cell sequencing. Differences in T cell exhaustion trajectories across different infection backgrounds suggest that the molecular mechanisms underlying T cell dysfunction induced by *Clonorchis sinensis* and hepatitis B virus differ [10].

4.3. Insect infection remodels stromal cells and forms a "criminal network"

In addition to immune cells, the tumor microenvironment contains a variety of non-immune stromal cells, including fibroblasts, endothelial cells, and others. These cells maintain tissue structural homeostasis in normal tissues but can be "activated" or "remodeled" in the tumor microenvironment, thereby supporting tumor growth.

There are characteristic stromal cell subpopulations in dual-infected liver cancer tissues: the endothelial cell subpopulation with high ENPP2 expression is significantly enriched, promoting angiogenesis and the inflammatory response through lysophosphatidic acid signaling; the fibroblast subpopulation with high COL1A1 expression is significantly increased, suggesting excessive activation of tumor-associated fibroblasts.

Analysis of cell-to-cell communication showed that the number of cell interactions was significantly higher in the double-infection group, in which the two signaling axes, DLL4-NOTCH4 and COL1A1-CD44, were significantly enriched, mediating angiogenesis and matrix adhesion, respectively. Spatial transcriptome data confirmed that these signals are highly co-localized in tissue space and have the structural basis for efficient interaction [1].

Studies have found that SMPD3 expression is significantly upregulated in liver cancer patients with *Clonorchis sinensis* infection and is associated with poor prognosis. In vitro experiments have confirmed that SMPD3 overexpression can enhance the proliferation, invasion, and tumorigenicity of liver cancer cells, suggesting that infection can reshape stromal cell function through the sphingolipid metabolism pathway and build a tumor-promoting microenvironment [11].

5. Conclusion

This article systematically reviews the molecular mechanisms by which *Clonorchis sinensis* infection promotes the occurrence and development of hepatocellular carcinoma. Parasite excretory/secretory products directly activate carcinogenic pathways (MAPK, PI3K-AKT, NOTCH) in liver cells, with synergistic effects in HBV co-infection. In the microenvironment of infection, liver cancer cells exhibit malignant phenotypes, including accelerated proliferation, resistance to apoptosis, and enhanced invasion and metastasis. The E2F1-CD24 transcription axis is the molecular mechanism, and CD24 can be a potential therapeutic target. At the same time, the tumor microenvironment reshaped by infection builds an immunosuppressive network through the enrichment of $SPP1^+$ macrophages and the depletion of $CD8^+$ T cells, which indirectly promotes the progression of liver cancer through matrix remodeling.

Limitations include few direct studies and a need for further verification. Future work should build more clinically relevant models, investigate co-infection mechanisms, and explore targets like CD24 and SPP1 using single-cell and spatial omics to guide precision therapy.

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