

RESEARCH ARTICLE

TGF- β -activated Kinase-1: A Potential Prognostic Marker for Clear Cell Renal Cell Carcinoma

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Abstract

Background: TGF- β -activated kinase-1 (TAK1) has been found to be over-expressed in a variety of solid malignancies and related to tumor growth. The aim of this study was to evaluate the expression level of TAK1 in clear cell renal cell carcinoma (ccRCC) and assess its value as a novel prognostic marker. **Methods:** TAK1 mRNA was assessed in 51 paired ccRCC tissues and adjacent normal tissues (ADTs) by real-time PCR. Tissue TAK1 protein was also assessed in 91 ADTs and 177 samples of ccRCC immunohistochemically for evaluation of relationships with clinical characteristics. **Results:** RT-PCR showed that TAK1 RNA level was significantly higher in ccRCC tissues than in the paired ADTs and immunohistochemistry confirmed higher expression of TAK1 protein in ccRCC samples compared with ADTs. TAK1 protein expression in 177 ccRCC samples was significantly correlated with T stage, N classification, metastasis, recurrence and Fuhrman grade, but not age and gender. Patients with low TAK1 levels had a better survival outcome. TAK1 expression and N stage were independent prognosis factors for the overall survival of ccRCC patients. **Conclusions:** Overexpression of TAK1 predicts a poor prognosis in patients with ccRCC, so that TAK1 may serve as a novel prognostic marker.

Keywords: Clear cell renal cell carcinoma - TAK1 - tumorigenesis - prognosis

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Introduction

Clear cell renal cell carcinoma (ccRCC), the most common form of kidney cancer, initially has an asymptomatic clinical course, 25-30% of patients present with metastatic disease at time of diagnosis. Worldwide incidence and mortality rates are rising at a rate of approximately 2-3% per decade (Jemal et al., 2011). Surgery and chemotherapy have limited or no effect, leaving ccRCC patients underserved in the realm of cancer treatment (Lam et al., 2005). About 70% of patients were present with localized or locally advanced renal cell carcinoma (LARCC), unfortunately, 20-40% of these patients would experience recurrence and metastasis (Lam et al., 2005), suggesting that surgical excision is insufficient. Once metastasis occurred, the patients with metastatic ccRCC would face a poor prognosis with a median survival of 6-10 months and a 10-20% 2-year survival rate (Janzen et al., 2003). In recent years, some prognostic markers for ccRCC, such as RALYL (Cui et al., 2012), CXCR4 (Li et al., 2011), Cdc25B (Yu et al., 2012), have appeared, but large-scale clinical application is impossible. Despite the continual progress in medical technology, the clinical characteristic of ccRCC is still difficult to predict (Wei et al., 2012). Therefore, novel diagnostic and prognostic markers of ccRCC would be very valuable in high-risk people and in those with existing

disease.

TGF- β -activated kinase-1 (TAK1) is a member of mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK), and is an indispensable signaling intermediate in several MAPK and NF- κ B pathways (Ninomiya et al., 1999; Takaesu et al., 2001; Shim et al., 2005). TAK1 activity has been related to a greater aggressiveness in several types of cancer (Kondo et al., 1998; Edlund et al., 2003; Kaur et al., 2005), but the underlying mechanisms is not clear until now. Moreover, the relationship between TAK1 expression and prognosis status is still ambiguous. In this study, we examined the expression and clinical significance of TAK1 in ccRCC and explored the association between TAK1 expression level and prognosis status.

Materials and Methods

Patients and tissue specimens collection

51 fresh ccRCC and matched normal tissue, surgically resected between 2011 and 2012 at the Department of Urology, Peking University Shenzhen Hospital, were used for real-time polymerase chain reaction (RT-PCR). All of these specimens were immediately immersed in RNAlater (Qiagen, Germany) after resection and subsequently stored at -80°C. Additionally, 177 paraffin-embedded RCC samples and 91 adjacent normal tissues

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from patients operated in our department between 2002 and 2012 were used for immunohistochemical assay to detect the expression level of TAK1 in ccRCC and normal tissue. All of these tumors were diagnosed and classified or reclassified according to the American Joint Committee on Cancer (AJCC) staging system. Clinical and pathological characteristics of these 177 patients are listed in Table 1. The study was approved by the institutional review board and ethical committee of Peking University Shenzhen hospital and all patients provided written informed consent.

Real-time polymerase chain reaction (RT-PCR) assay for mRNA of TAK1

After extracted from the fresh tissue with Trizol solution (Invitrogen; USA) and purified using the RNAeasy Maxi Kit (Qiagen, Germany) according to the manufacturer's protocol, 1 µg mRNA of each sample was used for reverse transcription with Omniscript RT kit (Qiagen, Germany). The reactions of qRT-PCR were performed and analyzed with the ABI PRISM 7000 Fluorescent Quantitative PCR System. Reaction mixture was set up in a total volume of 20 µl, consisting of 1 µl of cDNA template synthesized previously, 10 µl SYBR Green master mix (Invitrogen; USA), 1 µl of each primer (sense and antisense primer) and RNase-free water. Primers against TAK1 (p1: GGGGCCACCGTAAAACCGCT; p2: GCCTTGTCGTTTCTGCTGTTGGC), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (p1: AACGGGAAGCCCATCACCATCTT; p2: CAGCCTTGGCAGCACCAGTGG). Cycling parameters were set as 95°C for 2 min, followed by 40 cycles of 95°C (15sec), 55°C (30 sec), and 72°C (40 sec). Relative expression of TAK1 was normalized and the data was analyzed with the comparative threshold cycle ($2^{-\Delta\Delta CT}$, $\Delta\Delta CT = CTTAK1 - CTGAPDH$) method (Livak et al., 2001).

Immunohistochemical assay for TAK1

The immunohistochemical assay for TAK1 was performed according to standard procedures. Paraffin-embedded samples were cut into 5 µm sections and baked at 65°C for 1h, then deparaffinized in xylene and rehydrated in descending ethanol series, followed by antigen retrieval. This step was done by heating the sections in a microwave oven for 30 min in 0.01M citrate buffer (pH 6.0) antigen retrieval buffer. Then the slides were immersed in 3% hydrogen peroxide solution for 20 minutes, washed in phosphate buffered saline (PBS) triple for 5 minutes, treated in 10% bovine serum albumin for 30min in 37°C to block non-specific protein binding. For the immunostaining of TAK1, the specimens were treated with rabbit monoclonal antibody anti-TAK1 (Epitomics, California, USA) at 1:200 dilution and overnight at 4°C. Rinsed with PBS for 3 times, the samples were incubated with anti-Rabbit IHC Kit (Maixin Bio; Fujian, China) at 37°C for 30 min. Finally, the slides were stained with 3'3'-diaminobenzidine tetrahydrochloride (DAB) for 4 min, counterstained with hematoxylin, dehydrated, and mounted. Negative controls were performed with omission of the primary antibodies.

Staining evaluation of each sample was carried out by two independent observers blinded to clinicopathologic variables. Intensity of staining was graded: 0, no staining; 1, weakly stained; 2, moderately stained; 3, highly stained. Percentage of cells showing positive staining was graded: 1, 0-5%; 2, 6-25%; 3, 26-50%; 4, 51-75% and 5, 75%. All of these paraffin-embedded sections were given final scores based on the multiplication of staining intensity and percent of positive cells. In case of any discrepancy, specimens were reviewed by the two observers together and a score was agreed upon. The optimal cut-off value was calculated with log-rank test on the basis of a measure of heterogeneity in overall survival rates and final score of 4 was considered as high expression of TAK1 and 4 as low expression.

Statistical analysis

To analyze significance of differences in mRNA and protein expression of TAK1 between tumors and adjacent normal tissues, paired -sample t test was used. The relationships between expression of TAK1 and clinicopathologic variables were calculated using χ^2 test. Kaplan-Meier method and log-rank test were used to plot survival curves and test the statistical significance between stratified survival groups. To assess which covariates affect overall survival, we used univariate and multivariate Cox proportional hazards models. In all tests, $p < 0.05$ was considered statistically significant and all statistical analyses were performed with the SPSS 17.0 software package.

Results

RT-PCR analysis of TAK1 mRNA in 51 ccRCC tumor samples

Real-time PCR was performed to measure the expression of TAK1 mRNA in 51 ccRCC tumor tissues and ADTs. Compared with normal tissues, 45 ccRCC tumor tissues were significantly higher expression at mRNA levels ($p < 0.001$, paired-sample t test, Figure 1).

Immunohistochemistry analysis of TAK1 expression in 91 ccRCC samples and the paired ADTs

Immunohistochemistry method was applied to assess expression and subcellular localization of TAK1 protein

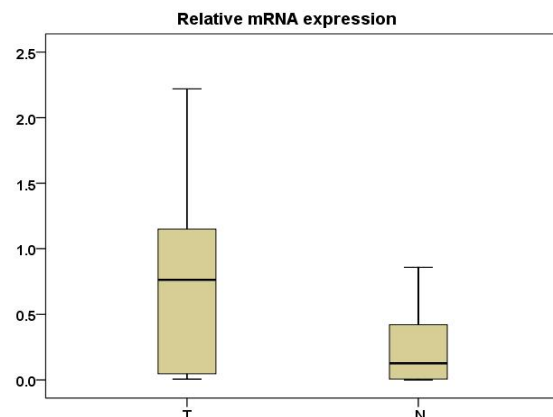


Figure 1. Real-time Quantitative RT-PCR Analysis of TAK1 Expression

Table 1. Association of TAK1 with Clinic-pathologic Characteristics in ccRCC Patients

| Parameters | n | TAK1 | | χ^2 | p |
|------------|-----|------|-----|----------|-------|
| | | high | low | | |
| Total case | 177 | 102 | 75 | 0.262 | 0.608 |
| Male | 96 | 57 | 39 | | |
| Female | 81 | 45 | 36 | | |
| Age(years) | | | | | |
| ≥ 50 | 119 | 74 | 45 | 3.089 | 0.079 |
| < 50 | 58 | 28 | 30 | | |
| T stage | | | | | |
| T 1 | 101 | 51 | 50 | 7.179 | 0.028 |
| T 2 | 55 | 34 | 21 | | |
| T 3/4 | 21 | 17 | 4 | | |
| N stage | | | | | |
| N0 | 146 | 79 | 67 | 4.224 | 0.04 |
| N+ | 31 | 23 | 8 | | |
| Metastasis | | | | | |
| No (M0) | 154 | 84 | 70 | 4.609 | 0.032 |
| Yes (M1) | 23 | 18 | 5 | | |
| Recurrence | | | | | |
| No | 131 | 68 | 63 | 6.751 | 0.009 |
| Yes | 46 | 34 | 12 | | |
| Fuhrman | | | | | |
| F 1/2 | 48 | 34 | 14 | 4.704 | 0.03 |
| F 3/4 | 129 | 68 | 61 | | |

Table 2. The Multivariate Cox Regression Analysis for the Overall Survival Rates of ccRCC Patients

| Risk factors | Relative Risk | 95% Confidence Interval | P value |
|-----------------|---------------|-------------------------|---------|
| T stage | 2.301 | 0.439-2.002 | 0.129 |
| N stage | 2.176 | 0.952-3.012 | 0.059 |
| M stage | 6.008 | 1.849-10.301 | 0.009 |
| Age | 0.731 | 0.624-1.589 | 0.247 |
| Gender | 0.565 | 0.750-1.341 | 0.411 |
| Fuhrman Grade | 8.499 | 2.802-9.573 | 0.092 |
| TAK1 expression | 0.722 | 0.259-0.816 | 0.031 |

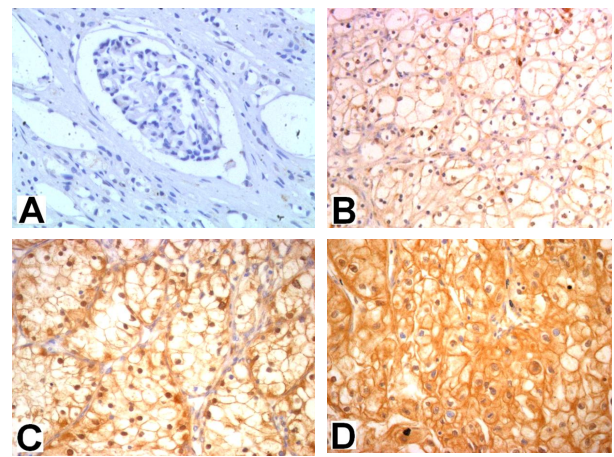
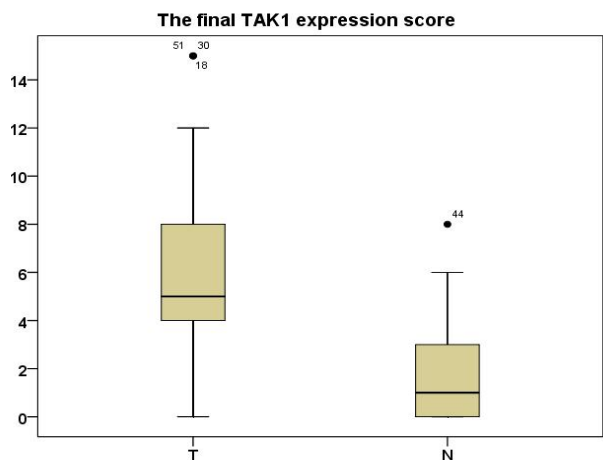
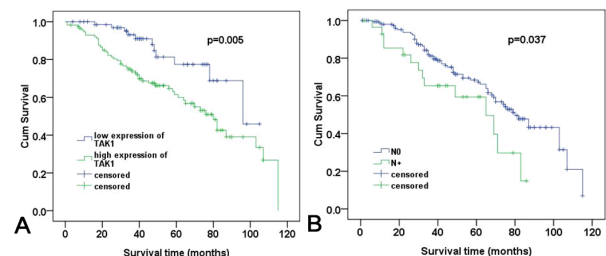
in 91 paraffin-embedded ccRCC tissues and paired ADTs. As shown in Figure 2, TAK1 staining was present mainly in the nuclei and cytoplasm (Figure 2). In normal renal tissue, TAK1 expression was negative (53/91, score =0) or at a low level (32/87, score <4). The TAK1 expression in 86 tumor tissue samples was higher than the ADTs ($p < 0.001$, paired-sample t test Figure 3).

Immunohistochemistry analysis of the relationships between TAK1 protein expression and clinical features in 177 ccRCC tumor samples

Immunohistochemistry analysis was performed in 177 paraffin-embedded ccRCC tumor samples to further assess the correlation between TAK1 expression and various clinic-pathological parameters. As shown in Table 1, low expression of TAK1 (score ≤ 4) was showed in 75 of the 177 tumor samples, high expression (score ≥ 5) was showed in another 102 samples. Increased expression of TAK1 in tumor samples was correlated with T stage, N classification, metastasis, recurrence and Fuhrman grade, while associations with age and gender were not found.

Survival analysis

To further investigate the prognostic value of TAK1

**Figure 2. Immunohistochemical Analysis of the Expression of TAK1 Protein****Figure 3. Increased Protein Expression of TAK1 in ccRCC****Figure 4. Survival Analysis**

expression in ccRCC, Kaplan-Meier analysis and the log-rank test were applied to assess the relationships between TAK1 expression level in ccRCC and prognosis status. We found that the level of TAK1 expression was correlated with the overall survival of ccRCC patients. People with higher level of TAK1 expression had poorer survival rates than those with lower level. The group of high expression TAK1 patients' means survival time was 69.910 months and the medians survival time was 79 months, but the low expression group's means and medians survival time was 86.547 months and 96 months. The log-rank test showed the survival rates were significantly different between these two groups (log-rank, $p=0.005$, Figure 4A). Furthermore, patients without regional lymph node metastasis (N0) had a high cumulative survival rates when compared with patients with regional lymph node invasion (N+) (log-rank, $p=0.037$, Figure 4B).

The multivariate Cox regression analysis indicated that TAK1 expression ($p=0.031$) and M stage ($p=0.009$) were independent prognosis factors for the overall survival of ccRCC patients (Table 2).

Discussion

The annual mortality of ccRCC is significantly higher than other tumors in the genitourinary tract (Schrade et al., 2008) and the clinical outcome remains frustrating despite of advances in clinical technologies (Lane et al., 2007). It is extremely significant to seek specific molecular biomarkers of ccRCC for early diagnosis and evaluation of prognosis.

Recently, more and more molecular markers emerged with their potential values in predicting patients' prognoses and molecular-targeted therapy. For instance, Cui et al. (2012) found that RALY RNA binding protein-like (RALYL) was independent prognosis factors for the overall survival of ccRCC patients, people with lower level of RALYL expression had a poorer survival rate than those with a higher level of RALYL. Li et al. (2011) found that high expression of CXC receptor 4 (CXCR4) was associated with not only increased risk for disease progression, but also worse OS of high-risk LARCC patients. Yu et al. (2012) proved that knockdown of *cdc25B* in ccRCC is associated with decreased malignant features and *cdc25B* was an attractive prognostic marker for this tumor. Some other biomarkers were also found to be promising molecular predictor of tumor recurrence, such as RNA-binding protein IMP3 (Jiang et al., 2006), carbonic anhydrase 9 (Tostain et al., 2010), and P53 (Shvarts et al., 2005). However, few utility of these molecular markers for ccRCC exist till now, probably because of lack of knowledge at the molecular level regarding the biology of renal cell carcinogenesis and progression (Wood et al., 2006; Nogueira et al., 2008).

TAK1, a mitogen-activated protein kinase kinase kinase (MAP3K7), was identified as a kinase that mediates TGF- β signaling to MAP kinases p38 and JNK (Yamaguchi et al., 1995). TAK1 participates not only in TGF- β signaling but also in many other molecular pathways (Delaney et al., 2006). It responds to a variety of upstream signals, including inflammatory molecules and developmental cues, therefore acting as a common effector in regulating cellular responses to stress signals coming from different molecular sensors.

TAK1 is also involved in BMP signaling (Shibuya et al., 1998) as well as in inflammatory and innate immune responses (Sato et al., 2005). TAK1 is activated by association with TAK1-binding proteins TAB1, TAB2, TAB3 and BIRC4/XIAP (Singhirunnusorn et al., 2005). In addition to activating MAP kinases (ERK, JNK, and p38), TAK1 is an important activator of NF- κ B transcription factor (Karin et al., 2005). TAK1 was proved to be a critical regulator of matrix-metalloproteinase-9 (MMP-9) expression (Safina et al., 2008). MMP-9/gelatinase-B contributes to the invasive and metastatic potential of cancer cells (Farina et al., 1998; Suarez et al., 2004; Safina et al., 2007), as well as to tumor angiogenesis, by promoting recruitment of endothelial cells (Bergers et al.,

2000) and pericytes (Chantrain et al., 2004).

In mouse models, TAK1 is found to be essential for immune cell differentiation and activation by mediating activation of MAPK and NF- κ B (Pasparakis et al., 2005; Sato et al., 2005; Liu et al., 2006; Wan et al., 2006). In epithelial cells, TAK1 is critically involved in cell survival, and ablation of TAK1 up-regulates TNF-induced epithelial cell death (Omori et al., 2006; Kajino et al., 2008; Omori et al., 2008). Ablation of TAK1 signaling diminishes activation of MAPK and NF- κ B pathways, and decreases antioxidant capacity (Omori et al., 2008).

Et al demonstrate that the TAK1-TAB2/TAB3 signaling axis is critical for carcinoma-induced bone lesions, mediating expression of pro-invasive and osteolytic factors. These findings identify the TAK1-TAB2 axis as a potential therapeutic target in bone metastasis (Safina et al., 2011). TAK1 might be responsible for the resistance of pancreatic cancer to the proapoptotic effect of chemotherapeutic agents by increasing the NF- κ B and activator protein-1 (AP-1)-mediated transcription of cIAP-2. Targeting the expression or the kinase activity of TAK1 can reverse the intrinsic resistance of pancreatic cancer to chemotherapy (Melis et al., 2011). TAK1 inhibitors may provide significant clinical benefit for the most recalcitrant form of colon cancer. Beyond tool compounds such as 5Z-7-oxozeaenol, synthetic TAK1 inhibitors have been tested in preclinical models (Melis et al., 2011).

Our study indicated the clinical significance of TAK1 in ccRCC. Real-time PCR in 51 ccRCC tumor tissues and the paired ADT samples showed a significant increase of TAK1 mRNA in ccRCC samples. Further immunohistochemistry analysis in 91 paired samples of ccRCCs and ADTs confirmed overexpression of TAK1 protein in tumor tissue. These results indicate TAK1 may play important roles in the initiation and progression of malignancies.

To further investigate the prognostic value of TAK1, immunohistochemical analysis was performed to evaluate the correlation between TAK1 expression and various clinic-pathological parameters. In this study, we showed that the increased TAK1 expression was significantly correlated with Fuhrman grade, stage, N classification, metastasis and recurrence. According to Kaplan-Meier analysis, TAK1 protein expression in ccRCC was significantly correlated with overall survival. Patients with high TAK1 expression level had a shorter survival time than those with a low level. The log-rank test revealed that the group with lower expression of TAK1 had a favorable prognosis than higher expression group. The TNM stage of ccRCC was closely related to prognosis (Levi et al., 2008). Consistent with this, in this study, TAK1 expression and N classification were independent prognosis factors for the overall survival of ccRCC patients by multivariate Cox regression analysis. Therefore, this study reveal that there were significant correlations between the TAK1 expression level and clinic-pathological parameters and may be a potential prognostic marker and therapeutic target for ccRCC.

Fuhrman's nuclear grading system is considered to be a reliable prognostic indicator for ccRCC (Fuhrman et al., 1982). But the multivariate Cox regression analysis in our

study did not show any relations between Fuhrman grade and prognosis, this may due to our limited sample volume and the observation error pathologists made.

As far as we know, this is the first study to evaluate the value of TAK1 as a clinically potential indicator for ccRCC progression, as well as a prognostic marker for patient survival. However, it should admitted that our study was a single hospital-based, retrospective study, and multi-centres or community-based prospective studies are required.

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