Up-regulated RFC2 Predicts Unfavorably Progression in Hepatocellular Carcinoma

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Research

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Abstract

Background: Replication factor C (RFC) is closely related to tumor progression and metastasis. However, the functional significance of RFC2 in hepatocellular carcinoma remains unclear.

Materials and methods: In order to solve this problem, the expression of RFC2 in liver cancer patients was analyzed through ONCOMINE, UALCAN, human protein atlas. Survival analysis was conducted using Kaplan-Meier plotter and GEPIA. GO and KEGG enrichment analyses were carried out. The protein-protein interaction (PPI) network was performed through Metascape.

Result: The transcription and protein level of RFC2 in HCC were overexpressed, which was significantly related to the clinical individual cancer stage and pathological tumor grade of HCC patients. In addition, in patients with liver cancer, higher RFC2 expression was found to be significantly correlated with shorter OS and DFS. Furthermore, the function of RFC2 in liver cancer was DNA replication, and its main mechanism was the phase transition of the cell cycle.

Conclusion: RFC2 might promote the development of liver cancer. It could also be used as a novel biomarker for the prognosis of liver cancer.

Introduction

Liver cancer is the second leading cause of cancer-related deaths worldwide (Slotta et al. 2015). Its morbidity and mortality have been on the rise in recent years (Ferlay et al. 2015). Hepatocellular carcinoma (HCC) is the main form of liver cancer (about 90%). Its main risk factors including chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcoholism, metabolic diseases, etc., which causes an inflammatory environment in the liver (Yang & Roberts 2010). Long-term inflammatory stimulation leads to chromosomal instability, genetic and epigenetic changes, which may lead to liver cancer (Dhanasekaran et al. 2016). In-depth research on the mechanism of the occurrence and development of liver cancer has been carried out for a long time, however, the molecular events of HCC are not yet fully understood. It is important and urgently needed to identify more valuable biomarkers for early diagnosis and survival prediction.

The fidelity of DNA replication is usually closely related to cancer progression. DNA damage repair and loss of checkpoint function can lead to genome instability. Replication factor C (RFC) is a primer recognition factor of DNA polymerase, which participates in DNA damage repair and checkpoint control during the cell cycle process (Pascucci et al. 1999; Sancar et al. 2004; Shimada et al. 1999), and is closely related to tumor progression and metastasis (Li et al. 2018).

Specifically, the RFC complex recognizes the primer on the template DNA and binds to its end. It can load proliferating cell nuclear antigen (PCNA) onto the DNA template to promote subsequent DNA replication (Mossi & Hübscher 1998). RFC consists of five subunits, including RFC1-5 (Bowman et al. 2004). Among them, the RFC2 gene encodes the third largest subunit (40 kDa) of the RF-C complex. The

cells with RFC2 mutation showed increased the incidence of spontaneous mitosis(Noskov et al. 1998). Studies have shown that RFC2 was significantly up-regulated in some tumor tissues, such as nasopharyngeal carcinoma (NPC) tissue(Xiong et al. 2011) and choriocarcinoma tissue(Cui et al. 2004). Besides, studies have shown that RFC2 was used to predict breast cancer progression and metastasis(Gupte 2018). These findings suggest that RFC2 might be one of the most important genes regulating cancer. However, the role of RFC2 in the development of HCC is still unknown.

In this study, we analyzed the expression of RFC2 in HCC patients, its relationship with clinical parameters, and survival analysis to solve this problem. In addition, we predicted the function and pathway of 100 similar genes to RFC2.

Materials And Methods

Ethics

The research has been approved by the Ethics Committee of the Sixth People's Hospital Affiliated to Shanghai Jiaotong University. All data is from an online database, which has obtained all written informed consent.

ONCOMINE

ONCOMINE database is a publicly accessible online cancer microarray database (www.oncomine.org) (Rhodes et al. 2004). In this study, the mRNA expression of RFC2 was obtained from the ONCOMINE database between different tumor tissues and their corresponding adjacent normal control samples. The differences in transcription expression were compared through students' t-test. The specific settings were as follows: fold change: 1.5, P value: 0.01.

UALCAN

UALCAN (<http://ualcan.path.uab.edu>) is a user-friendly and comprehensive web resource for analyzing cancer data, which is based on the TCGA database and contains a large amount of clinical data (Chandrashekar et al. 2017). In this study, UALCAN was used to analyze the relative transcriptional expression of RFC2 in HCC tissues and the relationship between RFC2 and clinicopathological parameters, including clinical stages and HCC grades. The t-test was used to compare the expression of RFC2 in HCC and normal tissues. P value < 0.05 was considered statistically significant.

Cancer Cell Line Encyclopedia (CCLE)

Cancer Cell Line Encyclopedia (<https://portals.broadinstitute.org/ccle>) is an online database jointly developed by the Broad Institute and the Novartis Institute of Biomedicine, Novartis Research Foundation, and the Institute of Genomics(Ghandi et al. 2019). This project aims to describe in detail the genetics and pharmacology information of a large number of human cancer models, comprehensive computational analysis that links different pharmacological vulnerabilities and genomic patterns and use cell line

integrated genomics to stratify cancer patients. In this study, the mRNA expression data of RFC2 in cancer cell lines was downloaded from CCLE. Then, we screened out the mRNA expression of RFC2 in liver cancer cell lines.

Human Protein Atlas

The Human Protein Atlas (HPA) (<http://www.proteinatlas.org/>) uses transcriptomics and proteomics techniques to study protein expression in different human tissues and organs from the RNA and protein levels.

HPA is mainly divided into three sections: Cell, Tissue and Pathology, which respectively show the expression of protein in cells, normal tissues and cancerous tissues(Thul et al. 2017; Uhlén et al. 2015; Uhlen et al. 2017). This study used immunohistochemical images to compare the protein expression of RFC2 between human normal tissues and HCC tissues.

Kaplan-Meier plotter

Kaplan Meier Plotter (<http://kmplot.com/analysis/>) is an online website dedicated to prognostic analysis(Nagy et al. 2018). In this study, the overall survival (OS) was carried out with Kaplan-Meier plotter analysis, and the prognostic value of the characteristic RFC2 with high expression found in HCC samples was evaluated. The log-rank P was calculated accordingly.

Gene Expression Profiling Interactive Analysis (GEPIA)

GEPIA (<http://gepia2.cancer-pku.cn/#index>) is a newly developed interactive web server that analyzes RNA sequencing expression data from TCGA and GTEx projects(Tang et al. 2017). In this study, we used GEPIA to find 100 similar genes for RFC2, which were used for subsequent functional analysis.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

GO and KEGG analyzed the functions and pathways of 100 similar genes in Metascape (<http://metascape.org/>) (Zhou et al. 2019). Terms with p value < 0.01, minimum count 3 and enrichment factor > 1.5 were collected through Metascape, and they were grouped according to the similarity of their members. GO enrichment analysis predicted the function of RFC2 and 100 similar genes(Ashburner et al. 2000). KEGG analysis predicted the metabolic and regulatory pathways of RFC2 and 100 genes similar to it from the perspective of molecular networks(Kanehisa & Goto 2000).

Protein-protein Interaction (PPI) Enrichment Analysis

The PPI network of 100 similar genes was obtained using the Metascape (Zhou et al. 2019). For a given gene list, PPI enrichment analysis was performed with the following databases: BioGrid, InWeb_IM, OmniPath(Li et al. 2017; Stark et al. 2006). The Molecular Complexity Detection (MCODE) algorithm was further applied to identify densely connected network components if the network contains 3 to 500 proteins.

Pathway and process enrichment analysis has been applied to each MCODE component respectively, and the three best score items obtained by P value are used as the function description of corresponding components.

Results

Overexpression of RFC2 in hepatocellular carcinoma

To explore the potential therapeutic value of RFC2 for HCC, ONCOMINE database (www.oncomine.org) and UALCAN database (<http://ualcan.path.uab.edu>) were used to analyze mRNA expression of RFC2, and Human Protein Atlas (HPA) (<https://www.proteinatlas.org/>) are used to analyze protein expression. The transcription of RFC2 in various types of cancer was first analyzed (Fig. 1A) and compared with normal tissues through ONCOMINE database. The mRNA expression of RFC2 was found to be markedly higher in liver cancer tissues in the data set. Specifically, in the Roessler Liver 2 dataset(Roessler et al. 2010) (Table 1), RFC2 was overexpressed in HCC tissues compared with normal tissues, with a fold change of 1.645 ($p = 8.11E-44$). Then, the mRNA expression of RFC2 was further measured through UALCAN based on level 3 RNA-seq and clinical data of 31 types of cancer from the Cancer Genome Atlas (TCGA) database, which was different from the ONCOMINE database. The mRNA expression of RFC2 was significantly upregulated in primary liver cancer tissues compared with normal samples ($p < 0.05$) (Fig. 1B). Besides, the protein expression of RFC2 was explored in liver cancer through the Human Protein Atlas (HPA). Immunohistochemistry images displayed that RFC2 protein was not detected in normal liver tissues, however, highly expressed in HCC tissues (Fig. 1C). In general, the transcription and protein of RFC2 are overexpressed in patients with liver cancer. In addition, to find the suitable liver cancer cell line for RFC2 research, the mRNA level of RFC2 in liver cancer cell lines was examined with the CCLE. JHH7 showed the highest RFC2 level and HEPG2 showed the lowest (Fig. 2).

Table 1
Significant overexpression of RFC2 mRNA expression between HCC and normal liver tissues in ONCOMINE database.

Types of HCC VS. Liver	Fold Change	P value	t-test	Ref
RFC2				
Hepatocellular Carcinoma	1.645	8.11E-44	16.175	Roessler Liver 2 ^[15]

Association of RFC2 with clinical stages and tumor grades of HCC patients

After the high mRNA and protein expression of RFC2 was found in HCC,the relationship between the mRNA expression levels of RFC2 and clinicopathological parameters was analyzed using UALCAN (<http://ualcan.path.uab.edu>). As shown in Fig. 3A, the mRNA expression of RFC2 was significantly correlated with the individual cancer stage in HCC, and patients in the advanced stage of tumor tended to

express higher mRNA expression of RFC2. The highest RFC2 mRNA expression was found in stage 3. The reason why the mRNA expression of RFC2 in stage 3 seemed to be higher than that in stage 4 might be due to the small sample size (only 6 HCC patients in stage 4). Similarly, as shown in Fig. 3B, the mRNA expression of RFC2 was significantly correlated with tumor grade, and as the tumor grade increased, the mRNA expression of RFC2 tended to be higher. In short, the above results indicated that RFC2 mRNA expression in HCC patients was significantly correlated with clinicopathological parameters.

Unfavourable effects of elevated RFC2 expression on survival

In addition, the Kaplan-Meier plotter (<http://kmplot.com/analysis/>) was used to analyze the prognostic information of RFC2 in HCC. As shown in Fig. 3C, the mRNA expression of RFC2 was significantly correlated with the prognosis of liver cancer patients. The results indicated that the higher expression of RFC2 mRNA was associated with unfavorable overall survival (OS) (HR = 1.59, 95% CI: 1.12 – 2.26 and $p = 0.0086$), and lower expression of RFC2 (HR = 1.59, 95% CI: 1.13 – 2.24 and $p = 0.0068$) was associated with better disease-free survival (DFS) in patients with liver cancer. These results showed RFC2 might be used as a useful biomarker for predicting the survival of HCC patients.

Functions and pathways of RFC2 and its similar genes in HCC

First, 100 genes similar to RFC2 was found through GEPIA 2.0 (<http://gepia2.cancer-pku.cn/#similar>). GO and KEGG enrichment analysis was performed for similar genes through Metascape (<https://metascape.org/gp/index.html#/main/step1>) to deduce possible enrichment functions and pathways. The enriched items in the list of 100 similar genes were shown in Fig. 4. The bar graph corresponded to the P value. Biological processes (BP), including GO:0006260 (DNA replication), GO:0051301 (cell division), GO:0010564 (regulation of cell cycle process) were significantly associated with RFC2 (Fig. 4A). RFC2 also prominently affected the cellular components (CC), such as GO:0098687 (chromosomal region), GO:0005657 (replication fork), GO:0005819 (spindle)(Fig. 4B). In addition, molecular functions (MF) such as GO:0003688 (DNA replication origin binding), GO:0140097 (catalytic activity, acting on DNA), GO:0003682 (chromatin binding) were remarkably regulated by RFC2 in HCC (Fig. 4C). KEGG analysis showed that the most important pathways included hsa03030 (DNA replication), hsa04110 (cell cycle), hsa00240 (pyrimidine metabolism), hsa03430 (mismatch repair) (Fig. 4D).

Protein-protein interaction analysis

PPI analysis was performed by Metascape (Fig. 5A). In the input list of 100 similar genes, the main interaction networks were DNA replication initiation (Fig. 5B); DNA replication (Fig. 5C); chromosome, centromeric region (Fig. 5D). In general, DNA replication initiation was the most important interaction network, and the proteins involved mainly included MCM2, MCM3, MCM4, MCM6, MCM7, MCM10, CDC6, CDK2, CDT1, CLSPND, DBF4 and ORC1.

Discussion

The fidelity of DNA replication is usually closely related to the progression of cancer, including liver cancer. The strict regulation of cell cycle growth signals controls the proliferation of normal cells. DNA damage repair and loss of checkpoint function can lead to genome instability, causing cancer cells to proliferate out of control. RFC is involved in DNA damage repair and checkpoint control during the cell cycle, and has been showed to be an important regulator of the malignant progression of many cancers(Cui et al. 2004; Koch et al. 2007; Xiong et al. 2011). Among them, RFC2 has been proven to promote a variety of tumor proliferation(Xiong et al. 2011) (Cui et al. 2004) (Gupte 2018). However, the biological function of RFC2 in HCC has not been explored. In this study, the expression, function, interaction pathway and prognostic value of RFC2 in HCC were analyzed for the first time to guide the future research of liver cancer.

Existing studies have shown that RFC2 is significantly upregulated in some tumors, such as nasopharyngeal carcinoma(Xiong et al. 2011), choriocarcinoma(Cui et al. 2004), and colorectal cancer(Hu et al. 2020). Our results found that RFC2 is overexpressed in HCC, which is consistent with previous studies. In addition, the protein expression level of RFC2 was further checked through the HPA database. The immunohistochemical staining intensity of tumor samples is much higher, which is consistent with mRNA expression.

Previous studies have shown that RFC2 is related to the progression and metastasis of breast cancer and can be used as a prognostic indicator for breast cancer patients. CRC patients with higher RFC2 levels showed poor clinicopathological symptom(Hu et al. 2020). In this study, the expression of RFC2 mRNA is significantly related to the clinical stage and tumor grade of liver cancer. Patients with advanced cancer and high tumor grade tend to express higher RFC2 mRNA. Further survival analysis showed that higher expression of RFC2 mRNA was associated with poor overall survival (OS) and disease-free survival (DFS).

Previous studies have found that the inhibition of RFC2 can enhance the cytotoxicity of temozolomide to glioblastoma(Ho et al. 2020). This article analyzed the functions and pathways of 50 similar genes of RFC2 in HCC patients. We found that DNA replication is the most significant functional enrichment term. In terms of protein interaction, the PPI network composed of proteins associated with the DNA replication origin was most affected (these proteins included: MCM2, MCM3, MCM4, MCM6, MCM7, MCM10, CDC6, CDK2, CDT1, CLSPND, DBF4 and ORC1).

Some limitations exist in our research. First, all the data in our study comes from online databases. In vivo and in vitro tests including larger sample sizes are needed to verify the findings in this article and explore the clinical application of RFC2 in the treatment of liver cancer. Secondly, the potential diagnostic and therapeutic role of RFC2 in liver cancer has not been evaluated, so further studies are needed to explore whether RFC2 can be used as a diagnostic marker or therapeutic target. Finally, although the possible pathways are analyzed in this article, further research is needed to explore the direct mechanism of RFC2 on HCC in the future.

Conclusions

In summary, our results show that the transcription and protein of RFC2 in HCC are overexpressed, which is significantly related to the clinical individual cancer stage and pathological tumor grade of HCC patients. In addition, in patients with liver cancer, higher RFC2 expression was found to be significantly correlated with shorter OS and DFS. RFC2 may be a prognostic biomarker for the survival of liver cancer patients. In addition, the function of RFC2 in liver cancer is DNA replication, and the main mechanism is cell cycle phase transition. The JHH7 and HEPG2 cell line may be suitable for the study of the mechanism of RFC2 in liver cancer. This study is the first to study the prognostic correlation of RFC2 in liver cancer, and we believe that the results are worthy of further study.

Abbreviations

HCC: hepatocellular carcinoma; RFC2: replication factor C; TCGA: cancer genome atlas; GO: gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; BP: biological processes; CC: cellular components; MF: molecular functions; OS: over survival; DFS: disease free survival;

Declarations

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Authorship contributions

Jianbo Wang conceived and designed the research. Zaixiong Ji and Jiaqi Li implemented the research, analyzed the data, and wrote this paper.

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Figures

Figure 1

Transcription of RFC2 in various types of cancer analyzed and compared with normal tissues through ONCOMINE database.

Figure 2

The mRNA level of RFC2 in liver cancer cell lines was examined with the CCLE.

Figure 3

The mRNA expression of RFC2 was significantly correlated with the individual cancer stage in HCC, and patients in the advanced stage of tumor tended to express higher mRNA expression of RFC2.

Figure 4

The enriched items in the list of 100 similar genes.

A

B

C

D

Figure 5

PPI analysis.

Supplementary Files

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