Original Article
Increased expression of tumor necrosis factor receptor-associated factor 6 in pancreatic cancer and its clinical significance

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Abstract: Objective: Tumor necrosis factor receptor-associated factor 6 (TRAF6) has been proved to play an important role in various malignancies. However, the role of TRAF6 in pancreatic adenocarcinoma (ADC) has not been clearly defined. The objective of this study was to assess the aberrant expression of TRAF6 and its clinicopathological features in pancreatic ADC. Methods: Immunohistochemistry was used to detect the expression of TRAF6 in 153 pancreatic ADC tissues. We also analyzed the association of TRAF6 expression with clinical pathological characteristics and the survival of pancreatic ADC patients. Furthermore, genomic alterations of TRAF6 were analyzed from The Cancer Genome Atlas (TCGA) via cBioPortal. Results: The expression level of TRAF6 was higher in pancreatic ADC tissues (54.2%) than that in normal pancreas (11.1%, P=0.001) and para-tumor pancreatic tissues (16.7%, P<0.001). The area under curve (AUC) of TRAF6 was 0.694 to diagnose pancreatic ADC. Positive correlations were also noted between TRAF6 expression and N stage (r=0.285, P<0.001), M stage (r=0.19, P=0.019) and TNM stage (r=0.334, P<0.001). Furthermore, cBioPortal provided OncoPrints of TRAF6 in pancreatic ADC (TCGA, provisional) and 8% of 185 cases showed different genetic alteration including amplification, mRNA upregulation, mRNA down-regulation, and missense mutation. Conclusion: TRAF6 is up-regulated in pancreatic ADC tissues and could serve as a tumor promoter in the initiation and progression of pancreatic ADC.

Keywords: TNF receptor-associated factor 6, pancreatic adenocarcinoma, immunohistochemistry, TNM stage

Introduction
Pancreatic adenocarcinoma (ADC) is the main histologic type of pancreatic cancer, which has the following characteristics: the treatment is difficult and the mortality rate is high. Most pancreatic ADC patients are diagnosed at an advanced stage with serious clinical symptoms, and distant metastases will then lead to poor prognosis. Thus, early detection and diagnosis of pancreatic ADC remains a clinical challenge [1-7]. Nowadays, though the systemic recurrence rate of pancreatic ADC is higher, the only potentially curative treatment of pancreatic ADC is still surgical resection, which can cover only small portion of the pancreatic ADC patients. Presently, people have been working on the molecule associated with pancreatic ADC and attempted to discover new target molecules for clinical diagnosis and treatment for pancreatic ADC [4, 5, 8-13]. However, the mechanism underlying pancreatic ADC initiation and progression has not been well elucidated.

Tumor necrosis factor receptor-associated factors (TRAFs) are an adapter protein in the cell, which act as a bridging protein to link cell surface receptors and the downstream signaling cascades, and eventually the process will lead to the release of proinflammatory cytokines
As the especial family member in the TRAFs, TRAF6 can not only activate signaling pathways but are also extensively involved in the inflammatory response and immune response. In the other hand, TRAF6 can be a signal transducer in the pathway of NF-κB by activating IKK in response to proinflammatory cytokines [15]. More importantly, studies have recently demonstrated that TRAF6 may have a close relationship with some neoplasms [16-25].

However, only one study has reported the clinical value of TRAF6 in pancreatic cancer. Rong Y, et al [18] revealed that the expression level of TRAF6 mRNA and protein was both increased in pancreatic cancer samples than that in the paired normal pancreatic tissues. However, the sample size was small (n=53) and the relationship between TRAF6 and disease progression has not been investigated. Thus, in the present study, both immunohistochemistry (n=237) and bioinformatics based on The Cancer Genome Atlas (TCGA, n=185) were performed to explore the clinicopathological implication of TRAF6 in pancreatic ADC.

### Materials and methods

#### Study design

The study included a total of 237 specimens of pancreatic tissues. All of pancreatic specimens were randomly selected from the Department of Pathology of the First Affiliated Hospital of Guangxi Medical University, Department of Pathology of the First Affiliated Hospital of Guangxi University of Science and Technology, or purchased from Fanpu Biotech, Inc (PAC481 and PAC961, Guilin, China). In this study, all of patients did not receive any radiotherapy or chemotherapy before surgery. The patients all agreed that these specimens could be applied for research work. All the pancreatic specimens in the current study were fixed by 10% formalin and embedded with paraffin according to the standard procedure in pathology department. Specimens of pancreatic tissues were clinically classified into ADC (n=153), para-tumor pancreatic tissues (n=66) and non-tumor normal pancreatic tissues (n=18, Table 1).

#### Immunohistochemical staining to detect TRAF6

All of specimens were performed according to the following process: treated with high pressure hotfix, retied by hematoxylin, differentiated by hydrochloric acid-alcohol, dehydrated by anhydrous ethanol, and sealed by neutral gum. Next primary and second antibodies were incubated and lastly 3.3’-Diaminobenzidine spectrophotometry (DAB) chromogenic was used as the color reagent. Immunostaining was performed with TRAF6 mouse monoclonal antibody from Santa Cruz Biotechnology Inc. (D-10, sc-8409, dilution: 1:300, Heidelberg, Germany). All steps were strictly carried out with the manufacturer’s protocols as previously reported [20].

#### Evaluation of immunostaining

The immunohistochemical staining of pancreatic specimens were evaluated by three authors (Lin Shi, Dan-ming Wei and Gang Chen) without the information about patient’s clinical manifestations. The average percentage of positive cells were used as criteria to score the TRAF6 expression semi quantitatively: no staining was marked as “0”, <30% as “1”, 30-70% as “2”, and >70% as “3”. The staining intensity was also recorded as following: no color was recorded as “0”; weakly stained with yellow as “1”, moderately stained with yellow-brown as “2” and strongly stained with dark brown as “3”). According to the sum of the scores: 0~2 were divided into negative group(-), 3~6 were divided into positive group(+) [20].

#### OncoPrints of TRAF6 in pancreatic ADC based on TCGA data via cBioPortal

OncoPrints was produced to present the genomic alterations directly from cBioPortal in pancreatic ADC (TCGA, provisional) containing 185 patients [26]. (www.cbioportal.org).
Statistical analysis

All of the data was analyzed statistically by SPSS22.0 software. Mann-Whitney U tests were used to compare the difference of TRAF6 expression with two categories for different parameters. Kruskal-Wallis H was selected for the comparison of the difference of TRAF6 expression among more than two categories for some parameters, for instance, four T stages and four TNM stages. Receiver Operating Characteristic Curve (ROC) was drawn with SPSS22.0 as well. Spearman's correlation test was performed to assess the correlation between TRAF6 expression and various clinico-pathological features. When the value of the $P$ with two-tailed was less than 0.05, the tests had statistical significance.

Results

HE and immunohistochemical staining

HE staining of the pancreatic tissues including ADC and non-cancerous pancreatic tissues was shown in Figure 1. Immunohistochemically, TRAF6 positive signaling was observable in the cytoplasm of pancreatic ADC cells and some areas of non-cancerous pancreatic tissues (Figure 2). The positive ratio of TRAF6 protein was 54.2% (83/153) in pancreatic ADC tissues, significantly higher than that in normal pancreas (11.1%, 2/18, $P=0.001$) and para-tumor pancreatic tissues (16.7%, 11/66, $P<0.001$, Table 1; Figure 2). Further, the area under curve (AUC) of TRAF6 was 0.694 (95% confidence interval: 0.626-0.762, $P<0.001$) to diagnose pancreatic ADC. From normal pancreas, para-tumor pancreatic tissue to pancreatic ADC, the TRAF6 expression also rose from 11.1%, 16.7% to 54.2%, showing an increasing trend ($r=0.367$, $P<0.001$).

The relationship between TRAF6 expression and clinical parameters in pancreatic ADC

As opposed to lymphatic metastasis group (46/65, 70.8%), the patients without lymph node metastasis (37/88, 42%) showed a signifi-
cantly lower TRAF6 expression level (P<0.001). With regard to tumor distant metastasis, higher level TRAF6 expression was found in metastasis group (9/10, 90%) compared to that in non-metastasis group (74/143, 51.7%, P=0.019). Furthermore, with TNM stage increased, the positive ratio of TRAF6 was growing from 38%, 53.3%, 68.4% to 90% for each stage (Table 2). Positive correlations were also found between TRAF6 expression and N stage (r=0.285, P<0.001), M stage (r=0.19, P=0.019) and TNM stage (r=0.334, P<0.001). No significant correlation was noted between TRAF6 expression and other characteristics (data not shown).

Survival analysis

The survival data was collected from 49 patients and the mean follow-up time was 15.913±1.754 months (range: 0.5-36.0 months). Thirty-five patients died during the follow-up period. The positive expression rate of TRAF6 among 49 patients followed-up was 59.2% (29/49). Kaplan-Meier was performed to evaluate the relationship between TRAF6 expression and survival of pancreatic ADC patient. However, no statistics significance was found between TRAF6 negative group (15±2.683 months) and TRAF6 positive group (16.221±2.214, P=0.754, Figure 3).

OncoPrints of TRAF6 of pancreatic ADC based on TCGA

OncoPrints of TRAF6 in pancreatic ADC (TCGA, provisional) was assessed by cBioPortal (www.cbioportal.org). Altogether 185 cases in TCGA were available for the OncoPrints. Genetic alteration of TRAF6 in 14 cases (8%) was noted including amplification, mRNA upregulation, mRNA downregulation, and missense mutation (Figure 4).

Discussion

In the current study, we investigated the clinico-pathological role of the TRAF6 expression in pancreatic adenocarcinoma.
TRAF6 in pancreatic adenocarcinoma

Table 2. Relationship between TRAF6 expression and clinical parameters in pancreatic adenocarcinoma patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>n</th>
<th>TRAF6 positive (n, %)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1a</td>
<td>2</td>
<td>1/2 (50%)</td>
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</tr>
<tr>
<td>T1b</td>
<td>43</td>
<td>22/43 (51.2%)</td>
<td></td>
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<tr>
<td>T2</td>
<td>77</td>
<td>42/77 (54.5%)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>31</td>
<td>18/31 (58.1%)</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>88</td>
<td>37/88 (42%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N1</td>
<td>65</td>
<td>46/65 (70.8%)</td>
<td></td>
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<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>143</td>
<td>74/143 (51.7%)</td>
<td>0.019</td>
</tr>
<tr>
<td>M1</td>
<td>10</td>
<td>9/10 (90%)</td>
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<tr>
<td>TNM</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>I</td>
<td>71</td>
<td>27/71 (38%)</td>
<td>0.005*</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>8/15 (53.3%)</td>
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<tr>
<td>IV</td>
<td>10</td>
<td>9/10 (90%)</td>
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</tbody>
</table>

* I vs II: P=0.276, I vs III: P=0.001, I vs IV: P=0.002, II vs III: P=0.278, II vs IV: 0.129, III vs IV: P=0.166.

Figure 3. Correlation of TRAF6 expression with survival of pancreatic adenocarcinomas. Kaplan-Meier test was performed.

pancreatic ADC, and found that TRAF6 might be related to the initiation and progression of pancreatic ADC.

TRAF6 is a potential factor in the development of various cancers as well as in immune and inflammation. The expression of TRAF6 has been reported to be overexpressed in both esophageal squamous cell carcinoma (ESCC) clinical samples and ESCC cell lines [16, 17]. TRAF6 was also overexpressed in gastric cancer [23], colon cancer [19, 25] and primary as well as metastatic melanoma tumors and melanoma cell lines [24]. Previously, we found that the expression of TRAF6 protein in both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) was markedly increased than that in non-tumorous lung tissues. Furthermore, the AUC of ROC for TRAF6 was 0.663, which indicated a moderate diagnostic value for lung cancer [20]. Taken together, TRAF6 may play an oncogenic role in several cancers. However, when concerning the clinical role of TRAF6 in pancreatic ADC, only one paper performed by Rong Y, et al [18] was available. Rong Y, et al [18] detected both of the mRNA and protein level of TRAF6 in 53 cases of pancreatic cancer samples and paired normal pancreatic tissues with real-time PCR and immunohistochemistry assay, respectively. They found that the expression level of TRAF6 was upregulated in pancreatic cancer samples. They further performed western blot analysis on six randomly selected pancreatic cancer tissues and paired normal tissues, as well as several pancreatic cancer cell lines (MIAPaCa2, BXPC3 and Suit2) and achieved concordant overexpression of TRAF6 in pancreatic cancer tissues and cells. In the current study, we confirmed the upregulation of TRAF6 protein level with a larger size of clinical samples with 153 cases of pancreatic ADC and the AUC was 0.694 with a moderate diagnostic effect. We also attempted to analyze the TRAF6 mRNA level based on TCGA data, however, since only four samples were available for the non-cancerous controls, we could not compare the difference of TRAF6 mRNA between 186 pancreatic ADCs and only four normal pancreases, which did not meet the requirements of the statistics. However, cBioPortal provided OncoPrints of TRAF6 in pancreatic ADC (TCGA, provisional) and 8% of 185 cases showed different genetic alteration including amplification, mRNA upregulation, mRNA downregulation, and missense mutation. The association between the genetic alteration of TRAF6 and pancreatic ADC remains to be investigated. Even though, our immunohistochemical verification with previous report of Rong Y, et al [18] strongly indicated that TRAF6 might play an essential part in the tumorigenesis of pancreatic ADC.

Besides the tumorigenic role of TRAF6, several studies have revealed that TRAF6 is closely related to the progression of malignancies,
including lung cancer [20], gastric cancer [23] and colon cancer [19, 25]. To our knowledge, we are the first to explore the relationship between TRAF6 expression and progression of pancreatic ADC. We found significant upregulation of TRAF6 in the more advanced periods of pancreatic ADC, including when patients had lymph node metastasis, distant metastasis and in TNM stage III or IV. Therefore, our finding supports that TRAF6 could also lead to tumour progression of pancreatic ADC. However, the follow-up data showed TRAF6 aberrant expression was not related to the patient survival based on only a small number of patients. Thus, larger sample size and longer following duration are considered necessary to study the influence of TRAF6 on patient survival of pancreatic ADC.

Considering the mechanism of TRAF6 in pancreatic ADC, Rong Y, et al [18] showed that over-expression of TRAF6 in pancreatic cancer-MIAPaca2 and BXPC3 cells could promote cell proliferation and migration. On the contrary, knock-down of TRAF6 could weaken the tumorigenicity of MIAPaca2 and Suit2 cells both in vitro and in vivo. Mechanistically, TRAF6 could regulate the expression of a series of multiple genes (c-Myc, CyclinD1, Snail, active Caspase 3, Bax, E-cadherin and cleaved PARP) involved in cell growth, apoptosis and migration. Chiu HW et al [22] also reported that down-regulation of TRAF6 could cause a large increase in apoptosis and autophagy in human pancreatic cancer cells. Hence, the above reports suggested several vital roles of TRAF6 in the carcinogenesis and progression of pancreatic ADC.

It is well-known that early diagnosis with body fluid can assist to reduce the mortality of pancreatic ADC effectively. Serum TRAF6 expression has been detected in breast cancer (BC) [21]. Median serum TRAF6 expression was 0.90 ng/ml in 13 triple-negative breast cancer (TNBC) patients, significantly higher than 0.63 ng/ml in 35 hormone receptor-(HR-) positive BC patients. Also, TRAF6 expression was obviously increased in the TNBC patients than that in the obese control group. Additionally, median serum TRAF6 expression levels rose remarkably in HR-negative patients than that in HR-positive patients. Thus, the study in BC proved that serum TRAF6 expression increased significantly in TNBC and HR-negative patients with non-metastatic BC, than that in HR- and human epidermal growth factor receptor 2 (HER2)-positive cases or the obese healthy controls, which suggested that increased TRAF6 expression may act as a poor prognostic biomarker in non-metastatic BC. Interestingly, Slotwinski R [27] also tested the TRAF6 level in peripheral blood leukocytes of 55 patients with pancreatic ADC using real-time polymerase chain reaction (RT-PCR) and found that the level of expression of TRAF6 gene was significantly elevated. Over-expression of TRAF6 gene may contribute to chronic inflammation and tumor progression by up-regulation of the innate antibacterial response. However, the clinical role of serum TRAF6 still needs to be confirmed with new designed study with larger sample size in pancreatic ADC.

In conclusion, the current study indicates that the upregulated expression of TRAF6 may play a vital part in the development and progression of pancreatic ADC. Moreover, TRAF6 may become a new biomarker for diagnosis and treatment of pancreatic cancer. Nevertheless, further study should be conducted to address the molecular mechanism of TRAF6 underlying the carcinogenesis of pancreatic ADC, as well as the clinical potential of serum TRAF6 detection.
TRAF6 in pancreatic adenocarcinoma

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Disclosure of conflict of interest

None.

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