RESEARCH COMMUNICATION

Expression of Tumor Necrosis Factor-alpha-induced Protein 8 in Pancreas Tissues and its Correlation with Epithelial Growth Factor Receptor Levels

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Abstract

Tumor necrosis factor (TNF)-alpha-induced protein 8 (TNFAIP8 or TIPE) is a recently identified protein considered to be associated with carcinogenesis. To investigate its expression pattern in pancreatic cancer patients and to analyse its correlation with clinicopathological significance and the expression levels of epithelial growth factor receptor (EGFR), immunohistochemistry was performed to detect the TNFAIP8 and EGFR proteins in pancreatic cancers, pancreatitis tissues, and healthy controls. The results showed stronger staining of TNFAIP8 protein in pancreatic cancer tissues compared with normal pancreas tissue. Furthermore, in 56 patients with pancreatic cancer, the expression levels of TNFAIP8 in patients with low tumor stage was higher than that with high tumor stage, and correlated with tumor staging and lymph node metastasis ($P<0.05$). Furthermore, TNFAIP8 expression positively correlated with EGFR levels ($r=0.671135$, $P<0.05$). These results indicate that TNFAIP8 may play important roles in the progression of pancreatic cancer.

Keywords: TNFAIP8 - pancreatic cancer - immunohistochemistry - EGFR

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Introduction

Pancreatic cancer (PC) is relatively common as it is the fourth leading cause of cancer related mortality. Most patients present with obstructive jaundice, epigastric or back pain, weight loss and anorexia. Analysis of overall survival shows that the prognosis of PC is still quite poor despite the fact that 1-year survival and 5-years survival have increased over the years (Sharma et al., 2011; Vincent et al., 2011). Its precipitating factors are drinking, diabetes mellitus, smoking, chronic pancreatitis and Genetic predisposition.

Treatment always concludes surgery, chemotherapy, TACE (Azizi et al., 2011), radiotherapy and palliative care teams, however its original cause is not clear yet (Hong et al., 2011). Tumor necrosis factor-α-inducible protein 8 (TNFAIP8 or TIPE) family are very recently identified proteins and induced by TNF-α stimulation and by activation of transcription factor NF-κB (Patel et al., 1997; Kumar et al., 2000). TNFAIP8 family consists of TNFAIP8, TIPE1 (TNFAIP8L1), TIPE2 (TNFAIP8L2) and TIPE3 (TNFAIP8L3), which share considerable sequence homology to regulate cell functions (Freundt et al., 2008; Sun et al., 2008). The early studies had showed that TNFAIP8 is an oncogenic factor that may play a role in tumor progression, and exogenous expression of TNFAIP8 enhances cell survival. Overexpression of TNFAIP8 contributes to enhanced DNA synthesis, cell proliferation and inhibition of activities of the apoptotic enzymes caspase 8 and caspase 3 (You et al., 2001; Romanuik et al., 2009). Human breast cancer cells transfected with TNFAIP8 increased proliferation, cell migration, and tumor growth rate (Kumar et al., 2004). Knocking down of TNFAIP8 expression in tumor cells reduces their tumorigenicity, suggesting that it may play a role in oncogenesis (Zhang et al., 2006). However, it is not known whether TNFAIP8 takes a part in the progress of PC, and if so, what role it may play. To address this issue, we compared the expression pattern of TNFAIP8 in pancreatic cancer and other pancreatitis tissues, and analyzed the correlation between the expression levels of TNFAIP8 protein and the tumor staging, lymph node metastasis, and expression of epithelial growth factor receptor (EGFR) protein. We found that TNFAIP8 may play an important role in the progression of pancreatic cancer.

Materials and Methods

Patients and tissues: This study was conducted with the approval of the Fundamental Laboratory of provincial hospital affiliated to Shandong University, China. Pancreas tissues were obtained from 78 patients (Male 42, Female 36), of pancreatic cancer 56 patients (well differentiated 14, moderately differentiated 21, poorly differentiated 23) who underwent complete resection in provincial hospital

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affiliated to Shandong University between 2003 and 2010, and normal pancreatic tissues 12 patients, Chronic pancreatitis tissues 10 patients. Follow up information was obtained from review of the patients medical records. None of the patients had received radiotherapy or chemotherapy before surgical resection, and all of them were treated routine chemotherapy after operation. The mean age of the patients was 53.5 years (rang 35-65 years old). The histological diagnosis and grade of the differentiation were evaluated using hematoxylin-eosin-stained sections according to the World Health Organization Guidelines of Classification. All 56 specimens were revaluated with respect to histological subtype, differentiation and tumor stage. The lymph node metastases were identified in 13 of the 56 pancreatic cancer patients. The TNM staging system of Union for International Cancer Control was used to classify specimens.

Immunohistochemistry: Surgically excised tumor specimens were fixed with 10% neutral formalin, embedded in paraffin, and 4-lm-thick sections were prepared. Normal Pancreas tissues were used as control. Immunostaining was performed using the avidin-biotin-peroxidase complex method (Jin Qiao, Zhong Shan, China). The sections were deparaffinized in turpentine, rehydrated with graded alcohol, and then boiled in 0.01M citrate buffer (pH 6.0) for 2 min with an autoclave. Hydrogen peroxide (0.3%) was applied to block endogenous peroxide activity and the sections were incubated with normal goat serum to reduce nonspecific binding. Tissue sections were incubated with TNFAIP8 rabbit polyclonal antibody (1:200 dilution; Abcam, Cambridge, MA, USA) and EGFR antibody (Zhong Shan, China) at 4 °C for one night, or with the isotype-matched control antibodies (rabbit IgG, Invitrogen, Zymed, USA, 1:200). Biotinylated goat antirabbit serum IgG was used as a secondary antibody. After washing, the sections were incubated with streptavidin–biotin conjugated with horseradish peroxidase, and the peroxidase reaction was developed with 3, 3¢-diaminobenzidine tetra hydrochloride. Counterstaining with hematoxylin was performed and the sections were dehydrated in ethanol

The Evaluation of immunohistochemical findings: The intensity of staining was measured using a computerized image system composed of a Leica CCD camera DM4000b (Leica Microsystems Imaging Solutions, Ltd, Cambridge, UK). The scores wan evaluated by Image ProPlus v6.0 from 4 different areas. Under highpower view, the pictures of four representative welds were captured by the Leica application suite v2.8.1 software (Leica Microsystems Imaging Solutions, Ltd.). The two biomarkers were counted by Image-Pro Plus v6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA). For reading of each antibody staining, we used the same setting for all the slides. Mean Integrated optical density (Mean IOD) in each picture was measured, and positive staining density was formulated as IOD/corresponding area of each picture.

Data analysis: All the analysis were performed with SPSS 13.0 for Windows (SPSS, Chicago, IL, USA). The χ^2-test was used to examine possible correlation between TNFAIP8 expression and clinicalpathologic factors. Spearman rank correlation coefficient determination was used to analyze the correlation among TNFAIP8 and EGFR. P<0.05 was considered to indicate statistical significance.

Results

The average staining intensities of TNFAIP8 is higher in pancreatic cancer than normal pancreatic tissue; however its expression in chronic pancreatitis is even higher than pancreatic cancer (Table 1). It was mostly found in the cytoplasm of acinous cell in pancreas tissues (Figure 1).

Clinical significance of TNFAIP8 protein expression in pancreas tissue

We found that TNFAIP8 express more in pancreas cancer than in normal pancreas tissue, however it express even more in Chronic pancreatitis tissues than in pancreas cancer. Distribution of TNFAIP8 status in pancreatic cancer according to clinicopathological was showed in Table 2. In our study, most of the pancreas cancer are adenocarcinoma, while other types are rare in our

Table 1. Distribution of Protein TNFAIP8 Status in Different Pancreatic Tissues

Characteristics	No. patients	TNFAIP8 MEAN IOD Value	P Value
Normal pancreatic tissues	12	0.01832±0.0099	p<0.05
Chronic pancreatitis tissues	10	0.25153±0.0815	
Pancreatic cancer	56	0.12562±0.0711	

Table 2. Distribution of TNFAIP8 Status in Pancreatic Cancer According to Clinicopathological Characteristics

Characteristics		No. Patients	TNFAIP8 MEAN IOD Value	P Value
Gender	Male	30	0.124518±0.0818	0.8719
	Female	26	0.127935±0.0914	
Differentiation	Well	14	0.0948±0.0719	0.0219
	Moderate	21	0.1404±0.0673	
	Poor	21	0.1742±0.0980	
TNM stage	I+II	44	0.1441±0.0534	0.0002
	III+IV	12	0.2013±0.0472	
Tumor status	T1	38	0.1339±0.0519	0.0045
	T2,T3,T4	18	0.1829±0.0687	
Nodal status	N0	40	0.1264±0.0582	0.0007
	N1	16	0.1962±0.0688	

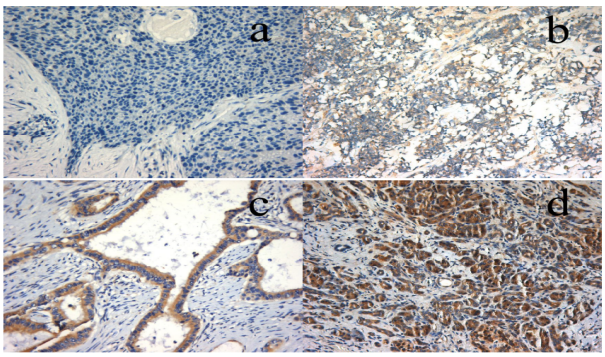

Figure 1. TNFAIP8 (a) negative control; (b) positive control (lung cancer); (c) pancreas cancer; (d) chronic pancreatitis

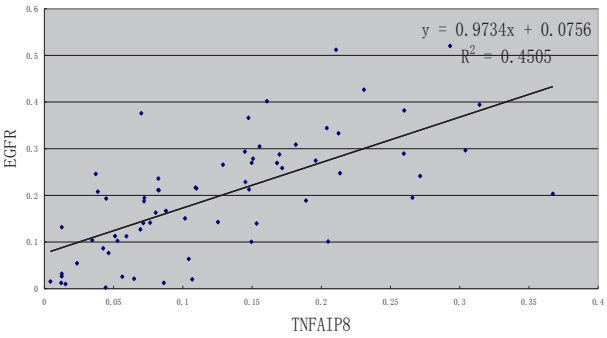

Chart 1. TNFAIP8 Correlation with EGFR

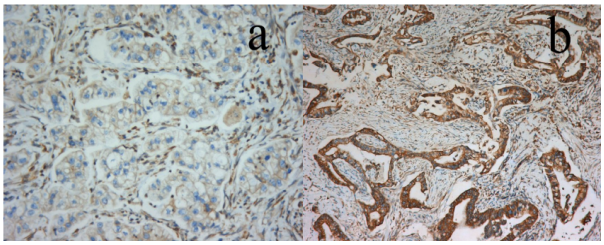

Figure 2. EGFR (a) low expression; (b) high expression

clinical care, so we didn't analyse the type's correlation with protein TNFAIP8. As in Table 2, we can see that the apparent difference were found between staining density of TNFAIP8 and differentiation, TNM stage, nodal status ($p<0.05$), while no difference with gender. In other words, patients with low TNM stage had more expression of TNFAIP8 than the ones with high TNM stage.

TNFAIP8 correlation with EGFR in pancreas

We also measured the IOD value of EGFR in pancreas tissues. With Spearman rank correlation coefficient determination, as in Chart 1,we found TNFAIP8 expression positively correlated to EGFR ($r=0.671135$, $p<0.05$). Always the slides with high TNFAIP8 expression had more EGFR expression (Figure 2).

Discussion

The previous research had demonstrated that Overexpression of TNFAIP8 was detected in a limited number of human breast cancer tissues and its expression enhanced cancer cell proliferation and invasion. Also it was found that TNFAIP8 express more in lung cancer tissues, non-small-cell lung cancer, NSCLC) than in corresponding normal tissues both at protein and mRNA level, and TNFAIP8 correlated with lymph metastasis, p-TNM stage, Ki-67 expression, and poor survival. Further study showed that depleting TNFAIP8 expression by small-interfering RNA inhibited growth and invasion in lung cell lines (Dong et al., 2010). However, the expression of TNFAIP8 as well as its correlation with clinical pathological factors has not been defined in pancreas cancers. In this study, we found that the level of TNFAIP8 in pancreas cancer tissues significantly higher than normal pancreases tissues at protein level and there was a close correlation between overexpression of TNFAIP8 and tumor stage, nodal status. Furthermore, TNFAIP8 overexpression correlated with EGFR in pancreas cancer. It implies that TNFAIP8 may play a important role in the progression of pancreatic cancer. However, we also found the TNFAIP8 express more in chronic pancreatitis than pancreatic cancer.

As we know, TNFAIP8 (gene symbol TNFAIP8), was originally identified by a comparison of the expression profile of a primary HNSCC cell line with its matched metastatic HNSCC-derived cell line from the same patient. Its expression can be induced by tumor necrosis factor-a (TNF-a) and by activation of the transcription factor nuclear factor-kappa B (NF-jB) in various cells. It was found that TNFAIP8 mRNA can be detected in many normal tissue of human and also can be found in kinds of cancer lines. Overexpression of TNFAIP8 is associated with enhanced cell survival and inhibition of activities of the apoptotic enzymes caspase-8 and caspase-3. TNFAIP8 protein expression, examined by western blot analysis, was found to be higher in several human breast carcinomas (10 cases) and renal cell carcinomas (9 cases) compared to matched normal adjacent tissues (Kumar et al., 2004). These data suggest that TNFAIP8 may function as an oncoprotein in human malignancies. To address this issue, we examined its protein levels in 78 pancreas tissues by immunohistochemistry. In the end, we found that TNFAIP8 expression was significantly in pancreatic cancer than normal pancreas tissues, which was consistent with the previous finding of TNFAIP8 overexpression in human malignant tumors. Moreover, TNFAIP8 positivity significantly correlated with lymph node metastasis and TNM stage in pancreatic cancer patients. As to high expression of TNFAIP8 in chronic pancreatitis tissues, we suppose the fact of mass inflammatory cells and other cells proliferate in the tissue may account for it. That is to say, TNFAIP8 protein also could promote the growth of kinds of inflammatory cells in inflammatory tissue and organ.

EGFR (ErbB-1) is one member of the ErbB protein family which consists of four structurally related receptor tyrosine kinases (ErbB-1, ErbB-2, ErbB-3, ErbB-4). It is a receptor for members of the epidermal growth factor family, and a cell surface protein that binds to epidermal growth factor. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation. Mutations in this gene are associated with lung cancer. Insufficient ErbB signaling in humans is associated with the development of neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease. EGFR (ErbB-1) was found in many human cancers and their excessive signaling may be critical factors in the development and malignancy of these tumors. Decreasing the expression level of EGFR protein contributes to EGFR targeted therapy of cancers (Ioannou et al., 2011; Xu et al., 2011). So EGFR is a legitimate therapeutic target, and there is a outline of endocytic escape of EGFRs in cancer with special attention towards advances in various approaches adopted for EGFR targeting (Aggarwal et al., 2011). In our study, we further detected the EGFR expression by immunohistochemistry at the same samples. The result we got is that TNFAIP8 expression positively correlated to EGFR ($r=0.671135$, $P<0.05$) with Spearman rank correlation coefficient determination. This means the samples with high TNFAIP8 protein expression always had high EGFR expression in pancreas cancer (PC). These

data may suggest that TNFAIP8 play a important role in the progression of pancreatic cancer (PC).

Regrettably, we did not study its expression at RNA and DNA level at the same time because of time and funds. So we will go on with the research at this subject to further analyse its role in the progression of pancreatic cancer (PC).

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References

Aggarwal S, Gupta S, Gupta MK, et al (2011). Possible role of epidermal growth factor receptors in the therapy of pancreatic cancer. *Crit Rev Ther Drug Carrier Syst*, **28**, 293-356.

Azizi A, Naguib NN, Mbalisike E, et al (2011). Liver metastases of pancreatic cancer: role of repetitive transarterial chemoembolization (TACE) on tumor response and survival. *Pancreas*, **40**, 1271-5.

Dong QZ, Zhao Y, Liu Y, et al (2010). Overexpression of SCC-S2 correlates with lymph node metastasis and poor prognosis in patients with non-small-cell lung. *Cancer Sci*, **101**, 1562-9.

Freundt EC, Bidere N, Lenardo MJ (2008). A different TIPE of immune homeostasis. *Cell*, **133**, 401-2.

Hong SM, Park JY, Hruban RH, et al (2011). Molecular signatures of pancreatic cancer. *Arch Pathol Lab Med*, **135**, 716-27.

Ioannou N, Dalgleish AG, Seddon AM, et al (2011). Anti-tumour activity of afatinib, an irreversible ErbB family blocker, in human pancreatic tumour cells. *Br J Cancer*, **105**, 1554-62.

Kumar D, Whiteside TL, Kasid U (2000). Identification of a novel tumor necrosis factor-alpha-inducible gene, SCC-S2, containing the consensus sequence of a death effector domain of fas-associated death domain-like interleukin-1beta-converting enzyme-inhibitory protein. *J Biol Chem*, **275**, 2973-8.

Kumar D, Gokhale P, Broustas C et al (2004). Expression of SCC-S2, a antiapoptotic molecule, correlates with enhanced proliferation antumorigenicity of MDA-MB 435 cells. *Oncogene*, **23**, 612-6.

Patel S, Wang FH, Whiteside TL, et al(1997). Identification of seven differentially displayed transcripts in human primary and matched metastatic head and neck squamous cell carcinoma cell lines: implications in metastasis and/or radiation response. *Oral Oncol*, **33**, 197-203.

Romanuik TL, Ueda T, Le N, et al (2009). Novel biomarkers for prostate cancer including noncoding transcripts. *Am J Pathol*, **175**, 2264-76.

Sharma C, Eltawil KM, Renfrew PD, et al (2011). Advances in diagnosis, treatment and palliation of pancreatic carcinoma: 1990-2010. *World J Gastroenterol*, **17**, 867-97.

Sun H, Gong S, Carmody RJ, et al (2008). TIPE2, a negative regulator of innate and adaptive immunity that maintains immune homeostasis. *Cell*, **133**, 415-26.

Vincent A, Herman J, Schulick R, et al (2011). Pancreatic cancer. *Lancet*, **378**, 607-20.

Xu S, Weihua Z (2011). Loss of EGFR induced autophagy sensitizes hormone refractory prostate cancer cells to adriamycin. *Prostate*, **71**, 1216-24.

You Z, Ouyang H, Lopatin D, et al (2001). Nuclear factor-kappa B-inducible death effector domain-containing protein suppresses tumor necrosis factor-mediated apoptosis by inhibiting caspase-8 activity. *J Biol Chem*, **276**, 26398-404.

Zhang C, Chakravarty D, Sakabe I, et al (2006). Role of SCC-S2 in experimental metastasis and modulation of VEGFR-2, MMP-1, and MMP-9 expression. *Mol Ther*, **13**, 947-55.