

# Relationship of the expression levels of XIAP and p53 genes in hepatocellular carcinoma and the prognosis of patients

ZHIQIN LI, CHUNFANG HAN and JING FENG

Department of Infectious Diseases, Weifang People's Hospital, Weifang, Shandong 261041, P.R. China

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**Abstract.** In this study, we measured mRNA and protein expression levels of X-linked inhibitor of apoptosis protein (XIAP) and p53 in hepatocellular carcinoma (HCC) and analyzed their relationships to clinicopathological parameters and the prognosis of the patients. Samples were obtained from tumors and tumor-adjacent normal tissues from 70 patients with HCC who were hospitalized in Weifang People's Hospital from January 2009 to December 2011. Quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC) were used to detect the mRNA and protein expression levels, respectively. The clinical data of patients who were followed for 5 years from the day of the tumor-resection surgery were collected in detailed clinical histories. Statistical analyses were used to find relationships between the XIAP and p53 levels and the clinical variables and 5-year survival of patients. Our qPCR results showed that the mRNA expression levels of XIAP and p53 in HCC tumors were significantly higher than those in tumor-adjacent normal tissues. At the same time, IHC results showed that the positive expression rates of XIAP and p53 in HCC in tumors were 81.4% (57/70) and 72.9% (51/70), respectively and their high expression was related to invasion, metastasis and tumor staging. The overall 5-year survival rate of the patients was 15.7% (11/70). Single factor survival analysis showed that both XIAP and p53 were influencing factors of the overall survival rate of patients with HCC ( $P < 0.01$ ). In conclusion, high expression levels of XIAP and p53 are closely related to clinicopathological parameters of patients with HCC, especially related to invasion, metastasis and tumor staging. XIAP and p53 levels can be used as reference values to guide the treatment of HCC and estimate the prognosis.

## Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor; its incidence ranks fifth and it has the third highest death rate among cancers (1). The pathogenesis of HCC is very complex; excessive alcohol drinking, viral hepatitis and nonalcoholic fatty liver have all been shown to induce carcinogenesis in normal liver cells (2-4). Studies on molecular physiology have found a dynamic balance between hepatocyte proliferation and apoptosis. When this balance is broken, apoptosis does not function well and tumor cell proliferation and even metastasis occur (5,6).

The inhibitor of apoptosis (IAP) family includes protein factors that inhibit apoptosis *in vivo* (7). Among the many factors of the IAP family, the activity of the X-linked IAP protein (XIAP) is the strongest. XIAP is mainly composed of *baculovirus* IAP repeat (BIR) and zinc finger domains. BIR domains can inhibit the functions of caspase-3 and -7, while the zinc finger domains inhibit the functions of caspase-9; together these domains have the capacity for inhibiting all the caspases that induce apoptosis (8). Additionally, studies have also shown that XIAP plays a key role in other biological processes such as cell signal transduction and protein ubiquitination (9).

The p53 genes were some of the first factors shown to control cell proliferation and apoptosis, thus inhibiting tumors. However, mutations in the wild-type p53 gene can lead to inhibition of tumor cell apoptosis (10). Studies have confirmed that mutation of p53 gene happens in 50% of tumor tissues in humans, so p53 gene is used as tumor biomarker (11).

The present study was designed to measure expression of XIAP and p53 in HCC patients and to identify their relationships to clinicopathological parameters and prognosis of patients. Quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC) were used to detect the expression levels of XIAP and p53 mRNA and proteins in HCC tumors and tumor-adjacent normal samples. Clinicopathological variables from the clinical history of 5 years from the day of surgery were used in the statistical analysis to calculate prognosis of patients.

## Materials and methods

*Specimens.* Frozen and paraffin specimens of tumor tissues from 70 patients with HCC and the accompanying tumor

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*Correspondence to:* Dr Jing Feng, Department of Infectious Diseases, Weifang People's Hospital, 151 Guangwen, Kuiwen, Weifang, Shandong 261041, P.R. China  
E-mail: feng\_jingwf@163.com

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adjacent normal tissues in 30 of those patients were used for the measurements. Patients admitted to Weifang People's Hospital (Weifang, China) from January 2009 to December 2011 were enrolled in the study. All patients were clinically and pathologically diagnosed with HCC and they all received surgical treatment for the first time and had no prior history of radiotherapy or chemotherapy. Patients with metastatic HCC, chronic hepatitis B or cirrhosis were excluded. In the end, there were 36 males and 34 females, aged 25-79 years with an average age of 47. The Clinical Ethics Committee of our hospital approved all the procedures in the study and all participating patients or their family members signed the informed consent forms.

*Detection of the expression levels of XIAP and p53 in specimens by qPCR.* Approximately 50 mg of frozen tumor tissues and tumor-adjacent tissues of patients were used to extract total RNA according to the method provided in the RNA isolation kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The UV-Vis spectrophotometer (Hitachi, Ltd., Tokyo, Japan) was used to detect the total RNA concentration and purity (when the absorbance value ratio of A260/A280 was 1.8-2.0, the RNA was deemed of good quality). cDNAs were obtained via reverse transcription according to the instructions in the reverse transcription kit (Invitrogen Life Technologies) and then the expression of XIAP and p53 mRNAs were detected following the method in the RT-PCR kit (Invitrogen Life Technologies) using cDNAs as templates. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences of XIAP, mutant p53 and GAPDH are shown in Table I, all primers were synthesized by Takara Bio (Dalian, China). The PCR conditions included 95°C for 10 min, then a total of 40 cycles of 95, 57 and 72°C for 30 sec and then a final extension at 72°C for 5 min. The relative expression levels were calculated using the  $2^{-\Delta Cq}$  method according to the following formula:  $\Delta Cq$  (target gene) =  $Cq$  (target gene) -  $Cq$  (control gene).

*Detection of the expression levels of XIAP and p53 proteins in patient specimens by IHC.* The procedure was carried out according to the instructions of the IHC SP-9001 kit (Beijing Zhongshan Golden Bridge Biological Technology, Beijing, China). Briefly, after dewaxing of the paraffin sections, the endogenous peroxidase was inactivated with 3% H<sub>2</sub>O<sub>2</sub>. The citrate buffer was used for thermal remediation and proteins were blocked with 10% goat serum. Primary rabbit polyclonal XIAP antibody (dilution, 1:500, cat. no. ab21278) and rabbit polyclonal p53 antibody (dilution, 1:500; cat. no. ab1431) were added and incubated at 4°C overnight. Then proteins were washed with phosphate-buffered saline (PBS) and secondary goat anti-rabbit (HRP) IgG antibody (dilution, 1:2,000; cat. no. ab6721) were added. All the antibodies were all purchased from Abcam (Cambridge, MA, USA). Under this condition, samples were incubated for 15 min. After that, proteins were washed with PBS and diaminobenzidine solution was used for development in the dark. Then hematoxylin was used for restaining the samples. Finally, pictures were taken under the light microscope (TE2000-U; Nikon, Tokyo, Japan).

Three representative regions from each sample were selected and the results of immunohistochemical staining were

Table I. Primer sequences of qPCR.

Gene	Primer name	Primer sequence
XIAP	Forward	5'-AACCTTGTGATCGTGCCT-3'
	Reverse	5'-ACCCTGGATACCATTTAGC-3'
p53	Forward	5'-TGCGTGTGGAGTATTTGGATG-3'
	Reverse	5'-TGGTACAGTCAGAGCCAACCTC-3'
GAPDH	Forward	5'-ATGGCACCGTCAAGGCTGAG-3'
	Reverse	5'-GCAGTGATGGCATGGACTGT-3'

XIAP, X-linked inhibitor of apoptosis protein; GAPDH glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative polymerase chain reaction.

evaluated by rating and grading the expression according to the staining intensity and the percentage of positive cells. The staining intensity was classified as stainless, brownish brown, tan and sepia (recorded as 0, 1, 2 and 3 points, respectively). In addition, the number of positive cells (magnification, x400) was classified as <5, 5-25, 26-50 and >50% (recorded as 0, 1, 2 and 3 points). In the end, the scores of the two indexes were added. Total points >3 were considered as positive for expression and ≤2 as negative for expression. The final scores were used in the statistical analyses.

*Relationship between the expression levels of XIAP and p53 in HCC tissues and the prognosis in patients.* The 70 patients in the study were divided into a XIAP positive or a XIAP negative group and also into a p53-positive or -negative group, according to the expression levels of XIAP and p53 in HCC tissues. There were no differences in statistical significance in terms of age, sex, patient condition and other factors between the two groups ( $P>0.05$ ). The relationship of XIAP and p53 with clinicopathological parameters was analyzed using the available recorded clinical data of patients. All the 70 patients were followed up for 5 years and the follow-up rate was 100%. The survival duration was recorded from the first day after surgery to the date of death of patients or the deadline of the follow-up. The statistical analysis was performed on a monthly basis.

*Statistical analysis.* SPSS 17.0 (IBM SPSS, Armonk, NY, USA) was used for statistical analyses in this study. Measurement data were expressed as mean ± standard deviation and the t-test was used for comparisons between groups. The enumeration data were compared between the two groups using the  $\chi^2$  test. Clinical prognostic data were calculated using the Kaplan-Meier survival analysis.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

*Detection of the expression levels of XIAP and p53 mRNAs in tissue specimens by qPCR.* qPCR results (Fig. 1) showed that the expression levels of XIAP and p53 mRNAs in HCC tissues were significantly higher than those in tumor-adjacent normal tissues and the differences were statistically significant ( $P<0.01$ ).

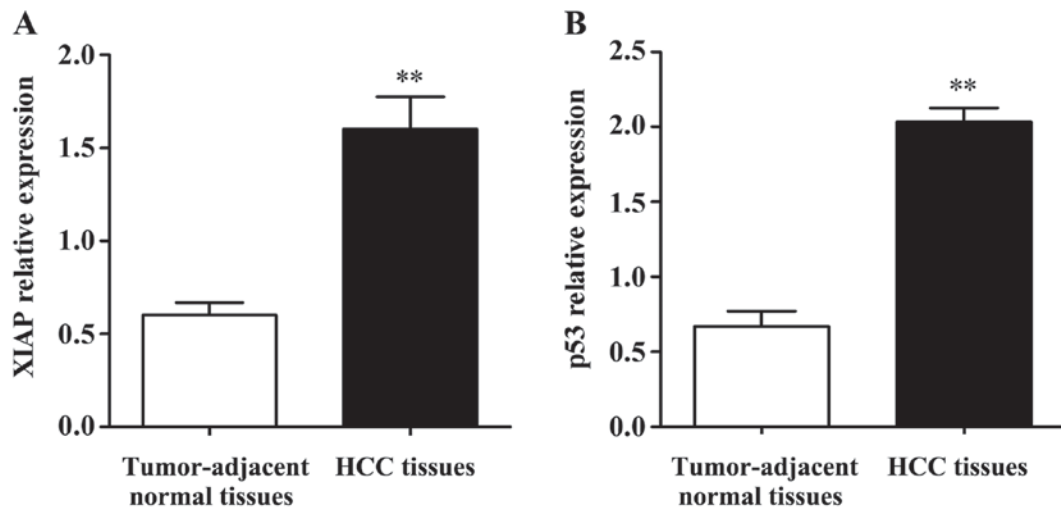


Figure 1. The expression levels of XIAP and p53 mRNAs in HCC tissues are significantly higher than those in tumor-adjacent normal tissues,  $**P<0.01$ . (A) The expression of XIAP mRNAs in tissue specimens. (B) The expression of p53 mRNAs in tissue specimens. XIAP, X-linked inhibitor of apoptosis protein; HCC, hepatocellular carcinoma.

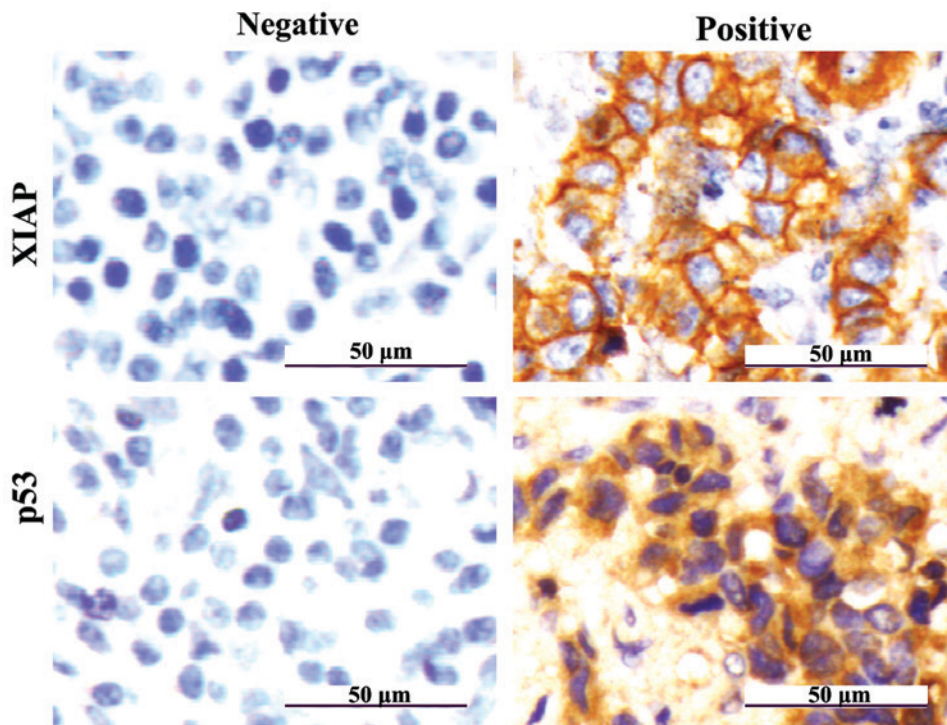


Figure 2. Detection of the expression of XIAP and p53 proteins in clinicopathological tissues by IHC. XIAP, X-linked inhibitor of apoptosis protein; IHC, immunohistochemistry.

*Detection of the expression levels of XIAP and p53 proteins in clinicopathological tissues by IHC.* In IHC results the color of stained XIAP and p53 by IHC was tan, with XIAP proteins mainly being present in the cytoplasm and partly in cell membranes and p53 proteins mainly present in the nucleus (Fig. 2).

The IHC scores were counted, the positive expression rates of XIAP in HCC and tumor-adjacent normal tissues were 81.4% (57/70) and 13.3% (4/30), respectively; and the difference was statistically significant ( $P<0.01$ ). The positive expression rates of p53 in HCC and tumor-adjacent normal

tissues were 72.9% (51/70) and 10.0% (3/30), respectively; and the difference was statistically significant ( $P<0.01$ ) (Table II).

*Relationship of clinicopathological parameters of HCC and the expression levels of XIAP and p53.* The statistical analysis results showing the relationship between clinicopathological parameters of HCC patients and the expression levels of XIAP and p53 are shown in Table III and Fig. 2. The  $\chi^2$  test showed that the positive expression of XIAP and p53 correlated with the occurrence of metastasis and higher tumor stage ( $P<0.01$ ), but were not correlated with sex, age or tumor size ( $P>0.05$ ).

Table II. Positivity of expression of XIAP and p53 proteins in HCC and tumor-adjacent normal tissues.

Group	Case	XIAP			p53		
		Positive expression	Positive expression rate (%)	P-value	Positive expression	Positive expression rate (%)	P-value
HCC tissues	70	57	81.4	<0.01	51	72.9	<0.01
Tumor-adjacent normal tissues	30	4	13.3		3	10.0	

XIAP, X-linked inhibitor of apoptosis protein; HCC, hepatocellular carcinoma.

Table III. Relationship between high expression levels of XIAP and p53 with clinicopathological parameters of HCC patients.

Clinical data	Case	XIAP			p53		
		Positive expression	Positive expression rate (%)	P-value	Positive expression	Positive expression rate (%)	P-value
Sex							>0.05
Male	36	30	83.3	>0.05	26	72.2	
Female	34	27	79.4		25	73.5	
Age, years							>0.05
≥50	42	34	81.0	>0.05	32	76.2	
<50	28	23	82.1		19	67.9	
Tumor size							>0.05
≥5 cm	39	31	79.5	>0.05	28	71.8	
<5 cm	31	26	83.9		23	74.2	
Invasion and metastasis							<0.01
Yes	46	43	93.5	<0.01	41	89.1	
No	24	14	58.3		10	41.7	
Tumor staging							<0.01
I-II	26	15	57.7	<0.01	11	42.3	
III-IV	44	42	95.5		40	90.9	

XIAP, X-linked inhibitor of apoptosis protein; HCC, hepatocellular carcinoma.

Table IV. Single factor analysis of the relationship of the expression levels of XIAP and p53 with the overall survival rate of HCC patients.

Group	Case	5-year survival case	5-year survival rate (%)	Wald (log-rank)	P-value
XIAP					<0.01
Positive	57	5	8.8	15.14	
Negative	13	6	46.2		
p53					<0.01
Positive	51	4	7.8	15.68	
Negative	19	7	36.8		

XIAP, X-linked inhibitor of apoptosis protein; HCC, hepatocellular carcinoma.

Single factor analysis of the survival time and prognosis of patients. A total of 70 patients with HCC were followed up for a maximum of 5 years. The number of 5-year survivors was 11 and the number of deaths was 59, as shown in Table IV.



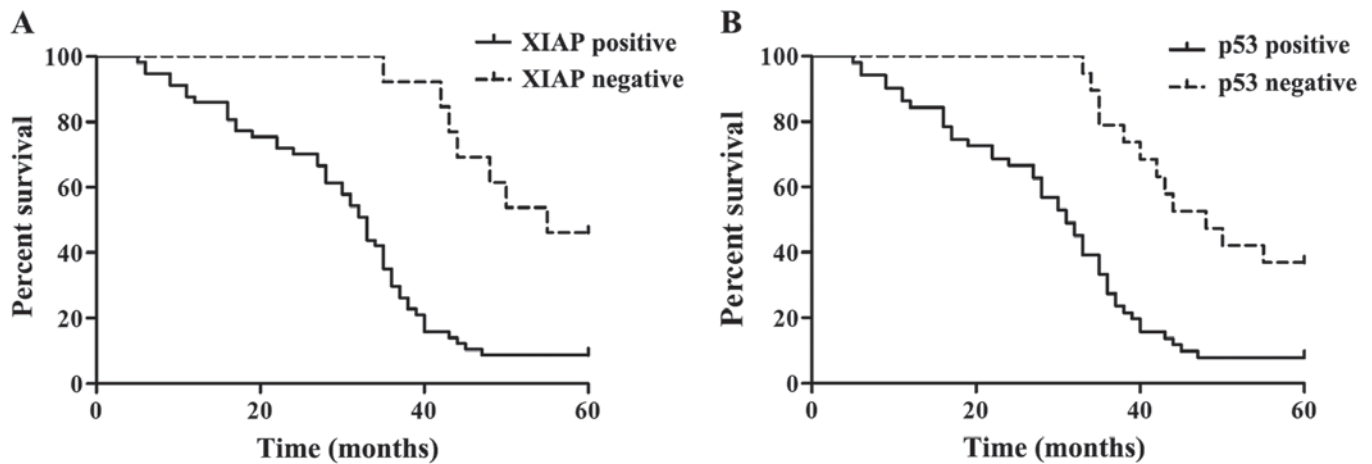


Figure 3. The expression of XIAP and p53 and Kaplan-Meier survival curve of patients with HCC. HCC patients with negative expression of XIAP and p53 have better survival prognoses. (A) The Kaplan-Meier survival curves of HCC patients with positive and negative expression of XIAP. (B) The Kaplan-Meier survival curves of HCC patients with positive and negative expression of p53. XIAP, X-linked inhibitor of apoptosis protein; HCC, hepatocellular carcinoma.

Fig. 3 shows the Kaplan-Meier survival curves of 70 patients with different expression of XIAP and p53. The results showed that HCC patients with negatively expressed XIAP and p53 had a relatively better survival prognosis. Differences in the overall survival curve were analyzed using the log-rank test, as shown in Table III. The results of single factor survival analysis showed that the effects of XIAP and p53 on the overall survival rate of patients with HCC were statistically significant ( $P < 0.01$ ).

## Discussion

HCC is one of the most common malignant tumors in China (12). Presently, more than 100 oncogenes have been found in studies, but the number of tumor suppressor genes is very small. Mutations, deletions and inactivations of tumor suppressor genes are events closely related to the occurrence and development of tumors (13).

XIAP, as an important member of the IAP family, can specifically inhibit the activity of caspase-3, -7 and -9, and inhibit the apoptosis of tumor cells (14). Clinical studies have shown that XIAP expression levels are significantly higher in a variety of malignant tumor tissues than in normal tissues (15).

The p53 gene is located on human chromosome 17p13 and is a kind of tumor suppressor and pro-apoptosis gene. Wild-type p53 plays a key role in cell gene transcription, cell cycle regulation, apoptosis, proliferation and differentiation (16,17). Studies have found that p53 gene mutations are closely related to the occurrence and development of HCC (18,19). The amount of wild-type p53 proteins is low in normal cells and their half-life periods are short, so IHC and other methods cannot detect them. However, the half-life periods of mutant p53 proteins are relatively longer and the amounts are relatively higher, allowing for their detection (20,21). Studies have found that mutant p53 proteins are highly expressed in tumor tissues of patients with breast cancer and the degree of malignancy of the cancer in these patients is high resulting in poor prognosis after surgery (22).

In order to find if tumor tissue levels of XIAP and p53 in HCC patients are also useful for prognoses, the expressions of XIAP and p53 mRNAs in tumor tissues of HCC patients were detected by qPCR. Our results showed the expression levels of XIAP and p53 mRNAs in HCC tissues were significantly higher than those in tumor-adjacent normal tissues. Furthermore, the IHC results showed that the positive expression rates of XIAP and p53 proteins in HCC tissues were 81.4% (57/70) and 72.9% (51/70), respectively, which were significantly higher than those in tumor-adjacent normal tissues. A further analysis, in combination with the clinicopathological features of patients, revealed that the expression of XIAP and p53 was related to the occurrence of metastasis and tumor staging; but not related to sex, age or the tumor size. Single factor Kaplan-Meier survival analysis was used to analyze the effects of different mRNA expression levels of XIAP and p53 on the overall survival rate curve of patients. The results showed the overall survival period of HCC patients with high expression levels of XIAP and p53 was relatively short.

A large number of experiments have shown that XIAP and p53 are closely related to the occurrence and development of a variety of tumors. A study found that XIAP protein levels in kidney clear cell carcinoma tissue specimens were high and the expression levels in the advanced stages were significantly higher than those in the early stages (23). Another study found the expression levels of p53 in 62 cases of primary HCC to be higher in cancer tissues than those in tumor-adjacent normal tissues (24). Our findings corroborate these studies and further confirmed that XIAP and p53 are highly expressed in HCC, and the expression of XIAP and p53 were related to the metastasis, staging and prognosis of HCC patients.

In conclusion, the positive expression of XIAP and p53 are closely related to the occurrence and development of HCC, especially to tumor metastasis and tumor staging. XIAP and p53 can be used as reference markers to guide the treatment of HCC and estimate its prognosis.

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