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High expression of XRCC5 is associated with metastasis through Wnt signaling pathway and predicts poor prognosis in patients with hepatocellular carcinoma

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Abstract. – OBJECTIVE: Metastasis is one of the main causes of leading recurrence and death in patients with hepatocellular carcinoma (HCC). XRCC5 can affect several types of human cancers. The aim of the present study is to explore the metastasis correlation between the expression level of XRCC5 protein in HCC cell lines with different metastatic potentials and the prognosis of patients with HCC.

MATERIALS AND METHODS: Hepatocellular carcinoma cell lines used in this study include MHCC97-H, MHCC97-L, HepG2, and HL-7702.

RESULTS: XRCC5 was widely expressed in HCC cell lines analyzed by Real-Time Reverse Transcription PCR and Western blotting. The levels of XRCC5 protein and mRNA were positively correlated with the migration and invasion capability of HCC cell lines. SiRNA-XRCC5 significantly suppressed the migration and invasion of HCC cells. Immunohistochemistry (IHC) results showed that high-expressed XRCC5 in patients with HCC was associated with advanced tumors, size, and microvascular invasion and lower overall survival time than the low-expressed. The expression levels of CTNNB1 and MMP9 decreased by knocked down XRCC5 which may promote the progression of HCC via the Wnt/β-catenin signaling pathway.

CONCLUSIONS: Our results suggest that the high expression of XRCC5 predicts poor prognosis in patients with HCC, and XRCC5 may be a potential biomarker to inhibit the invasion and migration of HCC.

Key Words:
XRCC5 protein, Hepatocellular Carcinoma Cells, Invasion, Migration.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and it has become the third leading cause of cancer-related death worldwide, following lung and stomach cancers; nonetheless, its incidence is still rising[1,2]. Due to the aggressive features of HCC, intrahepatic and extrahepatic metastases often occur in progressive and recurrent patients. However, only limited therapeutic options are available, thus leading to a poor five-year survival rate[3]. The metastasis of HCC is a comprehensive process that facilitates cancer cell transition from a primary lesion to a metastatic focus. Furthermore, the molecular pathogenesis of HCC metastasis remains largely unknown. Therefore, more accurate and reliable biomarkers need to be identified to understand the underlying mechanisms that facilitate this process. The identification of further biomarkers for therapeutic purposes in HCC is imperative.

Cancer has been widely accepted as a gene disease that is significantly influenced by gene abnormal expression and polymorphisms including lung cancer, gastric cancer, colorectal cancer[4-6]. The DNA repair systems are the genome caretakers, playing a critical role in the initiation and progression of cancers[7]. Recently, *XRCC5* is not only involved in DNA double-strand break repair, but also have been identified as crucial regulatory factor in the

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proliferation, invasion, and survival of various cancer cells[6,8,9]. Moreover, the T allele of the *XRCC5* G-1401T may be associated with the development of colorectal cancer and may be a novel useful marker for primary prevention and anticancer intervention[10]. However, the role and mechanism of *XRCC5* in HCC remain unclear.

In the present study, we detected the expression levels of *XRCC5* in HCC tissues and cell lines. Meanwhile, the correlations between the *XRCC5* expression and migration, invasion, and clinicopathological features were evaluated. In addition, we knockdown the expression level of *XRCC5* by transfecting siRNA and evaluated the invasion and migration ability of HCC cells. Subsequently, the expression levels of *CTNNB1* and *MMP9*, the relationship with *XRCC5* and overall survival of patients were analyzed by bioinformatics analysis in The Cancer Genome Atlas (TCGA) HCC dataset. Our findings reveal a novel mechanism showing that *XRCC5* promotes the progression of HCC through the Wnt signaling pathway, making *XRCC5* a potentially valuable anticancer target for the treatment of HCC.

Material and Methods

Cell Lines and Cell Culture

Hepatocellular carcinoma cell lines used in this study were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). These cells include MHCC97-H, MHCC97-L, HepG2, and HL-7702. These cells were cultured with Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma-Aldrich Corp., St. Louis, MO, USA). All mediums were supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Biowest, Nuaillé, France). All the cell lines were grown in 5% CO_2 at 37°C in incubators with 100% humidity.

Western Blot Analysis

The cells were lysed on ice for 30 minutes, and the lysed cell suspension was collected into a 1.5 mL centrifuge tube at 14,000 r/min, centrifuged at 4°C for 20 min. The BCA method was used to quantify the protein of each group, and the sample was prepared by using the 1x loading buffer. The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) by wet transfer method. On the membrane, 5% skim milk powder was blocked at room temperature for 2 hours, and the primary antibody *XRCC5* (ab80592, 1:5000), MMP-9 (ab73734, 1:1000), β-catenin (ab32572,1:5000), and GAPDH (ab9485, 1:2500) were incubated overnight in a 4°C refrigerator. All above antibodies were purchased from Abcam (Cambridge, MA, USA). The PVDF membrane was washed three times for 10 minutes each time and incubated with a secondary antibody for 1 hour at room temperature for chemiluminescence (ECL). The gel imager was used to observe the *XRCC5* protein levels of each group of cells.

Real-Time Reverse Transcription-PCR

Each cell line RNA was extracted, and reverse transcriptase was performed using the TRIzol (TaKaRa Bio, Dalian, China) and a Prime Script™ RT Reagent Kit (TaKaRa Bio, Dalian, China). The most efficient *XRCC5* primer sequence was screened for 5'-TGACTTCCTGGATGCACTAATCGT-3' (sense strand); 5'-TTGGAGCCAATGGTCAGTCG-3' (antisense strand); MMP9 primer sequence (sense strand: 5'-TGTACCGCTATGGTTACACTCG-3'; antisense strand:5'-GGCAGGGACAGTTGCTTCT-3'); CTNNB1 (β-catenin) (sense strand: 5'-TCCC TGAGACGCTAGATGAGG-3' and antisense strand: 5'-CGTTTAGCAGTT TTGTCAGCTC-3'. Internal reference β-actin (sense strand: 5'-GGCGGCACCATGTACCCT-3'; antisense strand: 5'-AGGGGCCGGACTCGTC ATACT-3').

After a pre-denaturation step at 95°C for 5 min, 40 cycles of PCR were performed as follows: 10 s denaturation at 95°C and 30 s annealing at 60°C. The fold amplification for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Scratch Assay

After the cells were grown significantly, 3×10^5 cells/m cells were seeded in 6-well plates. After the next day, the cells were gently smeared with a 20 μL sample gun. The scratch width was measured at 0 h and 72 h to calculate cell migration rate.

Transwell Invasion Assay

The invasion chambers were coated with BD Matrigel matrix according to the manufacturer's protocol (BD Biosciences, Franklin Lakes, NJ, USA). The cancer cells were seeded on top

of the Matrigel in the upper chamber, and the bottom chamber was filled with culture medium containing chemoattractant. The cells that invade through the Matrigel-coated membrane after 24 h were fixed with 4% paraformaldehyde, followed by staining with crystal violet. All experiments were conducted at least three times in triplicate.

Immunofluorescence Subcellular Localization

The cell-grown slides were washed three times with phosphate-buffered saline (PBS) in a culture plate for 3 minutes each time. The cells were fixed in 4% paraformaldehyde for 15 minutes and then permeabilized with PBS (containing 0.2% Triton X-100) for 20 minutes. Non-specific blocking was performed for 1 hour with 10% Goat serum. The *XRCC5* primary antibody was incubated in a wet box for 2 hours at room temperature. The secondary antibody was incubated for 1 hour in the dark. DAPI counterstained cells for 5 minutes. The cells were rinsed with PBS and observed under a fluorescence microscope.

Small Interference RNA and Transfection

The cells were seeded with 5×10^4 cells in 24-well plates, then incubated for 2 to 4 d in standard medium in the presence of 10 to 20 nmol/L siRNA directed against *XRCC5*. The siRNA *XRCC5* sequences used in this study were as follows: si*XRCC5*#1 (sense: 5'-GCCCUGGAAUACGAUGAAUTT-3', anti-sense: 5'-AUUCAUCGUAUUCCAGGGCTT-3'); si*XRCC5*#2 (sense: 5'-GAGCUGAGCUGUCG AUAUATT-3', anti-sense: 5'-UAUAUCGACAG CUCAGCUCTT-3'); si*XRCC5*#3 (sense: 5'-GCCGCAUCAUGAAG AUUGATT-3', anti-sense: 5'-UCAAUCUUCAUG AUGCGGCTT-3'); or control siRNA (sense: 5'-UUCUCCGAAC GUGUCACGUTT-3', anti-sense: 5'-ACGUGACA CGUUCGGAGAATT-3'). The cells were transfected using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunohistochemical Staining

The archival formalin-fixed and paraffin-wax-embedded tissue blocks of 74 hepatocellular carcinomas removed by surgery from 2008 to 2013 were retrieved from the Department of Pathology, Guizhou Provincial People's Hospital. The primary antibodies were directed toward *XRCC5* (rabbit monoclonal, 1:100; ab79391, Abcam, Cambridge, MA, USA). The serial sections of 5 μm were cut from the tissue blocks, deparaffinized in xylene, and hydrated in a graded series of alcohol. The staining was then performed using the EnVision+ anti-rabbit system (Dako Corporation, Santa Clara, CA, USA). The negative control staining was carried out by substituting nonimmune rabbit and phosphate-buffered saline (PBS) for the primary antibodies. This study was conducted with the approval of the Ethics Committee of Guizhou Provincial People's Hospital, China.

Bioinformatics Analysis

Gene expression and survival analysis (http://ualcan.path.uab.edu/cgi-bin/ualcan-res.pl) software programs were used to analyze The Cancer Genome Atlas (TCGA) HCC dataset.

Statistical Analysis

The statistical analysis was performed using the Statistical Product and Service Solution SPSS 19.0 software (IBM, Armonk, NY, USA). Data analysis was performed with the Student's *t*-test and ANOVA. Bonferroni test was used to validate ANOVA for pairwise comparisons. The comparison of count data was performed by the χ^2-test. The overall survival rate was calculated by Kaplan-Meier method. $p<0.05$ was considered statistically significant.

Results

Correlation of XRCC5 with Metastasis of HCC Cell Lines

To evaluate the correlation of *XRCC5* with metastasis of hepatocellular carcinoma, we detected their expression in HCC cells by qRT-PCR and Western blot. The results showed that the mRNA and protein levels of *XRCC5* in MHCC97-H cell line were significantly higher than those in the MHCC97-L, HepG2, and HL-7702 cell lines ($p<0.05$, Figures 1A-1C). Using the scratch assay and transwell invasion assay, we detected the migration and invasion of four cell lines. The number of invading cells crossing the matrigel basement membrane and scratch repair ability was evidently higher in MHCC97-H cell line than that in the other three cell lines ($p<0.05$, Figure 1D-1G). Immu-

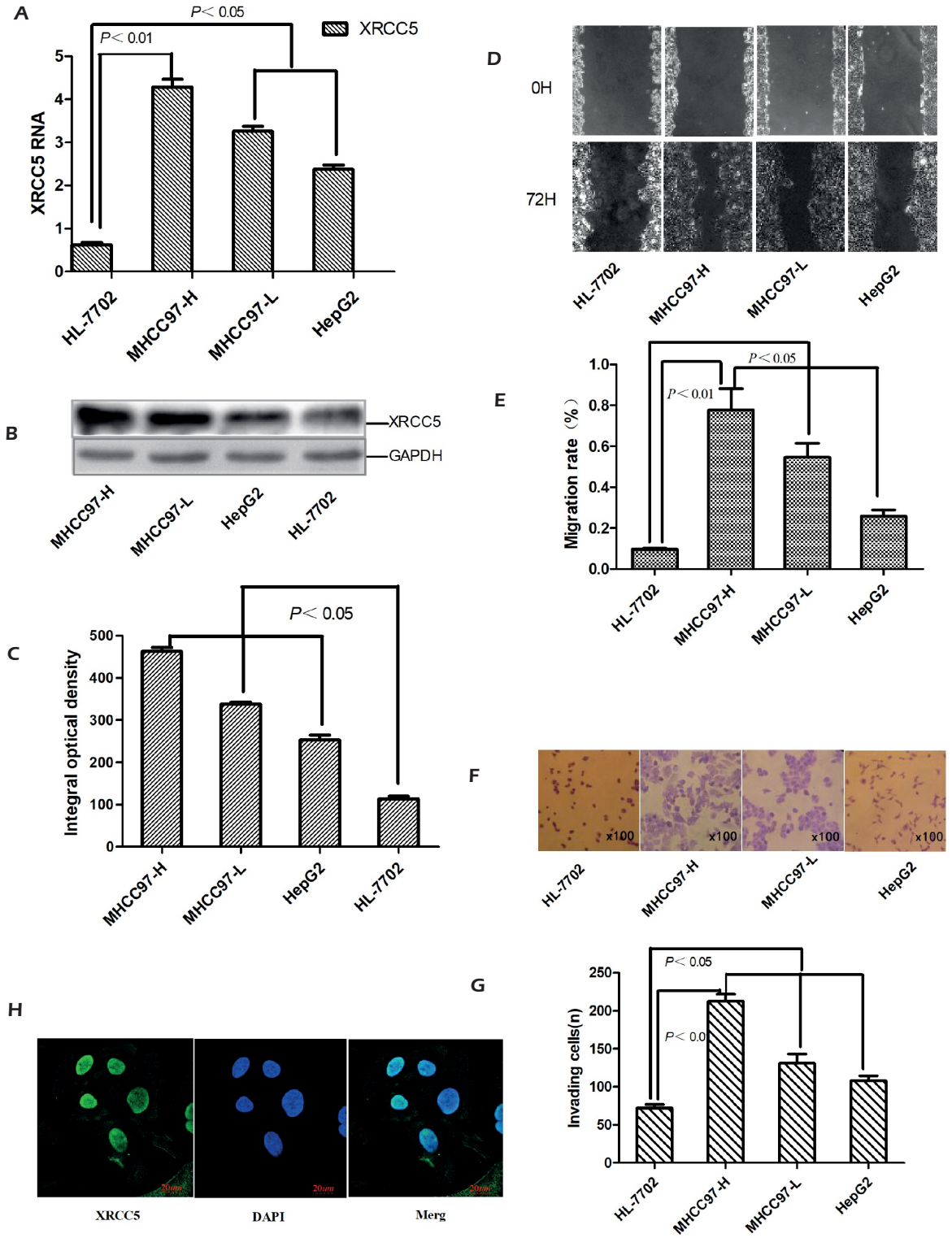

Figure 1. The expression levels of XRCC5 in HCC cell lines, subcellular localization and its relationship with invasion and migration. **A**, XRCC5 mRNA levels in HCC cells. **B-C,** Western blot (**B**) analysis of XRCC5 expression in HL-7702 cells and HCC cell lines; (**C**) XRCC5 has the highest expression level in MHCC97-H. **D-E,** Scratch assay (**D**) compares the migration ability after 72 h in HCC cell line; (**E**) MHCC97-H has the strongest migration ability than other three cell lines. **F-G,** The transwell invasion assay compared the invasive ability in HCC cell lines; (**G**) MHCC97-H has the highest number of transmembrane cells. **H,** Immunofluorescence detection of subcellular localization of XRCC5, which is located in the nucleus.

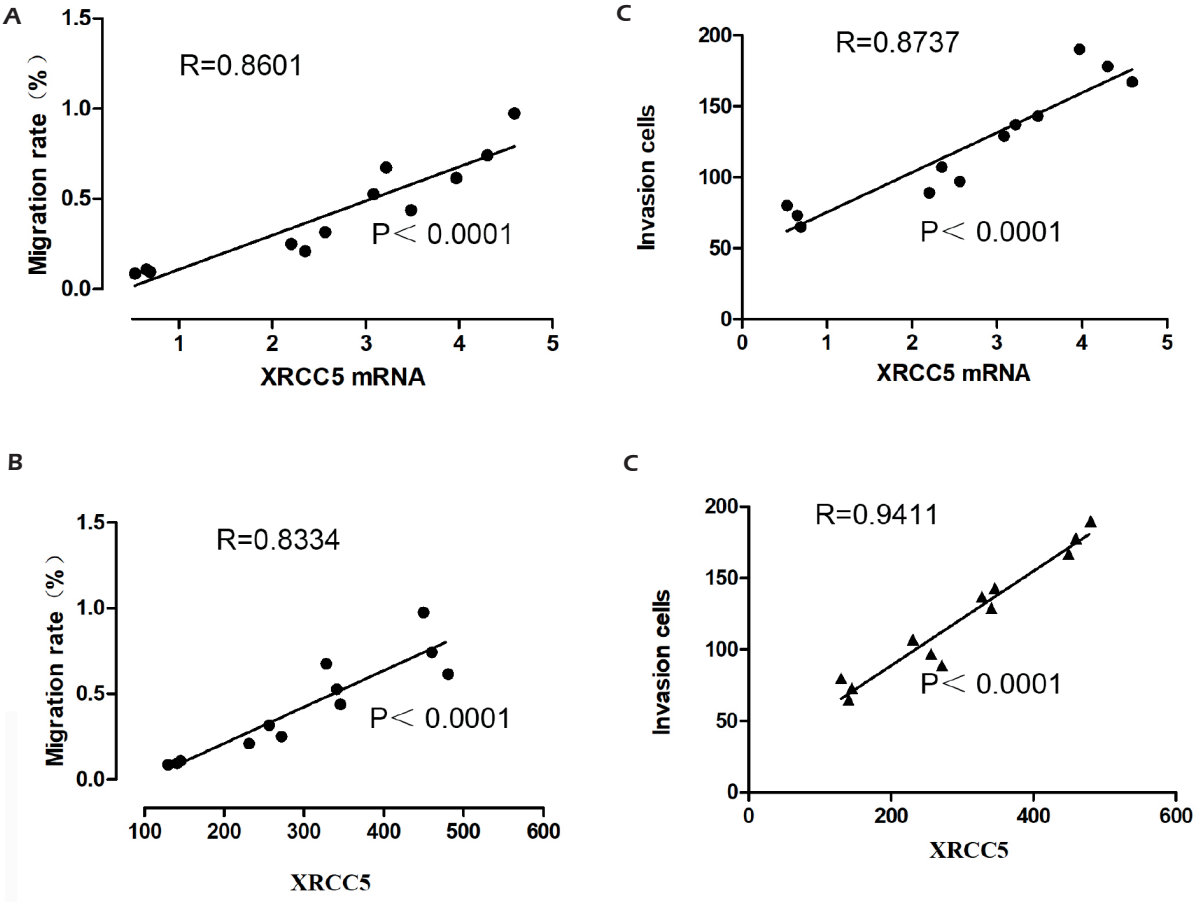

Figure 2. Correlation between the expression of XRCC5 protein, mRNA, the migration, and invasion ability of HCC cell lines. **A-C,** XRCC5 mRNA was positively correlated with the migration and invasion ability of HCC cell lines. **B-D,** XRCC5 protein is positively correlated with the migration and invasion ability of HCC cell lines.

nofluorescence technique found that *XRCC5* is mainly localized in the nucleus (Figure 1H). The linear correlation was used to analyze the correlation between the expression of *XRCC5* and the migration and invasion of the four cell lines. The results showed that the protein and mRNA levels of *XRCC5* positively correlated with the migration and invasion capability of HCC cell lines (p<0.05, Figure 2A-2D).

XRCC5 Expression Interference Impairs Migration and Invasion Capability of HCC Cells In Vitro

We performed a knockdown experiment using siRNAs targeting *XRCC5*, to further investigate roles of *XRCC5* in the invasion of HCC cells. MHCC97-H cell line was more aggressive than other cell lines, and then it was used to perform the following experiment. First, three independent siRNAs targeting *XRCC5* (si*XRCC5*#1, #2, and #3) were transfected to MHCC97-H cells. As shown in Figures 3A-3C, the knockdown effects of si*XRCC5*#2 were better than the other two siRNAs at both mRNA and protein levels. Next, using the si*XRCC5*#2, we performed the scratch assay and the transwell invasion assay in MHCC97-H cell lines, and found significant suppression of invasion and migration by siRNA#2 (p<0.05, Figures 3D-3G). No suppressive effect was found when we used the control siRNAs (Figures 3D-3G).

Overexpression of XRCC5 Predicts Poor Prognosis in HCC Patients

To explore the role of *XRCC5* in HCC and the mechanisms underlying the oncogenic function of *XRCC5*, we initially analyzed *XRCC5*

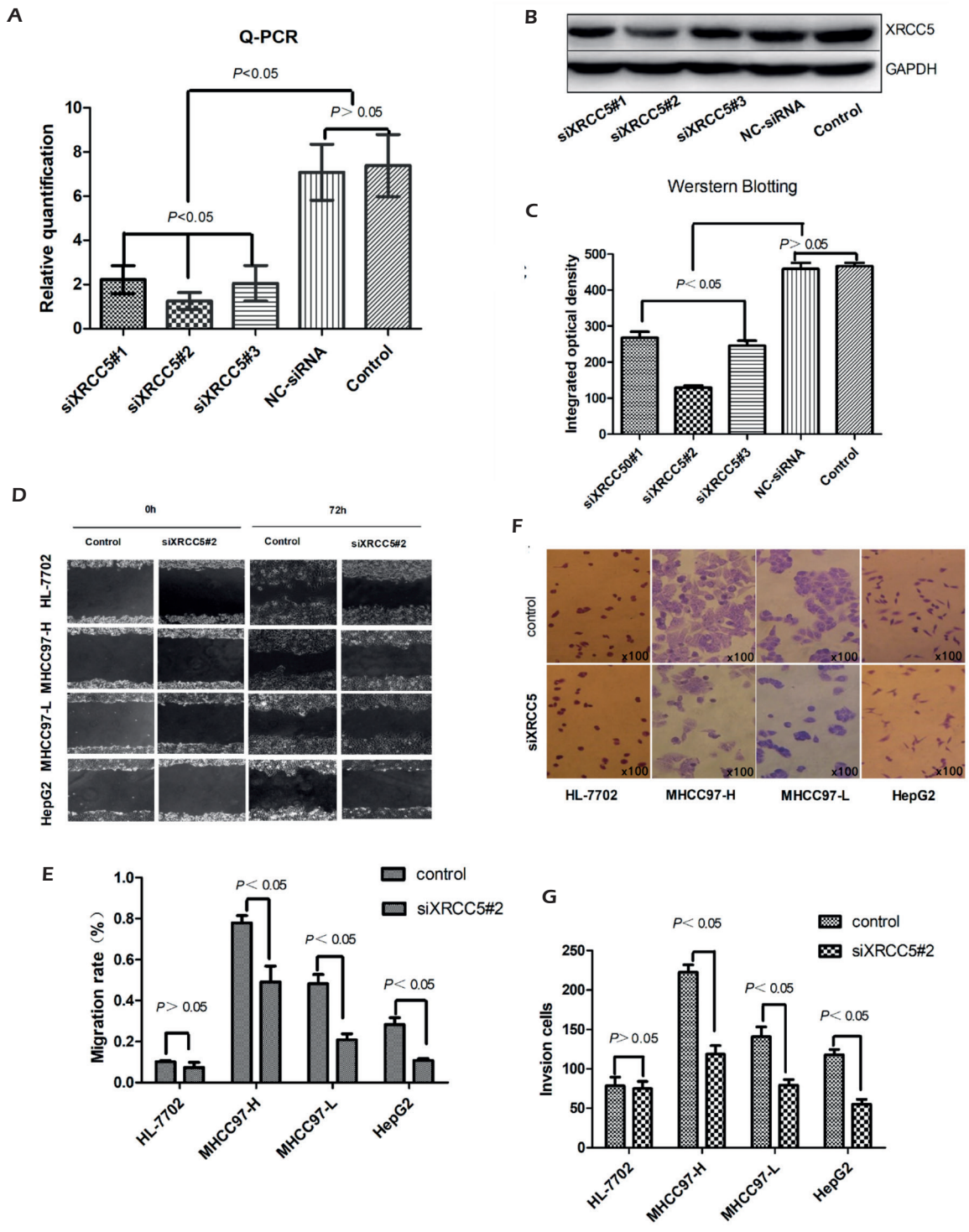

Figure 3. A, Changes in migration and invasion ability of HCC cell lines after interference with siXRCC5#2. Using different sequence of siRNA to interfere with MHCC97-H, siXRCC5#2 had the best interference effect (p<0.05). **B-C,** MHCC97-H was interfered with siRNA using different sequences, and the expression of XRCC5 protein was the lowest (p<0.05), compared with other interference sequences after interference with siXRCC5#2. **D-E,** After siXRCC5#2 interference, the scratch test showed that the migration ability of the hepatoma cell lines was inhibited after 72 hours (p<0.05). **F-G,** After interference with siXRCC5#2, the transwell assay found that the invasive ability of HCC cell lines was inhibited after 72 h (p<0.05).

Table I. Immunohistochemical score for XRCC5 in hepatocellular carcinoma.

Score	Patient number
1	27
2	18
4	29

mRNA expression between HCC specimens and normal controls in a publicly available HCC dataset (The Cancer Genome Atlas, TCGA), according to the method reported by Chandrashekar et al[11]. As shown in Figure 4A, *XRCC5* mRNA expression was significantly elevated in HCC tissues (n=371) compared to tumor-adjacent liver tissues (n=50). Notably, *XRCC5* mRNA expression was negatively associated with overall survival (p=0.00025, Figure 4B), suggesting that *XRCC5* gene expression might be an indicator of the risk of tumorigenesis in HCC. Subsequently, the expression level of *XRCC5* in patients' tumor tissues was detected by IHC (Figure 5). The patient distribution of *XRCC5* IHC score is shown in Table I. Patients were divided into *XRCC5* high and *XRCC5* low groups (Table II) according to the IHC score. High *XRCC5* levels of patients had advanced tumor stage (p=0.0007, Chi-square test), larger tumor size (p=0.0275, Chi-

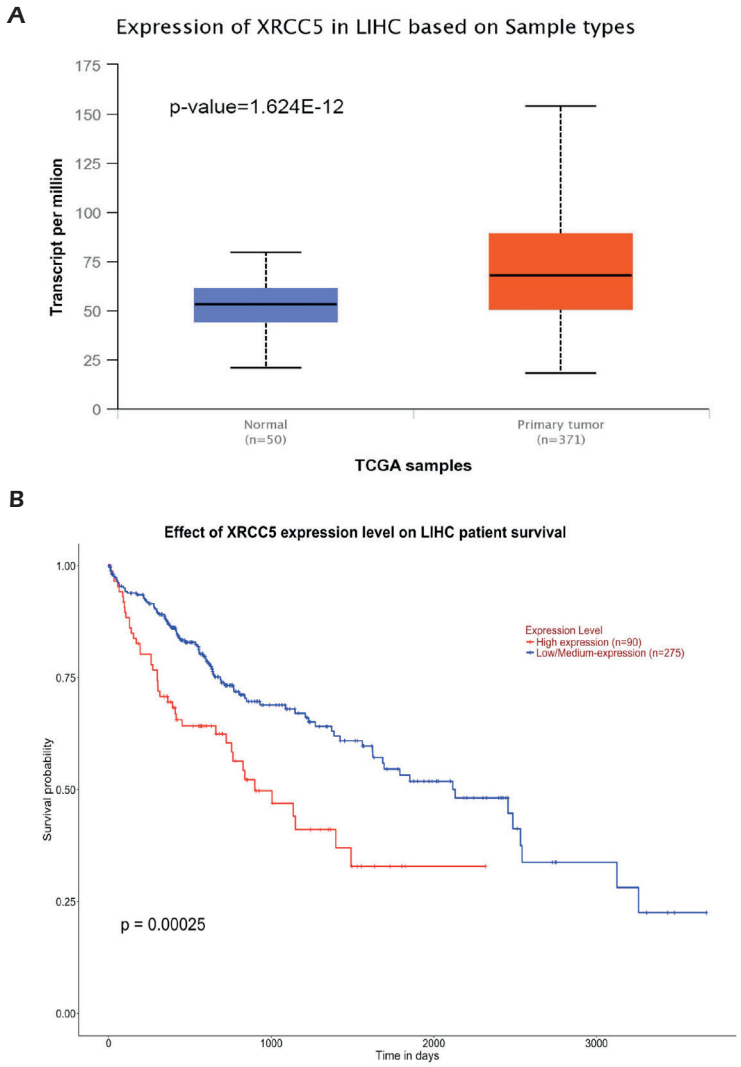

Figure 4. XRCC5 mRNA levels and overall survival of patients in the HCC TCGA dataset. **A,** XRCC5 mRNA levels in the HCC TCGA dataset. **B,** Overall survival of patients in TCGA HCC dataset with low *vs.* high levels of XRCC5 mRNA. LIHC: Liver hepatocellular carcinoma.

Table II. Clinicopathological characteristics of HCC patients according to XRCC5 expression.

Clinicopathological variable	XRCC5 expression		p-value
	Low	High	
All cases	27	47	
Gender			
Male	17	30	1.0000
Female	10	17	
Age (yr)			
< 60	12	27	0.3374
≥ 60	15	20	
TNM stage			
Early (I-II)	20	15	0.0007[a]
Late (III-IV)	7	32	
Tumor size (cm)			
Small (≤ 5)	18	19	0.0275[a]
Large (> 5)	8	29	
Microvascular invasion			
Present	6	26	0.0074[a]
Absent	21	21	
HBsAg			
Negative	4	23	1.0000
Positive	8	39	
Serum AFP level (ng/mL)			
< 400	22	31	0.1879
≥ 400	5	16	

square test), and high microvascular invasion rate (p=0.0074, Chi-square test; Table II). High *XRCC5* expression levels indicate that patients had significantly shorter OS than those with low *XRCC5* expression levels (p=0.0375; Figure 6). These data suggest that *XRCC5* is associated with tumor growth and invasion and it can also predict the prognosis of HCC patients.

XRCC5 Promotes HCC Progression via Wnt Signaling Pathway

To explore whether *XRCC5* promotes the progression of liver cancer through the Wnt signaling pathway, first, we searched the expression of CTNNB1 (β-catenin) and MMP9 in normal liver tissues and HCC tissues in the TCGA database. It was found that CTNNB1 and MMP9 were highly expressed in HCC compared with adjacent tissues (p<1E-12, p=2.726E-6, respectively) (Figures 7A, 7B), and their expression was significantly positively correlated with *XRCC5* (p=3.91E-36, R=0.56; p=0.007, R=0.13, respectively) (Figures 7C, 7D). Survival analysis found that the high expression of CTNNB1 and MMP9 predicted a poor prognosis (p=0.041, p=0.022, respectively) (Figures 7E, 7F). Subsequently, we knocked down *XRCC5* expression by siRNA#2 in MHCC97-H and found that CCTNB1 and MMP9 also decreased in mRNA and protein levels (p<0.05) (Figures 7G, 7H). Our results indicate that *XRCC5* may promote the progression of HCC *via* the Wnt signaling pathway.

Discussion

The mechanism of the development and progression of HCC is complex and largely unknown, and is associated with hepatitis C, hepatitis B virus, alcoholic, and non-alcoholic fatty liver disease[12,13]. Therefore, its limited treatment methods face enormous challenges[14-17]. Investigating and identifying the biomarkers of HCC, such as proteins, long-chain non-coding RNA, etc., is pivotal to provide biological targets for the precise treatment of HCC[18-20]. Many researchers[21,22] showed that *XRCC5*, as tumor promoters, promote tumor progression and is a poor prognostic biomarker. *XRCC5* is a subunit of the Ku protein complex, a rich, highly conserved DNA-binding protein found in prokaryotes and eukaryotes, that plays an important role in maintaining genomic integrity[21,22]. The *XRCC5* protein, also known as ku80, is encoded by the *XRCC5* gene, and forms a ku70/ku80 heterodimer with ku70. It is a DNA-dependent protein kinase complex (DNA-PK), which is best characterized by its central role as the initial DNA terminal binding factor in the "classical" non-homologous end joining (C-NHEJ) pathway[23,24]. It directly and indirectly interacts with several C-NHEJ factors and processing enzymes to participate in DNA double-strand break (DSB) repair as a scaffold for the entire DNA repair complex[25,26]. It is widely accepted that unrepaired or misrepaired DNA double strand breaks (DSBs) lead to the formation of chromosome aberrations[27]. There are also studies[28] showing that Ku is involved in the signaling of the DNA damage response (DDR) mechanism to regulate the activation of cell cycle checkpoints and activation of apoptosis. Ku plays an important role in maintaining the telomere stability. When telomere function is disrupted, the potentially fatal DNA damage

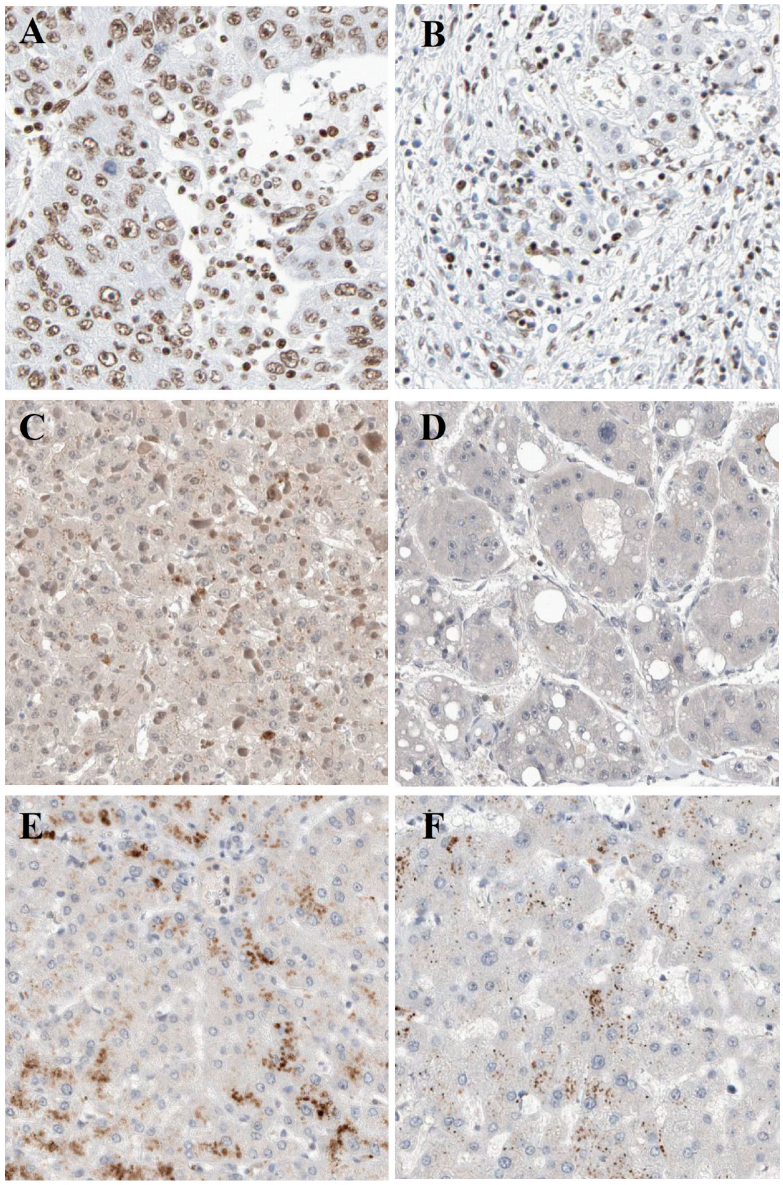

Figure 5. Expression of XRCC5 in HCC tissues and normal liver tissues. **A-D,** Expression of XRCC5 in hepatocellular carcinoma tissues: (**A**) strongly positive; (**B**) moderately positive; (**C**) weakly positive; (**D**) negative. **E-F,** Expression of XRCC5 in the normal liver group: (**E**) moderately positive; (**F**) weakly positive. SP method ×400.

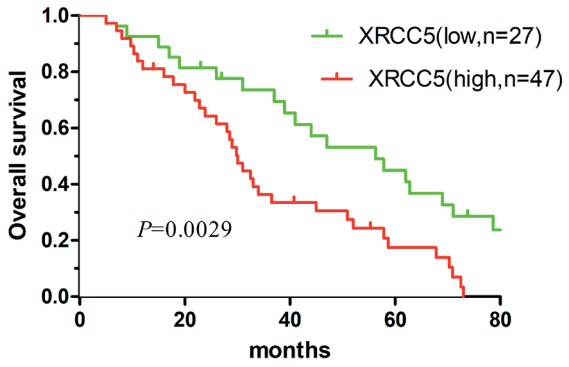

Figure 6. The relationship between XRCC5 and overall survival time. Survival analysis found that patients with high expression of XRCC5 protein had shorter overall survival time than patients with low expression of XRCC5 protein (p=0.0291).

reactions may occur, the DNA repair activities threaten the integrity of chromosome ends, and the extensive genomic instability may occur. Even chromosomal aberrations can lead to tumors[29,30].

In this study, we found that *XRCC5* was highly expressed in HCC cell lines and tissues; moreover, it was positively correlated with the invasion and migration ability of HCC cell lines. In addition, its expression level is significantly correlated with the patient's clinicopathological features (advanced tumor stage, tumor size, microvascular invasion). The analysis of the TCGA HCC dataset by bioinformatics also yielded consistent results. Several studies

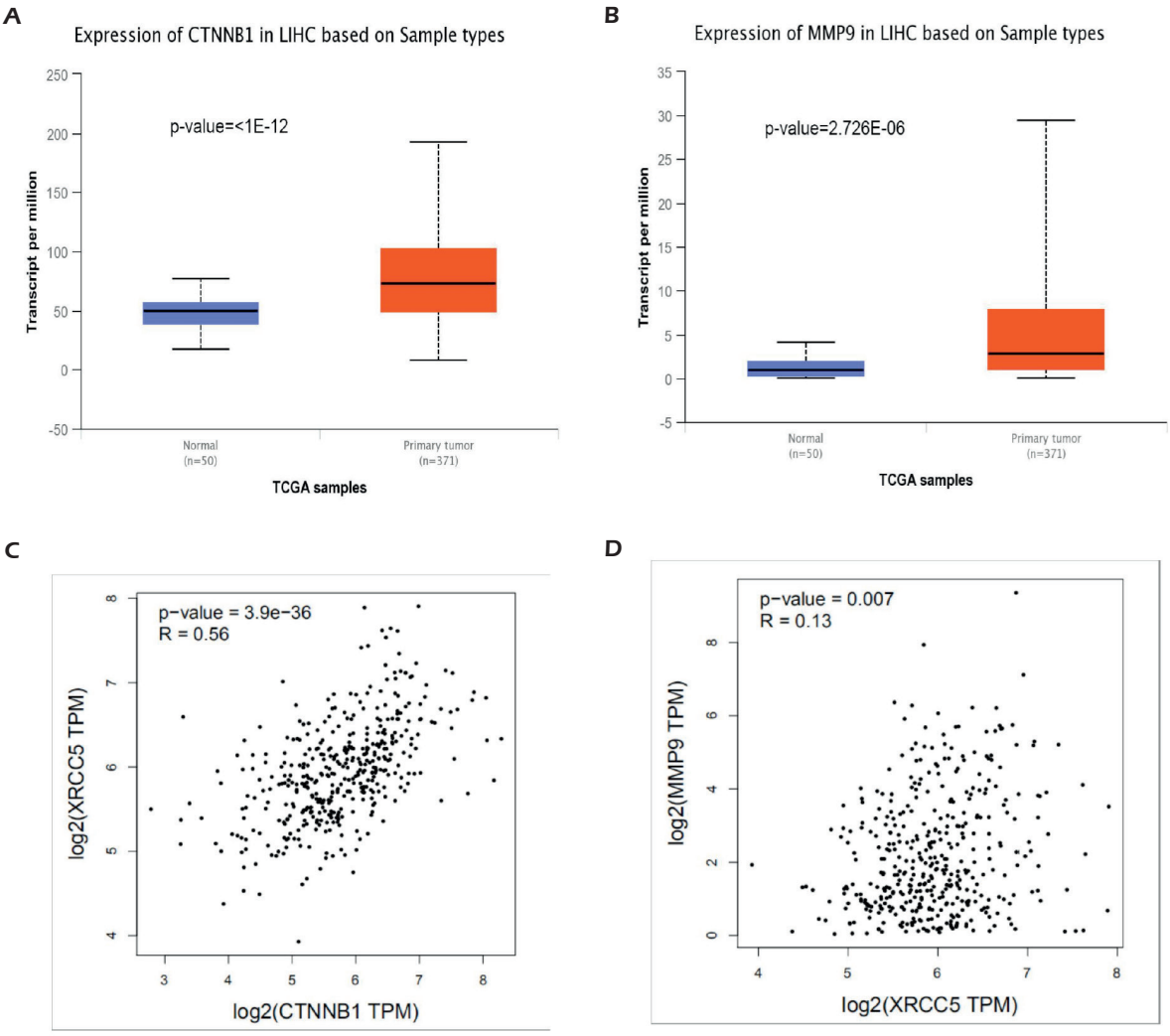

Figure 7. XRCC5 promotes HCC progression via Wnt signaling pathway. **A-B,** CTNNB1, MMP9 mRNA levels in the HCC TCGA dataset (normal *vs.* primary tumor). **C-D,** XRCC5 is significantly positively correlated with MMP9 and CTNNB1 mRNA levels in the HCC TCGA dataset.

Figure continued

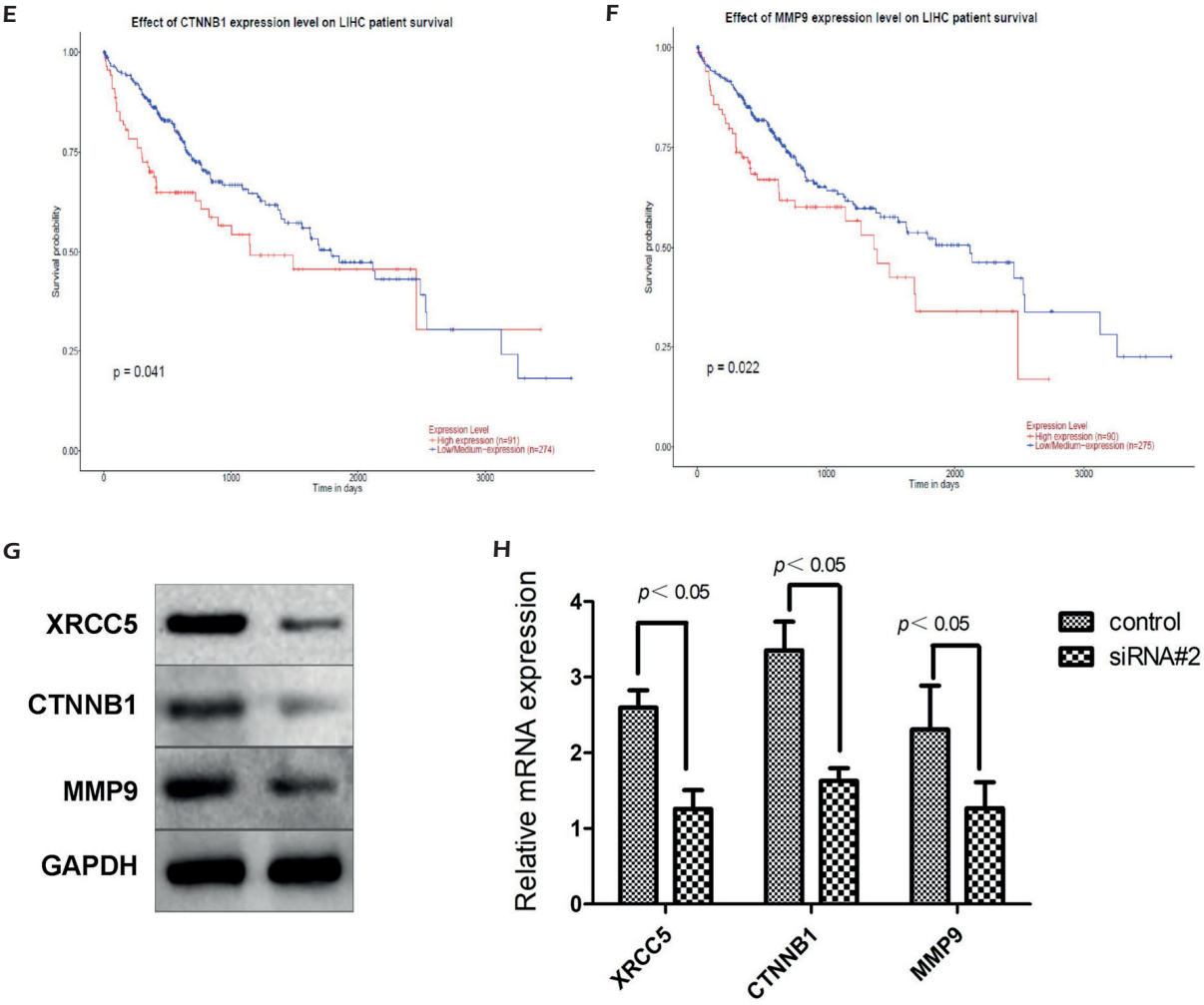

Figure 7. *Continued.* **E-F**, High expression of CTNNB1, MMP9 indicates poor overall survival time in the HCC TCGA dataset. **G-H,** SiRNA#2 was transfected into MHCC97-H and examined for mRNA and protein expression of XRCC5, MMP9, and CTNNB1. LIHC: Liver hepatocellular carcinoma.

showed that *XRCC5* probably played an important role in tumor progression. *XRCC5* was found overexpressed in lung cancer tissues. In non-small cell lung cancer cells, knocking down *XRCC5* can inhibit tumor characteristics *in vitro* and *in vivo*[31,32]. Tissue microarray immunohistochemical analysis of lung adenocarcinoma showed a strong positive correlation between *XRCC5* and COX-2 levels and clinicopathological variables. *XRCC5* overexpressing lung cancer patients had a poor prognosis, and *XRCC5* promoted COX-2 expression and tumor growth and may be a potential therapeutic target for lung cancer[33]. Xiao et al[33] have shown that DNA double-strand break (DSB) repair protein *XRCC5* has a sensitizing effect on cervical cancer cisplatin combined with radiotherapy.

Aberrant activation of the Wnt/β-Catenin signaling pathway is closely related to tumor development and progression. Wnt/β-catenin pathway is frequently upregulated in HCC and it is implicated in the maintenance of tumor initiating cells, drug resistance, tumor progression, and metastasis[34,35]. We knocked down the expression level of *XRCC5*, and then found that MMP9 and CTNNB1 were accordingly downregulated. This result suggested that MMP9 may be one downstream effector protein of *XRCC5*. Although it was unclear

whether *XRCC5* directly or indirectly binds to *MMP9* during regulation of gene expression, our finding suggested that *XRCC5* may regulate the migration of HCC cells *via* Wnt/β-catenin signaling. This evidence suggests that *XRCC5* plays a role in the progression of HCC and has potential value as a therapeutic target.

Conclusions

The research on the effect of *XRCC5* on invasion and migration of HCC cells and its mechanism could provide a useful target for the prognostic prediction in HCC and provides a potential target for the effective treatment of patients with HCC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Acknowledgments

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Authors' Contribution

Jie Ding conceived and designed the experiments; Zhenhua Liu and Ning Wang performed the experiments; Zhehua Liu and Fie-qing Wang analyzed the data; Zhen-hua Liu and Qi Dong prepared the figures; Zhen-hua Liu, Jie Ding, Ning Wang Fie-qing Wang and Qi Dong wrote the paper; all authors read and approved the final manuscript.

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