

Original Article

Altered TRAF3 and IKK α expression associated with advanced tumor stage and metastasis and poor prognosis of intrahepatic cholangiocarcinoma

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Abstract: Objective: Tumor necrosis factor receptor associated factor 3 (TRAF3) and I κ B Kinase alpha (IKK α) are both thought to be involved in human carcinogenesis. This study analyzed their expression in intrahepatic cholangiocarcinoma for association with clinicopathological data, and survival of patients. Methods: The expression of TRAF3 and IKK α in 50 ICC specimens, 50 adjacent normal tissues and 16 normal bile duct tissues were detected by immunohistochemistry and in situ hybridization. Results: The amount of TRAF3 expression in ICC specimens was statistically lower than that in normal epithelial tissues and adjacent normal tissues ($P < 0.01$); The amount of IKK α expression in ICC specimens was statistically higher than that in normal epithelial tissues and adjacent normal tissues ($P < 0.01$). The degree of TRAF3 and IKK α expression in ICC tissues were correlative and presents negative correlation ($P < 0.05$). Expression of TRAF3 and IKK α was related with pathological TNM stage and lymph node metastasis ($P < 0.01$), while not related with age, sex, tumor size, HBsAg or AFP. The overall survival were significantly longer for patients with TRAF3 higher expression or IKK α lower expression than those with NIAK1 lower expression or IKK α higher expression. Multivariate analysis identified that TRAF3 and IKK α were both independent prognostic factors for survival time. Conclusion: Altered TRAF3 and IKK α expression associated with advanced tumor stage and metastasis and poor prognosis of intrahepatic cholangiocarcinoma.

Keywords: Cholangiocarcinoma, IKK α , TRAF3

Introduction

Intrahepatic cholangiocarcinoma (ICC) is epithelial adenocarcinoma that originates from the secondary bile duct and its branches, and it is the second largest liver cancer rank only second to hepatocellular carcinoma. The highest rate of ICC is occurs in Asian countries especially China. In recent years, the morbidity of ICC is still showing a rising trend [1, 2]. Although patients can achieve more satisfactory results by early surgical treatment [3], but it is difficult to achieve due to the lack of specific inspection [4] and the characteristics of ICC such as symptoms hidden, rapid progression and high malignancy degree. Thus, sensitivity and specificity of molecular markers are important in improving the early diagnosis and prognosis evaluation of ICC patients.

The related studies confirmed that abnormal expression of TRAF3 and IKK α were associated

with the invasion of several malignancies such as breast cancer and prostate cancer [5, 6]. But to our knowledge, there was no relevant report for TRAF3 and IKK α expression and their connection in ICC. Through the research on these two factors, we can further understand their role in ICC and other malignant tumors. Through comparison of survival differences, we may use them to predict the prognosis of patients. TRAF3 is one member of the family of TNF receptor associated factors, which is widely existed in the organization, and it is participated in the physiological function such as innate immunity and acquired immunity. IKK α is a subunit of the IKK complex, which plays an important part in non-canonical pathway of NF- κ B. It is worth mentioning that NF- κ B pathway is closely related to inflammation, and as we know, the stimulation of chronic inflammation is closely related to multiple malignancies. Our study was implemented to detect the expression of TRAF3 and IKK α in ICC, it will analyze

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Table 1. Human mRNA gene sequence of TRAF3 and IKK α

| Name | mRNA gene sequence |
|--------------|---|
| TRAF3 | (1) 5'-CAGATCTATTGTCGGAATGAAAGCAGAGGTTGTGC-3' (2) 5'-TTTAAGCGCTATGGCTGCGTTTTTCAGGGGACAAA-3' (3) 5'-ATCCGGCCCTTCCGGCAGAACTGGGAGGAAGCAGA-3' |
| IKK α | (1) 5'-ATTCGATATTTGCATGAAAACAAAATTATACATCG-3' (2) 5'-GATCACATTTTGAATTTGAAGATAGTACATCCT-3' (3) 5'-GATTGTGTAATTATATTGTACAGGACAGCAAAT-3' |

their correlation with each other and with clinical parameters and prognosis, exploring their role in ICC.

Materials and methods

Subjects and postoperative follow-up

After informed consent, 50 patients were diagnosed as ICC and 16 patients were diagnosed as hepatic hemangioma (as a control group) from January 2005 to May 2012 and had undergone surgical operation in The First Affiliated Hospital of Anhui Medical University (AHMU, China) were included in this study. 50 cases of intrahepatic cholangiocarcinoma tissues and their adjacent tissues along with 16 cases of normal bile duct epithelium tissues were selected. Our study was carried out under the consent of the ethics committee of AHMU. All patients included in this study were not treated with radiotherapy and chemotherapy before surgical operation. Surgical resected specimens were examined pathologically using the TNM staging formulated by International Union against Cancer (UICC). The clinical determination of the critical value of preoperative serum AFP level was 400 $\mu\text{g/L}$ selection. All patients with intrahepatic cholangiocarcinoma had complete clinical data in this study. The overall survival time (OS) of patients was recorded by regular outpatient follow-up and telephone follow-up. The follow-up period of our study ended in August 2015. Patients died from other reasons or lost to follow-up were defined as censored cases.

Immunohistochemistry

The immunohistochemical method was carried out according to the kit instructions. Under room temperature, 3% hydrogen peroxide was rinsed for 8 min to exhaust the activity of endogenous peroxidase. Under 4°C tempera-

ture, rabbit TRAF3 polyclonal antibody and IKK α polyclonal antibody (Abcam Biotechnology, USA) were applied at ideal working concentration of 1:150. Sections were immunohistochemically stained with goat anti-rabbit polymers which marked with horseradish peroxidase after sufficient PBS rinses. At the last, stained TRAF3 protein and IKK α protein were visualized by dripping diaminobenzidine, and then sec-

tions were counterstained with Mayer's hematoxylin. In the negative control group, experiment was conducted using PBS buffer solution instead of the antibody under the same experimental conditions. The slides were then dehydrated and sealed with coverslips following a standard procedure.

In situ hybridization

The oligonucleotide probe of TRAF3 mRNA and IKK α mRNA were designed by Boster Biotechnology (Wuhan, China). The corresponding sequence was shown in **Table 1**. Brief introduction is as follows. Under 37°C temperature, 3% pepsin (diluted by citric acid) was dropping on the sections preparation for 20 min to expose the nucleotide fragment. After fixation with 1% paraformaldehyde for 10 min at room temperature, the slides were rinsed for 3 \times 5 min in PBS. Then sections were applied overnight at 4°C temperature after dropping hybridization solution. Sealing fluid, digoxin and peroxidase was dripped in succession. After abundant PBS rinses, sections were stained. Finally, the slides were dehydrated and sealed with coverslips following a standard procedure.

Evaluation of immunohistochemical staining and in situ hybridization

The outcomes of immunohistochemical staining were evaluated and scored independently by two pathologists with no prior knowledge of the information of the patients. Firstly, the intensity of the staining was scored: no color is 0 point, Light yellow recorded as 1 point, light brown recorded as 2 points, brown recorded as 3 points, dark brown recorded as 4 points. Then the percentage of positive cells was scored: Negative record as 0 point, positive cells rate is less than or equal to 15% recorded as 1 point. 16%-50% recorded as 2 points, 51%-85% recorded as 3 points, more than 85% recorded as 4 points. Finally, after multiplying the two score, the outcome is obtained: 0~2

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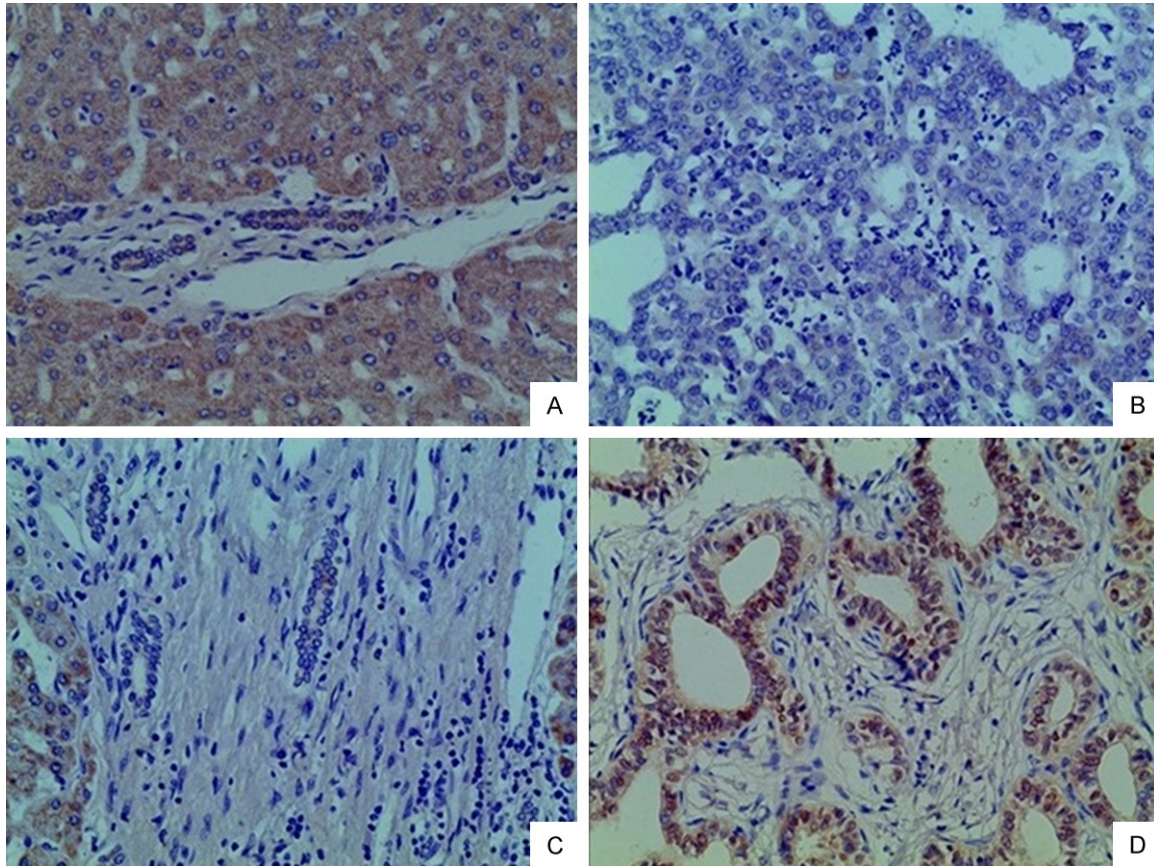


Figure 1. Immunohistochemical staining of TRAF3 and IKK α protein in ICC specimen tissues, adjacent normal tissues or normal epithelial tissues. A. Showed TRAF3 protein staining was strong in adjacent normal tissue and in normal tissue samples; B. Showed negative staining of TRAF3 in ICC tissue; C. Showed negative staining of IKK α protein in adjacent tissue and in normal tissue samples; D. Showed IKK α staining was strong in ICC tissues (PV \times 400).

points were classified as negative, 3~4 points classified as weak positive, 5~8 points classified as positive, 9~16 points classified as strong positive.

The outcomes of in situ hybridization were evaluated and scored independently by two pathologists with no prior knowledge of the information of the patients. Firstly, the intensity of the staining was scored: no color is 0 point, the weak color mark is 1 point, the medium color mark is 2 points, and the strong color mark is 3 points. Then the percentage of positive cells was scored: Positive cells reached 11%-50% was recorded as 2 points, 51%-80% was recorded as 3 points, more than 80% was recorded as 4 points. Finally, after adding the two score, the outcome is divided into 4 grades: no matter how the color intensity, positive cell rate is less than or equal to 10% were classified as negative, 2 or 3 points were classified as weak

positive, 4 or 5 points were classified as positive, 6 or 7 points were classified as strongly positive.

Statistical analysis

Methods SPSS19.0 statistical software package was used for statistical analysis. Chi-square test was employed to estimate the correlation between various clinical data and the degree of these two proteins expression. The correlation of the expression between TRAF3 and IKK α was determined using Spearman's rank correlation coefficient. Kaplan-Meier survival analysis was used to evaluate the differences of prognosis between the different levels of TRAF3 and IKK α expression as well as other clinical parameters. The significant survival curves were inspected by the log-rank test. Independent prognostic indicators were detected by multivariate analysis through establish-

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Table 2. Clinicopathological correlation of TRAF3 and IKK α expression in 50 patients with ICC

| Parameters | No. | TRAF3 expression | | χ^2 | P | IKK α expression | | χ^2 | P |
|------------------------|-----|------------------|----|----------|-------|-------------------------|----|----------|-------|
| | | + | - | | | + | - | | |
| Age (years) | | | | | | | | | |
| ≤50 | 18 | 8 | 10 | 0.231 | 0.630 | 9 | 9 | 1.719 | 0.190 |
| >50 | 32 | 12 | 20 | | | 22 | 10 | | |
| Gender | | | | | | | | | |
| Male | 27 | 13 | 14 | 0.483 | 0.487 | 17 | 10 | 0.023 | 0.879 |
| Female | 23 | 7 | 16 | | | 14 | 9 | | |
| Capsule integrity | | | | | | | | | |
| Yes | 20 | 10 | 10 | 1.389 | 0.239 | 12 | 8 | 0.057 | 0.812 |
| No | 30 | 10 | 20 | | | 19 | 11 | | |
| Tumor size | | | | | | | | | |
| ≤5 cm | 22 | 9 | 13 | 0.014 | 0.907 | 11 | 11 | 2.401 | 0.121 |
| >5 cm | 28 | 11 | 17 | | | 20 | 8 | | |
| Venous cancer embolism | | | | | | | | | |
| Yes | 16 | 5 | 11 | 0.751 | 0.386 | 12 | 4 | 1.688 | 0.194 |
| No | 34 | 15 | 19 | | | 19 | 15 | | |
| Lymph node metastasis | | | | | | | | | |
| Yes | 22 | 4 | 18 | 7.792 | 0.005 | 18 | 4 | 6.549 | 0.010 |
| No | 28 | 16 | 12 | | | 13 | 15 | | |
| TNM stage | | | | | | | | | |
| I~II | 20 | 13 | 7 | 8.681 | 0.003 | 8 | 12 | 6.848 | 0.009 |
| III~IV | 30 | 7 | 23 | | | 23 | 7 | | |
| HBsAg | | | | | | | | | |
| Negative | 31 | 12 | 15 | 0.483 | 0.487 | 19 | 12 | 0.017 | 0.895 |
| Positive | 19 | 8 | 15 | | | 12 | 7 | | |
| AFP | | | | | | | | | |
| <400 | 30 | 10 | 20 | 1.389 | 0.239 | 20 | 10 | 0.693 | 0.405 |
| ≥400 | 20 | 10 | 10 | | | 11 | 9 | | |

ing the cox proportional hazards regression model. $P < 0.05$ was the standard of statistical significance for our experiment.

Results

Patient characteristics

Among the 50 patients diagnosed as ICC (male 27, female 23), the mean age was 55 ± 9.4 years old (range 35-74 years old). Among them, 20 cases had tumor capsule integrity, and 30 cases were incomplete. The maximum diameter of tumors was less than or equal to 5 cm in a total of 22 cases, and the remaining 28 cases with diameter greater than 5 cm. Lymph node metastasis was found in 22 patients (44.0%). According to clinical TNM stage, the pathologi-

cal stages were I (n=4), II (n=16), III (n=7), and IV (n=23).

Among the 50 patients diagnosed as ICC, the median survival period was 23 months (range 2-63 months). Two patients died after surgery, and one patient died from other causes. Thirty-two patients died due to the progression of malignancy.

In order to detect the expression level of TRAF3 and IKK α protein, 50 ICC tissue specimens, 50 adjacent normal tissues and 16 normal bile duct tissues were detected by immunohistochemistry. The TRAF3 and IKK α protein were mainly localized in the cytoplasm, brownish yellow or brown particles (**Figure 1**). Among them, the expression level of TRAF3 protein in normal

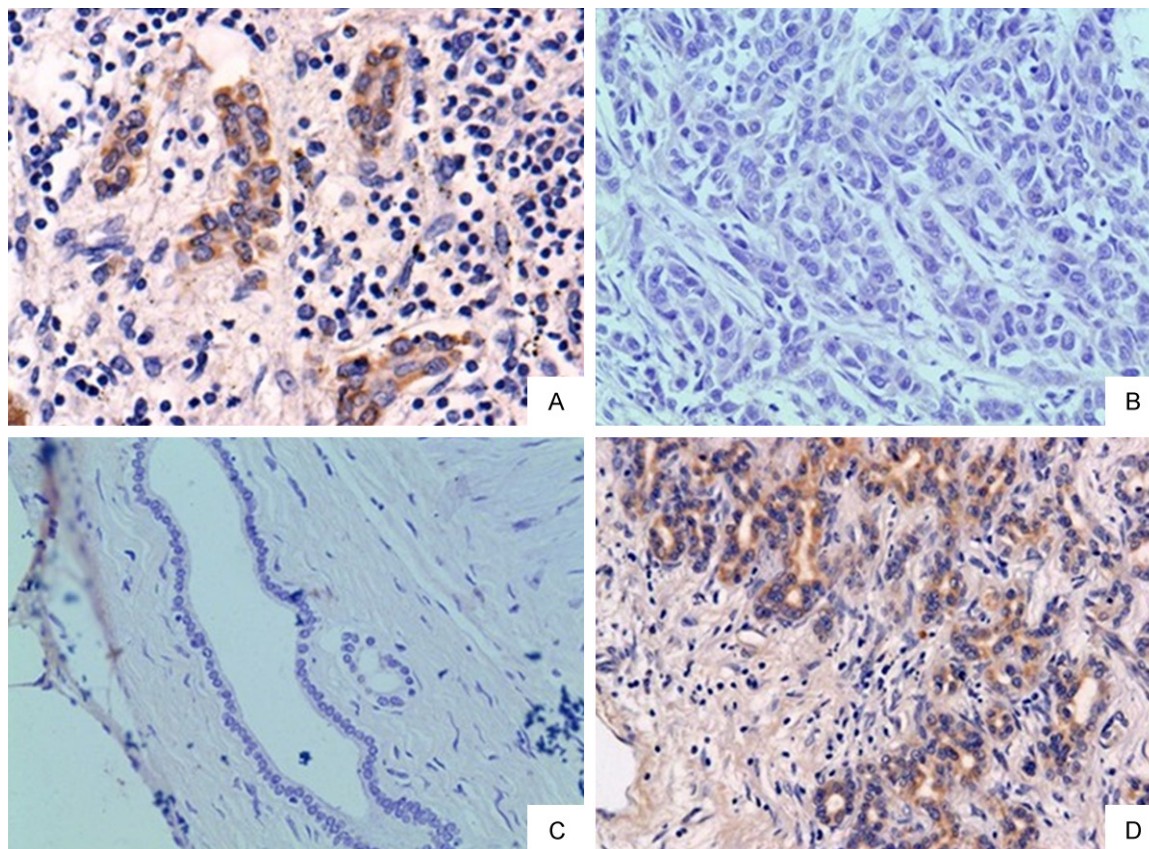


Figure 2. In situ hybridization staining of TRAF3mRNA and IKK α mRNA in ICC tissues, adjacent tissues or normal tissues. A. Showed TRAF3mRNA staining was strong in adjacent tissue or in normal tissue samples; B. Showed negative staining of TRAF3mRNA in ICC tissue; C. Showed negative staining of IKK α mRNA in adjacent tissue or in normal tissue samples; D. Showed IKK α mRNA staining was strong in ICC tissues. ISH data and ICH data used the same morphology of tissue specimens (PV \times 400).

epithelial tissues and adjacent normal tissues was higher than that in tumor specimen tissues. On the contrary, the expression level of IKK α protein in tumor specimen tissues was higher than that in adjacent normal tissues and normal tissues. The differences were both statistically significant ($P < 0.01$).

Association of the TRAF3 and IKK α protein expression with clinicopathological data

Among the 66 patients with immunohistochemical study, 50 patients were diagnosed as ICC and 16 patients were diagnosed as hepatic hemangioma. The correlation of TRAF3 and IKK α protein expression levels in different intrahepatic bile duct tissues and clinical parameters were statistically analyzed. As summarized in **Table 2**, the expression levels of TRAF3 and IKK α protein were significantly associated with clinical TNM stage and lymph node metastasis

($P < 0.01$). However, there is no obvious relationship between the expression of these two proteins and clinicopathological data such as gender, age, capsule integrity, tumor size, HbsAg or AFP ($P > 0.05$).

Expression of TRAF3 mRNA and IKK α mRNA in intrahepatic bile duct tissues

We also carried out in situ hybridization study to verify the results mentioned above. As illustrates in **Figure 2**, different levels of TRAF3-mRNA expression were detected in tumor tissues, adjacent tissues or normal tissues. Expression of TRAF3mRNA in normal epithelial tissues and adjacent normal tissues was statistically higher than that in ICC tissues. Expression levels of IKK α mRNA in intrahepatic bile duct tissues were also different, but contrary to the former, expression level of IKK α mRNA in tumor specimen tissues was statistically higher than

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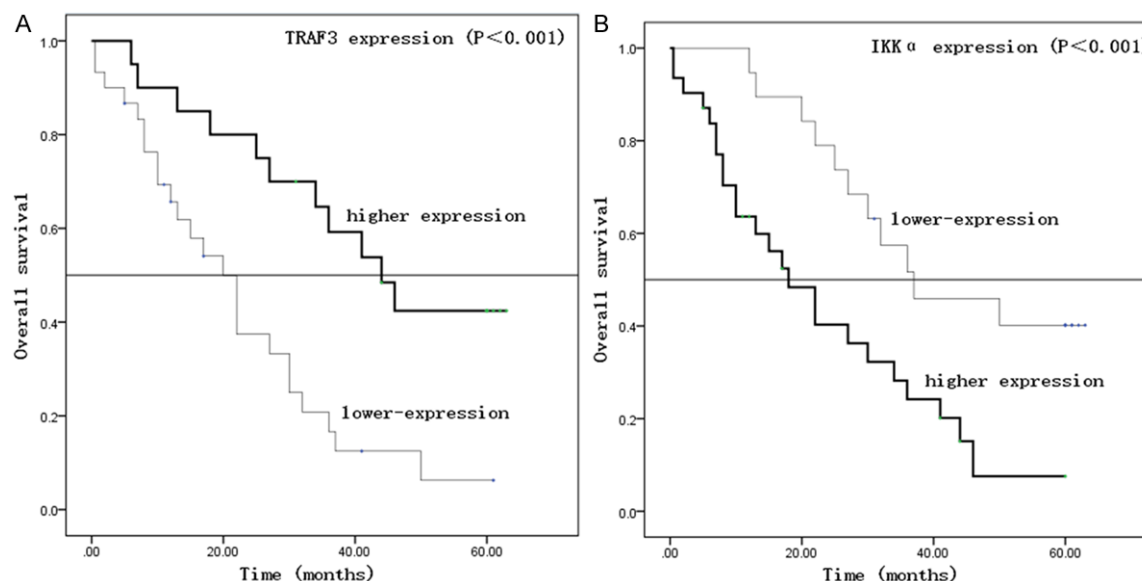


Figure 3. Kaplan-Meier survival analysis of OS in ICC patients, according to the TRAF3 and IKK α expression levels.

Table 3. The overall survival Cox proportional hazards model analysis

| Variable | B | SE | Wald | Relative risk (95% CI) | P value |
|-----------------------|--------|-------|--------|------------------------|---------|
| Univariate | | | | | |
| Age (years) | | | | 1.541 (0.765-3.106) | 0.227 |
| Gender | | | | 0.631 (0.325-1.226) | 0.174 |
| Capsule integrity | | | | 0.571 (0.289-1.128) | 0.107 |
| Tumor size | | | | 1.153 (0.589-2.256) | 0.678 |
| HBsAg | | | | 0.710 (0.351-1.438) | 0.341 |
| AFP | | | | 0.802 (0.406-1.587) | 0.527 |
| TNM stage | | | 4.944 | (2.319-10540) | <0.001 |
| Lymph node metastasis | | | 4.336 | (2.088-9.006) | <0.001 |
| TRAF3 | | | | 0.316 (0.151-0.663) | 0.002 |
| IKK α | | | | 2.706 (1.299-5.635) | 0.008 |
| Multivariate | | | | | |
| TNM stage | 1.274 | 0.399 | 10.198 | 3.575 (1.636-7.812) | 0.001 |
| TRAF3 | -1.285 | 0.421 | 9.332 | 0.277 (0.121-0.631) | 0.002 |
| IKK α | 1.240 | 0.411 | 9.901 | 3.456 (1.543-7.738) | 0.003 |

Survival analysis

Kaplan-Meier survival analysis indicated that ICC patients with higher TRAF3 expression had reduced risk of overall mortality ($P<0.01$; **Figure 3A**). While with higher IKK α expression had a relatively higher mortality rate ($P<0.01$; **Figure 3B**). Further confirmed by Log-Rank test, the expression levels of the two proteins expression were closely related to the overall survival time of patients with HCC (Log-Rank $P=0.001/0.005$).

that in adjacent normal tissues and normal epithelial tissues. The outcomes were consistent with the results obtained by immunohistochemistry.

Spearman's correlation coefficient

To further study the correlation between TRAF3 and IKK α , we use Spearman's rank correlation coefficient to analyze the expression of these two proteins. The results suggest that, TRAF3 protein expression is in negative correlation with the expression of IKK α protein ($\chi^2=4.089$, $R_s=-0.286$, $P<0.05$).

Univariate analysis and multivariate Cox regression analysis

In order to further study the factors affecting the mortality of patients with ICC, we employed univariate analysis of clinical data for prognosis. Univariate analysis showed that significant indicators associated with survival analysis included TNM stage ($P<0.001$), lymph node metastasis ($P<0.001$), TRAF3 low-expression ($P=0.002$) and IKK α overexpression ($P=0.008$). However, gender, age, capsule integrity, tumor size, HbsAg or AFP had no prognosis value on OS of patients with ICC. To further understand

the independent factors for the OS of patients, a multivariate Cox proportional hazards model was adjusted for TNM stage, lymph node metastasis, TRAF3 and IKK α expression. Our results demonstrated that clinical TNM stage, TRAF3 and IKK α expression were independent factors affecting the prognosis of ICC patients. TRAF3 is a protective factor while IKK α is a risk factor affecting the prognosis of patients with ICC (Table 3).

Discussion

Intrahepatic cholangiocarcinoma (ICC) is the second largest malignant tumor of liver cancer, which accounts for the 3% of the digestive tract tumor [7]. In spite of recent years, with the progress of diagnosis technology as well as application of combined radiotherapy and chemotherapy, the prognosis and quality of life of patients with ICC have been improved, but the long-term survival rate is still relatively low and the recurrence rate is still relatively high after surgery. Thus more sensitivity and specificity of molecular markers are helpful to the early diagnosis and evaluation of prognosis of patients with ICC.

TRAF3 is one of the 7 TRAF families (TNF receptor associated factors TRAFs) that have been found [8, 9]. Members of the family are important intracellular signal transduction factors which can regulate a large number of signaling pathways such as NF- κ B signal pathway, and then to mediate a variety of functions such as cell survival, apoptosis, proliferation, inflammation and immunity [10, 11]. Especially, TRAF3 is one of the most diverse members of the TRAFs, not only to participate in the regulation of innate immunity and acquired immunity, but also can positively regulate the production of type I interferon and negatively regulate of protein phosphatase [12, 13]. Related research found that lack of TRAF3 can cause abnormal opening of the non-canonical signaling pathway of NF- κ B, further to clarify that TRAF3 is an important mediated factor in the regulation of inflammatory pathway [14]. Recent research had indicated that TRAF3 has an important influence not only in the inflammatory pathway but also in the genesis and progression of tumor. Different from other TRAF family members, TRAF3 is considered to be one of the factors that can promote tumor cell apoptosis. TRAF3 is expressed in most normal human tissues, but low expres-

sion in breast cancer, lymphoma and other malignancies. We had not seen the relevant reports about the expression of TRAF3 in intrahepatic cholangiocarcinoma before this study. Our study confirmed that TRAF3 also showed low expression in intrahepatic cholangiocarcinoma and closely related to clinical TNM stage and lymph node metastasis. Our results cohered with the expression of TRAF3 in other malignancies, and it also further confirmed that TRAF3 plays a negative regulatory role in the occurrence and development of malignancies.

IKK α is an important part of the IKK complex, which has broad biological functions. One of the most important function of IKK α is the regulation of NF- κ B pathway, especially the non-canonical signaling pathway of NF- κ B. Studies clarify that NIK (NF- κ B inducing kinase) can interact with TRAF3 protein, which triggers the degradation of the former. When TRAF3 is lacking, NIK protein gradually accumulated in the cells and then lead to the phosphorylation of IKK α and eventually lead to the abnormal opening of the non-canonical signaling pathway of NF- κ B [14]. NF- κ B plays a key role in many important physiological functions, such as inflammation, cell survival and apoptosis. The abnormal activation of NF- κ B is closely related with the tumorigenesis [15]. Related research also confirmed that abnormal expression of IKK α is closely related to the invasion of malignancy and the metastasis of lymph node. For example, Affara's research indicates that overexpression of IKK α is associated with the metastasis ability of prostate cancer [16], and other documents also confirmed that the low expression of IKK α can inhibit the invasion and metastasis ability of prostate cancer and breast cancer [5, 6]. This is also consistent with the results we got in intrahepatic cholangiocarcinoma.

Our study demonstrated that the degree of TRAF3 expression in intrahepatic cholangiocarcinoma specimen tissues was statistically lower than that in adjacent normal tissues and normal epithelial tissues, on the contrary, the expression level of IKK α in intrahepatic cholangiocarcinoma specimen tissues was statistically higher than that in adjacent normal tissues and normal epithelial tissues. Through comparison with the clinical pathological parameters, we found that the expression of these two factors were closely related to the clini-

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cal TNM stage and lymph node metastasis ($P < 0.01$). Therefore, TRAF3 and IKK α are likely to become potential molecular markers for the diagnosis of intrahepatic cholangiocarcinoma.

In addition, we found TRAF3 and IKK α were negatively correlated by Spearman's rank correlation analysis. This is also consistent with the mechanism which IKK α is negatively regulated by TRAF3 in non-canonical signaling pathway of NF- κ B. It is worth mentioning that the stimulation of chronic inflammation is closely related to multiple malignancies [17-19], and chronic inflammation in the biliary system is an important incentive for the carcinogenesis of the intrahepatic bile duct epithelial cells [20]. Our previous study has found that NF- κ B as an important factor in mediating chronic inflammation was also expressed abnormally in intrahepatic cholangiocarcinoma, but the specific mechanism has not been reported. Whether the occurrence of intrahepatic cholangiocarcinoma is associated with the mechanism which downregulation of TRAF3 led to activation of IKK α and eventually caused the abnormal opening of the non-canonical signaling pathway of NF- κ B is worthy of further in-depth study.

Currently, the evaluation of the prognosis is still on the basis of clinical data such as pathological staging and imageological examination. However, it is difficult to accurately predict the prognosis of patients with the current clinical parameters. Advances in human molecular biology provide us enlightenment that exploration of molecular abnormalities can help early diagnosis and prediction of prognosis. Our study proved that the degree of TRAF3 and IKK α expression were closely related to the prognosis of ICC patients. According to the postoperative follow-up we found that patients with higher TRAF3 expression or lower IKK α expression had a better prognosis. According to the multivariate Cox regression model analysis, the expression levels of TRAF3 and IKK α are both independent factors affecting the prognosis of the patients, in which TRAF3 is the protective factor, IKK α is the risk factor.

In conclusion, our study confirmed that the abnormal expression of TRAF3 and IKK α were closely related to the aggressive behavior of human ICC, indicating these two factors as potential biomarkers for predicting prognosis and evaluation of treatment in ICC. However, TRAF3 and IKK α were expressed abnormally in

various malignant tumors, they might not be specific biomarkers for a particular tumor. But they would be appropriate markers of various malignant tumors. The next step of our work is to ensure that the potential of TRAF3 and IKK α in the diagnosis and treatment can be applied. Thus, the related experiments in vivo and in vitro conditions have been performed in our laboratory.

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Disclosure of conflict of interest

None.

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