High expression of TRAF4 in hepatocellular carcinoma as an independent negative survival and recurrence predictor

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Objective: To identify potential tumor markers for the development and recurrence of hepatocellular carcinoma (HCC), this research studied the relationship between the expression of the tumor necrosis factor receptor-associated factor 4 (TRAF4) and tumor angiogenesis together with its survival time of HCC patients.

Methods: The expressions of TRAF4, vascular endothelial growth factor and CD34 were performed upon 90 patients with curative liver resection between August 2006 and November 2009 by immunohistochemical method in locally advanced HCC and adjacent non-tumoral liver. The expression of TRAF4 was determined by the Spearman rank correlation. Their prognostic factors on disease free survival (DFS) and overall survival (OS) were guaranteed by Kaplan-Meier and Cox regression analyses. The detection of the levels of vascular endothelial growth factor and CD34 was fulfilled in 90 cases of HCC.

Results: TRAF4 expression was both significantly higher in HCC than in surrounding non-tumor tissues (57.8% vs. 22.2%; P<0.001) and significantly correlated with tumor size and tumor staging. High TRAF4 was correlated with reduced DFS rate (P=0.001) and overall OS rate (P<0.001) and were displayed in Kaplan-Meier survival analysis.

Conclusions: TRAF4 is involved with multifarious clinicopathologic features. TRAF4 expression, as an independent adverse prognostic factor, DFS and OS in HCC, is associated with increased tumor angiogenesis. The combined detection of TRAF4 in locally advanced HCC is a trustworthy predictive factor for the tumor development and recurrence.

1. Introduction

Hepatocellular carcinoma (HCC), the sixth most common cancer and the third most common cause of cancer mortality worldwide[1], is a major problem worldwide, had more than 748 000 new cases and responsible for more than 695 000 deaths in 2008[2]. Appropriate markers for early diagnosis of this tumor are still lacking and liver transplant, interventional radiology treatment, and chemoembolization for unresectable HCC remain the main choices for HCC therapy today, even if the survival probability is limited[3]. Most patients with resection have a high tendency to have recurrent diseases, particularly those with larger tumors or tumors displaying vascular invasion[4]. High recurrence rate and low long-term survival rate are still common in patients with unresectable HCC, so more effective and safer treatments are urgently needed[5]. Many patients with HCC suffer from severe prognosis because of the lack of early specific diagnosis[6]. Alpha fetoprotein is the most commonly used to diagnose liver cancer. Similarly, descarboxyprothrombin, also known as prothrombin induced by vitamin K absence, levels promote in most patients with HCC[7]. The screening of more biomarkers for progression and...
2. Materials and methods

2.1. Tissue samples

There were 90 consecutive cases of locally advanced HCC (Karnofsky ≥ 60%/Child-Pugh ≤ 12) and paired adjacent normal hepatic tissues were collected from patients undergoing curative liver resection during August 2006 to November 2009 at Anhui Provincial Hospital in our single-center retrospective study. Locally advanced HCC were defined as one nodule was larger than 5 cm or more than 3 nodules were larger than 3 cm in diameter. All the patients had neither other malignant tumors nor any anticancer therapies before surgery. The tumor differentiation degree is defined based on the Edmondson classification system. All cases had complete clinical data including disease-free survival time (DFS) and overall survival time (OS). The pathological diagnosis was squamous cell carcinoma and the double blind method was applied. The duration of follow-up was from surgery to death or deadline (September 20th, 2013). All patients detected by TRAF4 signed informed consent. The study was approved by the ethics committee of Provincial Hospital of Anhui Medical University.

2.2. Immunohistochemistry

The detection of the expression levels of TRAF4 protein was achieved by immunohistochemistry staining using Envision kit method. A primary rabbit polyclonal anti-TRAF4 antibody, bought from Abcam Ltd, Shanghai, China, was used at a working concentration of 1:300. In short, 4 microns thick slices were divided from all formalin fixed paraffin embedded specimens. Dewaxing, after rinsing by heating the specimen of incubated antigen repair in pH 6 citrate buffer, was completed with a microwave oven. The sections, washed three times in a buffer, were then incubated with primary antibody above room temperature for 2 h following blocking endogenous peroxidases. Then the coloring of the sections was completed after incubating with secondary antibody (mouse antirabbit IgG, Zhongshan Jinqiao Co., Beijing, China) at room temperature for 30 min. The positive tissue slices and glass slides treated with phosphate buffer saline were positively and negatively controlled.

2.3. Result evaluation

An independent and blind estimation of the stained section was fulfilled by two experienced pathologists. The staining intensity grading were negative, 0; light yellow, 1; brownish yellow, 2; brown, 3. Staining positive tumor cells were classified into positive cells <5%, 0; positive cells 5%-25%, 1; positive cells were 25%-50%, 2; positive cells >50%, 3. The score=grade in stain intensity × grade in percentage of positive cell. Scores more than 4 was regarded as high expressions; On the other hand, low and even no expressive slices were contained in low expression groups.

2.4. MVD assessment

The staining of vascular endothelial cells with CD34 antibody and the calculation of CD34 positive MVD were achieved. Five distinctly prominent vascular (hot) regions were selected, and the number of ships is at a high multiple (400×). An average count was calculated by average measurements. An independent evaluation of all the biopsies was done by two pathologists who did not have the same pathology, and the differences were resolved by consensus.

2.5. Statistical analysis

SPSS software (version 13.0) was used to analyze the data. The expression of TRAF4 was correlated with clinicopathologic characteristics by χ² or Kruskal-Wallis analysis. The detection of TRAF4 expression was performed by Spearman rank correlation test and the comparison of the correlation between survival records and the expression of proteins was conducted by Kaplan-Meier curve and log-rank test. The assessment of Multivariate analysis was realized by using the Cox regression model. All P < 0.05 was viewed as being full of statistical significances.

3. Results

3.1. TRAF4 expression in HCC and its relationship with clinical pathological factors

In this study, TRAF4 expression was mainly found in tumor cytoplasm around HCC cells (Figure 1), only a weak or no TRAF4 expression was shown in healthy hepatic tissues according to immunohistochemistry detection. The positive rate of TRAF4 expression was 57.8% (52/90) in HCC cancer tissues and 22.2% (20/90) in adjacent non-tumoral hepatic tissues showing significant difference between cancer tissues and cancer adjacent hepatic tissue.
tissues \((P<0.001)\) (Table 1). Besides, significant relationship was found between the positive expression of TRAF4 in HCC and tumor size \((P=0.001)\) and tumor stage \((P<0.05)\). However, no significant relationship was found between TRAF4 expression and gender, age, differentiation of grade, venous invasion \((P>0.05\), Table 2).
3.4. Survival analysis of TRAF4

Kaplan Meier analysis showed that DFS time in locally advanced HCC patients with positive expression of TRAF4 was shorter than those with negative expression rate of TRAF4 (median: 23.0 months vs. 49.6 months, \( P<0.001 \); Figure 4A). Similarly, OS time of patients with positive TRAF4 expression was shorter than that with negative TRAF4 expression (29.0 vs. 53.3 months, \( P<0.001 \); Figure 4B).

What’s more, a significant relation between the prognosis and TRAF4 expression (\( P<0.001 \)) according to univariate analysis. It was shown that TRAF4 was an independent prognostic factor for HCC according to multivariate Cox regression analysis (Table 5).

![Figure 4](image_url)

**Figure 4.** Kaplan-Meier analysis of DFS and OS curves of patients with HCC based on TRAF4 expression as positive or negative. A. DFS curve of patients with HCC based on TRAF4 expression. B. OS curve of patients with HCC based on TRAF4 expression.

**Table 4**

Univariate analysis of clinicopathologic features associated with survival time.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>DFS HR 95% CI P</th>
<th>OS HR 95% CI P</th>
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<tr>
<td>TRAF4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>52</td>
<td>2.026 1.095-3.749 0.024</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>38</td>
<td>2.062 1.095-3.749 0.024</td>
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<td>Gender</td>
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<tr>
<td>Male</td>
<td>78</td>
<td>1.813 0.959-3.427 0.293</td>
<td>1.771 0.937-3.345 0.078</td>
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<tr>
<td>Female</td>
<td>12</td>
<td>1.439 0.730-2.836 0.067</td>
<td>1.437 0.726-2.843 0.298</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>≤50</td>
<td>25</td>
<td>1.813 0.959-3.427 0.293</td>
<td>1.771 0.937-3.345 0.078</td>
</tr>
<tr>
<td>&gt;50</td>
<td>65</td>
<td>1.439 0.730-2.836 0.067</td>
<td>1.437 0.726-2.843 0.298</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>36</td>
<td>1.439 0.730-2.836 0.067</td>
<td>1.437 0.726-2.843 0.298</td>
</tr>
<tr>
<td>&gt;5</td>
<td>15</td>
<td>1.813 0.959-3.427 0.293</td>
<td>1.771 0.937-3.345 0.078</td>
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<td>Differentiation grade</td>
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<tr>
<td>well/moderate</td>
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<td>3.406 1.303-9.380 0.038</td>
<td>1.813 0.959-3.427 0.293</td>
</tr>
<tr>
<td>poor</td>
<td>33</td>
<td>3.406 1.303-9.380 0.038</td>
<td>1.813 0.959-3.427 0.293</td>
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<tr>
<td>Venous invasion</td>
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<td></td>
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<tr>
<td>Yes</td>
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<td>1.813 0.959-3.427 0.293</td>
</tr>
<tr>
<td>No</td>
<td>84</td>
<td>3.406 1.303-9.380 0.038</td>
<td>1.813 0.959-3.427 0.293</td>
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<tr>
<td>Tumor stage</td>
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<tr>
<td>I + II</td>
<td>44</td>
<td>1.4110 28.799 0.000</td>
<td>20.798 36.705 0.000</td>
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<tr>
<td>III</td>
<td>46</td>
<td>1.4110 28.799 0.000</td>
<td>20.798 36.705 0.000</td>
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</table>

HR: hazard ratio; CI: confidence interval.

4. Discussion

TRAF4, commonly over-expressed in cancer and initially identified as a gene amplified and over-expressed in breast carcinoma, located at 17q11.2 which was a region of amplification devoid of known oncogenes[14], might be involved in the development and progression of primary breast cancer and metastasis. TRAF4 protein high expression was limited to cancer cells and the subcellular localization was consistently cytoplasmic in a large majority of cases. It is considered that TRAF4 is the only traffic regulator and regulated by tumor suppressor p53, which is analyzed in p53 regulated gene microarrays[15]. TRAF4 may play a role in the p53 mediated apoptotic signaling of cellular stress responses. In response to TGF-β manner, TRAF4 was observed to be recruited to the TGF-β receptor complex[11]. TRAF4 increases NF-κ B activation through the glucocorticoid-induced TNF-R (GITR), a receptor expressed on T cells, B cells and macrophages[16]. Immunohistochemical experiments showed a strong cytoplasmic TRAF4 staining, but mainly localized in the basal epithelial cells[17]. The analyses have suggested that high TRAF4 expression is an indicator of poor outcomes in cancer patients. No extensive studies have yet been carried out to establish the significance of TRAF4 expression in diagnosis or prognosis.

HCC, a most common human malignancy, makes up for 70%-85% of the primary liver tumors and contributes to cancer-related death as a third leading cause worldwide[18,19]. Because HCC are highly vascular tissues and angiogenesis matters a lot in its development, progression, and metastasis, angiogenesis is a key process to tumor progression[18]. Accordingly, the prevention or delay of the development of HCC can be accomplished by the interference with angiogenesis. Tumor angiogenesis is a complex process of multi-factor regulation[20]. Kind of important roles are played by angiogenesis in many cancers, including HCC by VEGF, which, highly expressed in HCC, was the most studied angiogenic factor in vascular endothelial cell carcinoma. Besides, VEGF imposes key effects in stimulating angiogenesis and in enhancing vascular permeability[21]. MVD is a commonly used parameter to evaluate tumor neovascularization formation level number. MVD is usually quantized by immunohistochemical staining of endothelial cell marker. CD34 is a commonly used marker of endothelial cells[22],
and it might stand for MVD in HCC more accurately due to the fact that MVD by CD34 staining is an independent prognostic factor of patient survival after resection of HCC[23].

To examine the involvement of TRAF4 in tumor angiogenesis in HCC, the association between the expression of TRAF4, VEGF, CD34 and the expression of MVD in HCC tissues was studied. The expression of VEGF showed a significantly positive correlation. In addition, HCC cases with high TRAF4 expression had a significantly greater MVD than those with low TRAF4 expression. These results indicate the expression of TRAF4 in the angiogenesis in HCC may be associated with VEGF.

TRAF4 as an independent prognostic factor for advanced HCC is the most important finding of my study. Survival analysis also showed significantly shorter DFS and OS on patients with TRAF4-high expression than those with TRAF4-low expression. That TRAF4 was an independent poor predictor of DFS and OS was confirmed in univariate and multivariate analyses in following surgical resection of HCC patients.

Our present findings provide evidence that the detection of TRAF4 as a positive prognostic marker in the expression of liver cancer is very important. We believe that TRAF4 protein, by immunohistochemical examination, the diagnosis of liver cancer markers can improve the early detection of HCC and the detection rate and prognosis of patients with HCC.

Overall, the occurrence and development of advanced HCC is displayed in our research, which shows that TRAF4 is closely related to the abnormal tumor. Therefore, the prognostic value of combined detection of TRAF4 may provide a potential malignant tumor strategy.

Conflict of interest statement

All authors declare that they have no potential conflicts of interests.

References


