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Original Article

High expression of ERCC5 predicts a poor prognosis in hepatocellular carcinoma

Xueyong Zheng[1*], Ke Chen[1*], Xiaolong Liu[1], Guangyi Jiang[1], Hui Liu[2]

[1]Department of General Surgery, Institute of Minimally Invasive Surgery, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, No. 3, Qingchun Road, Hangzhou 310016, China; [2]Biomedical Research Center and Key Laboratory of Biotherapy of Zhejiang Province, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou, Zhejiang, China. [*]Equal contributors.

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Abstract: Human cells exposed to environmental or endogenous carcinogens can develop DNA damage. This DNA damage may contribute to a susceptibility to cancer; therefore, it is important to repair these defects. The nucleotide excision repair pathway (NER) is a versatile DNA repair pathway that eliminates a wide variety of helix-distorting base lesions induced by environmental or endogenous carcinogenic sources. The excision repair cross-complementation group 5 (ERCC5) gene is a central component of NER. Ectopic expression of ERCC5 has been linked to different types of cancers, including hepatocellular carcinoma (HCC). However, previous reports, mainly based on mRNA level and the role of ERCC5 in cancer, remain conflicting and unclear. In this study, we examined 104 cases of HCC for immunohistochemistry to explore the role of ERCC5 protein in HCC. We found the expression of ERCC5 protein was significantly increased in tumor tissues compared to paracancerous ones ($P<0.01$). The percentage of positive staining of ERCC5 in tumor tissues was 28.8% (30/104), while only 4.8% (5/104) in paracancerous tissues. Patients with low ERCC5 expression levels had a better overall survival rate and remained disease-free longer (both $P<0.01$). In addition, univariate and multivariate analysis showed a high expression of ERCC5 protein and large tumor size predict a poor prognosis for patients with HCC ($P<0.05$).

Keywords: ERCC5 (XPG), hepatocellular carcinoma, DNA damage, nucleotide excision repair (NER)

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy around the world, fifth in men and seventh in women, respectively [1]. However, it contributes to approximately 11% of cancer-related deaths, making it the second most common cause of cancer-related deaths worldwide according to the World Health Organization (WHO) [2]. Liver resection remains the only potentially curative treatment available for early stage HCC. Unfortunately, due to advanced disease and underlying hypohepatia, only 15% of cases are eligible for curative treatments [3]. Therefore, it is important to understand the molecular mechanism of the development and progression of HCC to improve the therapeutic effects.

It has been well documented now that cancer is a complex disease affected by environmental factors, hereditary susceptibility, and gene-environment interactions, etc. [4]. Human cells exposed to environmental carcinogens can cause DNA damage. On the other hand, reduced DNA repair capacity may lead to DNA damage accumulation, which has been linked to genetic susceptibility to cancer [5-7]. The incidence of cancer could be increasing unless this DNA damage can be repaired properly and efficiently [4]. Understandably, DNA repair pathways would play an important role in maintaining genomic integrity and stability. The nucleotide excision repair pathway (NER) is one of the primary pathways by which mammalian cells remove DNA lesions caused by both endogenous and exogenous carcinogenic sources [5, 8]. The NER system is most frequently associated with cancer [9], and it has been reported to play a critical role in protecting against human cancers [10].

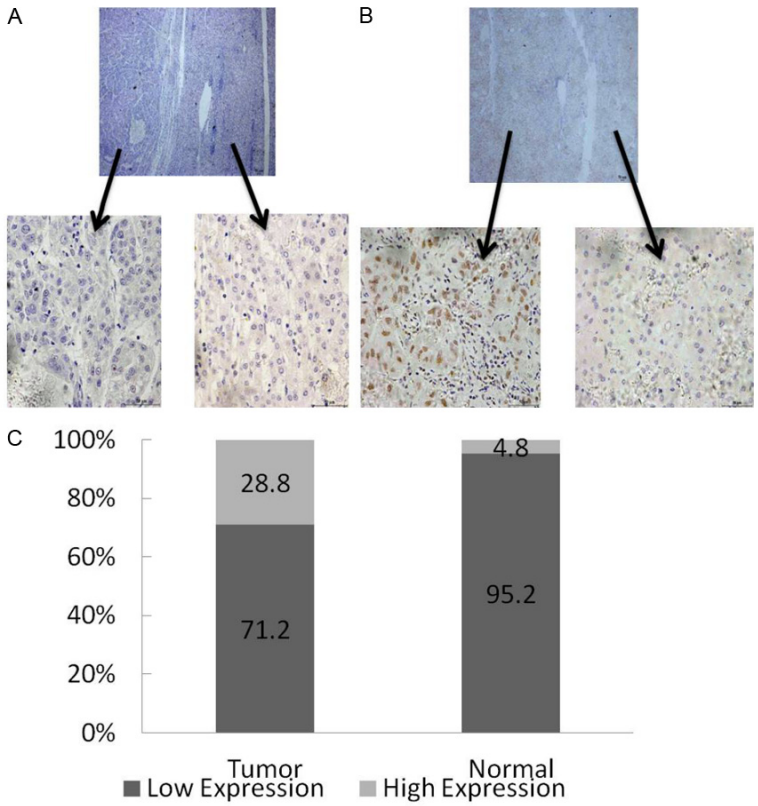

Figure 1. Expression of ERCC5 in HCC patients. A. Representative low-expression of ERCC5 in tumors; B. Representative high-expression of ERCC5 in tumors; C. The ERCC5 protein was significantly more highly expressed in tumor tissues than in paracancerous tissues (P<0.001).

The excision repair cross-complementation group 5 (ERCC5) gene, also named as xeroderma pigmentosum group G (XPG) gene, is a key member in the NER pathway [4]. In terms of mechanism, ERCC5 is required to format the 3' incision during NER repair [7, 11]. Research indicates that ectopic expression of ERCC5 is responsible for several cancers, such as gastric cancer, breast cancer, squamous cell carcinoma, and HCC [4]. However, almost all studies available are based on RNA level, and the association between ERCC5 and the risk of cancer remains conflicting. Therefore, the role of ERCC5 in the development of cancer merits further study. In this study, we examined the immunohistochemistry (IHC) of 104 HCC cases to elucidate the relationship between the ERCC5 protein and HCC.

Materials and methods

Patients and tissue samples

This study was conducted with the approval of the Ethical and Scientific Committees of Sir Run Run Shaw Hospital, Zhejiang University (Hangzhou, China). Patients were all informed that the resected specimens would be kept and possibly used for scientific research. All patients were promised that their personal privacy would be protected.

A total of 104 HCC patients who underwent hepatectomy between January 2006 and December 2010 were enrolled, among which 86 were men and 18 were women. The ages ranged from 28 to 79 years, and the median age was 60 years. Patients receiving preoperative radiotherapy, chemotherapy or immunotherapy before surgery were excluded. For all the samples, we used each own paracancerous tissue as a normal control. The tumor stages were graded according to the 7th edition of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) system. These patients were followed up for at least 60 months or until their deaths.

Immunohistochemistry

Immunohistochemistry was performed using the GTVision™ detection kit (Gene Tech, Shanghai), according to the manufacturer's instructions. Specimens obtained from surgical resection were fixed in 10% formalin prior to being processed in paraffin. All the hematoxylin-eosin stained sections were reviewed and confirmed by two experienced pathologists according to the WHO classification guidelines, and a representative section for each case was selected for immunohistochemical analysis.

The selected sections were dewaxed, hydrated with dimethylbenzene and a gradient concentration of alcohol, and then washed with deionized water and phosphate-buffered saline (PBS). Next, an antigen retrieval process was performed with 0.01 M citrate buffer (pH 6.0, Química Contemporânea, Diadema, Brazil) before blocking endogenous peroxidase activi-

Table 1. Clinical characteristics of HCC patients

Variable		ERCC5 expression		*P* Value
		Low (%)	High (%)	
Age	<60 years	56 (75.7)	24 (80.0)	0.635
	≥60 years	18 (24.3)	6 (20.0)	
Gender	Male	11 (14.9)	7 (23.3)	0.301
	Female	63 (85.1)	23 (76.7)	
HBsAg*	Negative	11 (14.9)	5 (16.7)	0.773
	Positive	63 (85.1)	25 (83.3)	
AFP (ng/mL)	<400	45 (60.8)	19 (63.3)	0.811
	≥400	29 (39.2)	11 (36.7)	
Liver cirrhosis	No	33 (44.6)	14 (46.7)	0.848
	Yes	41 (55.4)	16 (53.3)	
Tumor size	<5 cm	41 (55.4)	22 (73.3)	0.090
	≥5 cm	33 (44.6)	8 (26.7)	
Tumor number*	Single	65 (87.8)	26 (86.7)	1.000
	Multiple	9 (12.2)	4 (13.3)	
Tumor differentiation	Well/moderately	41 (55.4)	13 (43.3)	0.264
	Poorly	33 (44.6)	17 (56.7)	
Tumor thrombi*	No	65 (87.8)	25 (83.3)	0.539
	Yes	9 (12.2)	5 (16.7)	
TNM stage*	I+II	67 (90.5)	27 (90.0)	1.000
	III+IV	7 (9.5)	3 (10.0)	
BCLC stage*	A	68 (91.9)	26 (86.7)	0.469
	B	6 (8.1)	4 (13.3)	

*Distribution compared by Fisher's exact test.

ty by 0.3% hydrogen peroxide for 15 min. Sections were incubated in preimmunized goat serum for 0.5 h, and then incubated overnight at 4°C refrigeration using the following primary antibody: Anti-RNF40 (Atlas, rabbit polyclonal IgG, 0.2 mg/ml, 1:750 dilution, cat. No. HPA041330). The next day, after rewarming, sections were incubated with a secondary antibody of GTVisionTM/HRP, Rabbit/Mouse (ENV) reagent. Finally, ChemMateDAB+chromogen were used to visualize the reaction, followed by counterstaining with hematoxylin.

Evaluation of staining

The slides' staining intensity and the percentage of positive cells were evaluated by two independent investigators three times. A positive expression result was indicated by brown-yellow or brown granular deposits at the corresponding antibody expression sites. The positive expression of RNF40 is located in the nucleus. The intensity of staining was scored in the following four categories: 0, negative; 1, weak; 2, moderate; and 3, strong staining. Similarly, the percentage of positive cells was also scored in 3 categories: 1, 0%-25%; 2, 25%-50%; 3, 50%-100%. The two scores were combined to obtain an immunoreactivity score (IRS) value of RNF40 expression ranging from 0 to 12. The specimens were divided into two categories according to the IRS: <6, low expression and 6-12, high expression.

Statistical analysis

Statistical analysis was carried out using the SPSS 22.0 software package (SPSS, IBM, Chicago, IL, USA) by a third analyst, who did not participate in the experiment. The relationship between RNF40 expression and clinicopathologic features was estimated by Chi-square analysis or Fisher's exact test. The Kaplan-Meier method was used to analyze the survival curve, and the differences were conducted using the log-rank test. Disease-free survival (DFS) was defined as the time from surgery to the time of HCC recurrence, and overall survival (OS) was defined as the time from surgery to death by any cause. Cox's proportional hazards regression model was utilized for univariate and multivariate analyses. Multivariate analysis was performed depending on the Cox proportional hazards model for the variables with P<0.05 examined in the univariate analysis. The estimated relative risks of dying were expressed as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). P<0.05 was considered statistically significance.

Results

Expression of ERCC5 in HCC

The ERCC5 protein was mainly located in the nucleus. Representative immunostaining results of ERCC5 in HCC tissues were shown in **Figure 1A**, **1B**. The percentage of positive stain-

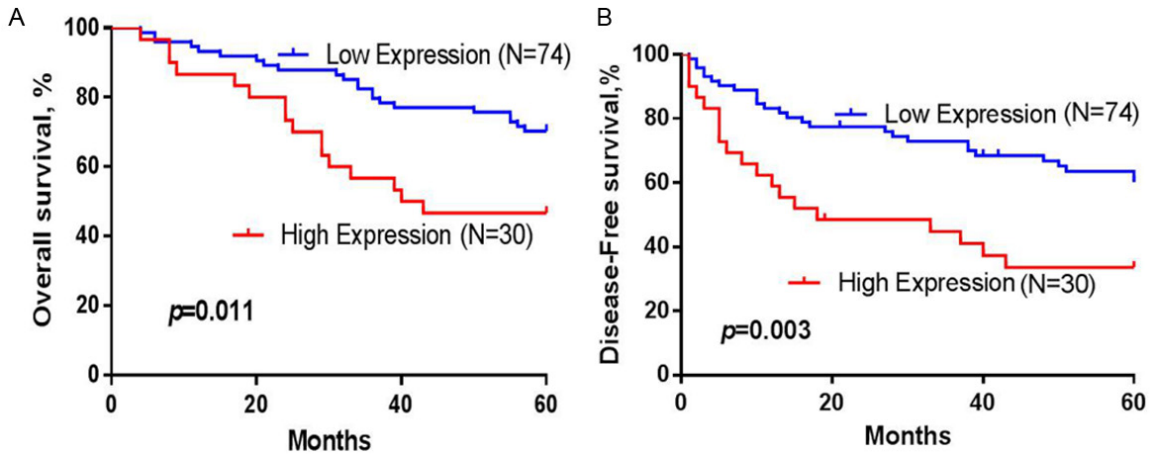

Figure 2. A. The 5 year, overall survival rate of patients with high ERCC5 expression was significantly lower than that with low ERCC5 expression (log-rank, P=0.011). B. The 5 year, disease-free survival rate of patients with high ERCC5 expression was significantly lower than those with a low ERCC5 expression (log-rank, P=0.003).

Table 2. Univariate and multivariate analysis of prognostic factors for overall-survival of HCC patients

Variable		Univariate		Multivariate	
		HR (95% CI)	P	HR (95% CI)	P
Age	≥60	1.02 (0.48-2.15)	0.96		
Gender	Female	0.89 (0.39-2.02)	0.778		
HBsAg	Yes	0.81 (0.36-1.85)	0.623		
AFP	≥400	1.34 (0.71-2.56)	0.367		
Liver cirrhosis	Yes	1.20 (0.63-2.29)	0.579		
Tumor size	≥5 cm	2.39 (1.26-4.53)	0.008	2.63 (1.29-5.37)	0.008
Tumor number	Multiple	1.17 (0.46-2.99)	0.746		
Tumor differentiation	Poorly	1.28 (0.68-2.43)	0.441		
Tumor thrombi	Yes	2.14 (0.98-4.67)	0.057		
TNM stage	III+IV	3.28 (1.43-7.49)	0.005	2.13 (0.88-5.18)	0.094
Expression Group	High	2.26 (1.18-4.31)	0.014	3.00 (1.52-5.90)	0.002

ing in tumor tissues and paracancerous ones was 28.8% (30/104) and 4.8% (5/104), respectively. In addition, ERCC5 was found to be more highly expressed in tumors than in paracancerous tissues with a significant difference according to the IRS value (P<0.01) (**Figure 1C**).

Relationship between ERCC5 expression and clinicopathological features in HCC patients

To evaluate the association between the ERCC5 protein and HCC progression, we analyzed the correlation between ERCC5 expression and clinicopathological parameters of HCC cancers (**Table 1**). No significant differences of association were detected between ERCC5 expression and gender, age, hepatitis B virus infection, AFP level, liver cirrhosis, tumor size, tumor stage, tumor thrombosis, tumor differential stage, or TNM stage. In addition, Kaplan-Meier survival results indicated a low expression of ERCC5 led to a significantly better outcome, both in overall survival time and in disease-free survival time (**Figure 2**, both P<0.01).

Univariate analysis explained that tumor size (P<0.01), TNM stage (P<0.01) as well as ERCC5 expression (P<0.01), was associated with overall survival time (**Table 2**). Univariate analysis also indicated a similar outcome of disease-free survival time (**Table 3**). According to those data above, we have a preliminary conclusion that ERCC5 could be a valuable prognostic factor in HCC. Therefore, multivariate analysis was performed depending on the Cox proportional hazards model for the variables with *P* value <0.05 examined in the univariate analysis. And the results showed that ERCC5 expression (HR: 3.00, 95% CI 1.52-5.90, P<0.01 vs. HR: 2.54, 95% CI 1.36-4.72, P<0.01, respectively) and tumor size (HR: 2.63, 95% CI 1.29-5.37, P<0.01 vs. HR: 2.23, 95% CI 1.15-4.35, P=0.02, respectively) were independent

Table 3. Univariate and multivariate analysis of prognostic factors for disease free survival of HCC patients

Variable		Univariate		Multivariate	
		HR (95% CI)	P	HR (95% CI)	P
Age	≥60	1.09 (0.56-2.11)	0.797		
Gender	Female	1.16 (0.52-2.59)	0.724		
HBsAg	Yes	1.06 (0.48-2.38)	0.882		
AFP	≥400	1.02 (0.55-1.87)	0.95		
Liver cirrhosis	Yes	1.52 (0.83-2.76)	0.174		
Tumor size	≥5 cm	2.07 (1.16-3.70)	0.014	2.23 (1.15-4.35)	0.018
Tumor number	Multiple	1.38 (0.59-3.26)	0.46		
Tumor differentiation	Poorly	1.12 (0.63-1.99)	0.703		
Tumor thrombi	Yes	3.44 (1.65-7.21)	0.001	2.38 (1.09-5.20)	0.029
TNM stage	III+IV	3.45 (1.53-7.76)	0.003	1.97 (0.78-4.8)	0.15
Expression Group	High	2.34 (1.30-4.22)	0.005	2.54 (1.36-4.72)	0.003

prognostic factors for both overall and disease-free survival in HCC (**Tables 2** and **3**). In addition, tumor thrombosis presented as an independent prognostic factor for disease-free survival in HCC both in univariate and multivariate analyses (**Table 3**).

Discussion

Biologically, DNA determines the whole genetic structure as well as many predispositions and appearances. Therefore, it is thought to be the key to life. On the other hand, it has been well described that genomic assaults are abundant due to environmental and endogenous factors [10]. Previous reports indicated DNA damage occurs at an estimated frequency of approximately 20,000-50,000 lesions per cell per day in human beings [7, 12, 13]. This means that up to 10-40 trillion damaged DNA lesions accumulate per second in one human body [7]. Accumulative DNA damage is one of the most common causes of cancer [6, 10, 14-18] because DNA damage accumulation can cause mutations by activating (pre-) onco-genes, silencing tumor suppressor genes or other indispensable genes, and leading to the loss of homeostasis [7]. Defects in DNA repair pathways are therefore also thought to accelerate tumorigenesis.

There are five major DNA repair pathways: homologous recombinational repair (HRR), nonhomologous end joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) [4, 19]. Among them, NER is a versatile DNA repair pathway that eliminates a wide variety of helix-distorting base lesions accumulated in cells [5, 8] and safeguards genome integrity [20].

The ERCC5 gene belongs to the FEN1/XPG family of endonucleases and plays a crucial role in the NER pathway [4, 21]. The NER repair pathway is divided into three steps: the recognition step, the unwinding step, and the incision step [22]. During the incision step, ERCC5 is recruited around the damaged DNA 3' region, cutting on the 3' side to repair DNA [7, 11, 22, 23]. The ectopic expression of ERCC5 has been linked to some types of cancer [4]. In this study, we found that expression of ERCC5 protein predicted a poor prognosis in HCC. We found that a low expression level of ERCC5 indicated a better overall survival and disease-free rate. However, there is no significant difference between the ERCC5 protein level and age, gender, hepatitis B virus infection, liver cirrhosis, tumor size, tumor thrombosis, differential stage or tumor stage. To the best of our knowledge, this is the first report elucidating the role of ERCC5 in HCC based on protein.

Further investigation is still needed for the association between ERCC5 because the risk of cancer remains uncertain [24]. One possible explanation of ERCC5 protein variants is due to a single nucleotide polymorphisms (SNP). Previous studies indicated that ERCC5 SNP can influence the DNA repair ability, thus disturbing the susceptibility to cancer [25-29]. A meta-analysis published recently indicated that ERCC5 gene polymorphism rs873601 was significantly associated with overall cancer risk, while the polymorphisms rs751402 and rs2296147 might not contribute to the overall cancer risk [4]. In addition, Wang et al. reported the ERCC5 rs873601 genotype had a decreased risk for HCC when compared with wide-type. Patients with ERCC5 rs873601 also showed a higher

mRNA expression of the ERCC5 gene, but the underline mechanism is still unclear [22]. Interestingly, Yoon et al. reported individuals with the inherited ERCC5 rs751402 CC genotype might experience significant protection against HCC, while those with T alleles were found to have a higher risk [30].

Our study still had some limitations. Firstly, the sample was restricted, thus limiting the interaction analysis. Secondly, although we found ERCC5 expressed in HCC tumor tissues and predicted a poor prognosis, we do not know the detail protein variants in this study. Finally, the exact role and mechanism of the ERCC5 protein in HCC is still not explained. Therefore, further functional studies will be needed to verify our findings.

Conclusion

Our study showed that the status of ERCC5 expression might be a prognostic factor for HCC patients, and targeting this molecule would be a potential strategy for the treatment of HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Xueyong Zheng, Department of General Surgery, Institute of Minimally Invasive Surgery, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, No. 3, Qingchun Road, Hangzhou 310016, China. E-mail: 3306053@zju.edu.cn

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