RESEARCH COMMUNICATION

Expression and Clinical Significance of Tbx2 in Pancreatic Cancer

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Abstract

TBX2 is one of the family of genes encoding developmental transcription factors, characterized by a 200 amino acid DNA binding domain (T-box), found to be related to malignant phenotypes of mammary cancer. However, the role of TBX2 in pancreatic cancer progression remains unclear. Therefore, the present study was conducted to investigate the expression and clinical significance of TBX2 in pancreatic cancer. Immunohistochemistry was carried out on paraffin-embedded sections of pancreatic cancer and normal pancreatic tissues. In addition, semiquantitative RT-PCR and Western blots were carried out to analyze mRNA and protein expression of Tbx2 in 6 pairs of freshly resected pancreatic cancer and their adjacent nontumorous tissue. TBX2 expression was significantly increased in pancreatic cancer tissue (29/48 or 60.4%). The expression level of Tbx2 had a significant positive relationship with tumor differentiation degree, higher TNM stage and distant metastasis. Also, mRNA and protein expression of Tbx2 were found to be at higher levels in almost all cancer tissues compared to adjacent tissues. In conclusion, Tbx2 protein might play an important role in the process of the development and metastasis of pancreatic cancers and high-level Tbx2 expression might be related to malignant potential.

Key Words: Tbx2 - pancreatic cancer - carcinogenesis

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Introduction

Pancreatic cancer is the fifth leading cause of cancer-related deaths in the United States, Europe and Japan(Greenlee et al., 2001; Jemal et al., 2002; 2003). Although some progress has been made in surgery, chemotherapy, and radiation therapy in recent decades, pancreatic cancer continues to be a formidable disease and is associated with a very high incidence of fatality. Most patients diagnosed with pancreatic cancer die within 12 months, the 5-year survival for pancreatic cancer is typically below 5% (Chen et al., 2007; Singh et al., 2007). The only curative treatment for pancreatic cancer is surgical resection, and it is feasible in less than one half of the patients (Richter et al., 2003). Clearly, there is a pressing need to understand more about pancreatic cancer pathogenesis and to develop an effective treatment for pancreatic cancer. Therefore, searching for new and sensitive biomarkers which can be used in clinic for the better detection and better intervention of pancreatic cancer is an important task.

TBX2 belongs to a gene family which encodes a group of transcription factors characterized by a highly conserved DNA-binding motif (T-box) and its unusual mode of DNA recognition(Qi et al., 2008). There is high-level TBX2 expression in mammary cancer (Rowley et al., 2004) and increasingly attention is being drawn to the possibility that it promotes the development of pancreas cancer. To further investigate the possible role of TBX2 in human pancreatic cancer progression, we compared mRNA and protein expression of Tbx2 in human pancreatic cancer specimens and analyzed the relationships between the expression levels and clinical features of the patients. Our results revealed significant Tbx2 expression in pancreatic cancer tissues, which may contribute to the progression of pancreatic cancer.

Materials and Methods

Tissue Specimens

From May 2006 to March 2008, paraffin-embedded blocks of 48 surgically resected primary infiltrating pancreatic adenocarcinomas at the hospital of China Medical University, were included in the present study. In addition, 12 nontumoral tissues containing normal ducts were obtained, all from these patients who received partial pancreatomy for benign tumors were used as normal controls. Clinical and pathological data were obtained from the Surgical Pathology Files, including age, gender, race, tumor size, tumor location, lymph node status, and TNM stage. Of the 48 patients, there were 33 men and 15 women. Median age at the time of surgery was 57 years (range 41–82 years). Tumor stage and histopathological grading were recorded according to the classification of

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the International Union Against Cancer. There were 15 stage I, 12 stage II, 15 stage III, and 6 stage IV tumors. Histological grades for the patients were as follows: 14 patients, grade I; 15, grade II; and 19, grade III. None of the patients had received chemotherapy or radiation therapy.

For Western blot examination, freshly resected tumor and adjacent nontumorous tissue specimens from 6 patients with pancreatic cancer were immediately frozen in liquid nitrogen and stored at -80°C until use. Histopathological analyses confirmed the malignant and surrounding normal tissues.

Immunohistochemical Staining

Expression of Tbx2 in pancreatic cancer and normal tissue was detected by the standardized avidinbiotin peroxidase staining technique as usual. Briefly, after being deparaffinized in xylene and rehydrated in alcohol, paraffin-embedded tissue sections were incubated in 3 ml/l of H_2O_2 to block endogenous peroxidase activity. Each slide was incubated with normal goat serum for 30 min at room temperature, and incubated with primary antibody overnight at 4 °C. The slides were washed in phosphate-buffered saline (PBS; pH 7.4). After incubation with biotinylated mouse anti-goat IgG for 30 min at 37°C, each slide was rinsed in phosphatebuffered saline and incubated in the avidinbiotin peroxidase complex for 30 min at 37 °C. The peroxidase was visualized with 3, 3'-diamino-benzidinetetrahydrochloride (DAB) solution and then counterstained with hematoxylin. Negative controls for immunostaining replaced the primary antibody with PBS. All sections were examined microscopically and scored by two independent pathologists in a blinded fashion without knowledge of clinical and pathological information. The reagents were bought from Beijing Zhongshan Biological Technology Ltd. Corp., China.

Immunohistochemical Staining Evaluation

Expression of Tbx2 was evaluated according to the ratio of positive cells per specimen and staining intensity. The ratio of positive cells per specimen was evaluated quantitatively and scored 0 for staining of <1%, 1 for staining of 2-30%, 2 for staining of 31-70%, and 3 for staining of 71-100% of the cells examined (Marsh et al., 1998) . Intensity was graded as following: 0, no signal; 1, weak; 2, moderate, and 3, strong staining. A total score of 0–6 was finally calculated by the following formula: total score = ratio of positively staining cells (score) + intensity of immunoreactivity (score), and graded as negative (I; score: 0), weak (II; 1–2), moderate (III; 3–4), and strong (IV; 5–6).

Western Blot Analysis

Equal amounts of extracted human pancreatic tissue protein were separated by 12% SDS–PAGE and the protein bands were electrotransferred to PVDF membranes. Expression of TBX2 was analyzed using corresponding specific primary antibodies (anti-TBX2 polyclonal antibody), followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG for TBX2 and β-actin antibody. The specific protein band was visualized by enhanced chemiluminescence (ECL, Amersham-PharmaciaBiotech, Beijing, China). Autoradiograms were quantified by densitometry (software: Bio Image IQ). Relative protein levels were calculated compared to the β-actin standard. Each experiment was performed in triplicate. All examined gene expression levels were quantitatively analyzed and expressed as ratios to β-actin.

Table 1. Primers and reaction parameters for RT-PCR of TBX2 and β-actin

Product	Sequence	T	C	Size
TBX2				
Forward: 5'- GGGATCAATTCCACACGTACG -3'		55	30	320
Reverse: 5'- AAAACCGTGTTTGTCCGAGATG -3'				
β-actin				
Forward: 5'-AGCGGGAAATCGTGCGTG-3'		54	25	287
Reverse: 5'-CAGGGTACATGGTGGTGCC-3'				

T, annealing temperature, ˚C; C, cycle number; Size, bp

Table 2. Immunohistochemical Analysis of Tbx2 Expression in Pancreatic Cancer and Normal Tissue

Tissue	Total cases	Tbx2 expression				Positive	p value
Non-cancer tissue	12	10	2	0	0	0(0%)	<0.01
Pancreatic cancer	48	9	10	18	11	29(60.4%)	

RNA Extraction and Semiquantitative RT-PCR

A total RNA of pancreatic cancer tissues was extracted using Trizol (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. DNase was used to decrease the contamination of genomic DNA. The quantity and purity of the RNA prepared from each sample was determined by electrophoresis and the ratio of the optical density at 260 nm to that at 280 nm.

PCR primers were designed by the Primer Premier 5.0 and the reaction conditions of PCR are listed inTable 1. PCR was performed in a thermal cycler (Touchgene) using Platinum Taq DNA Polymerase (Invitrogen, USA). Amplification of the RNAs without prior reverse transcription (RT) reaction was used as a negative control. PCR products were visualized by ethidium bromide staining of a 2% agarose gel. Relative mRNA levels were calculated relative to the β-actin standard. Each experiment was performed in triplicate. All examined gene expression levels were quantitatively analyzed and expressed as ratios to β-actin values.

Statistical Analysis

Each experiment was repeated at least three times. Bands from Western blot were quantified by densitometry (software: Bio Image IQ). Relative protein or mRNA levels were calculated by referring them to the amount of alpha-actin.

The $\chi 2$ test and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests were adopted. These analyses were performed using SPSS 13.0 for Windows (SPSS, Chicago, Ill., USA). Probability values of less than 0.05 were considered statistically significant.

Table 2. Clinicopathological Associations of Tbx2 Expression in Grades of Pancreatic Cancers

Category		Total	Tbx2 expression				Positive	p value
			I	II	III	IV		
Gender	Male	33	8	6	13	7	20 (60.6)*	0.688
	Female	15	1	4	6	4	10 (66.7)	
Age	<50	16	2	4	7	3	10 (62.5)	0.835
	≥50	32	7	6	11	8	19 (59.4)	
Tumor diameter								
	<3cm	19	4	4	8	3	11 (57.9)	0.772
	≥3cm	29	5	6	10	8	18 (62.1)	
Location of primary tumor								
	Head	33	8	5	12	9	21 (63.6)	0.499
	Body and Tail	15	1	5	6	2	8 (53.3)	
Differentiation								
	Well	14	3	6	2	3	5 (35.7)	0.015
	Mod	15	4	3	5	3	8 (53.3)	
	Poor	19	2	1	11	5	16 (84.2)	
TNM		27	6	8	7	6	13 (48.1)	0.049
		21	3	2	11	5	16 (76.2)	
Metastasis								
	No	30	7	9	8	6	14 (46.7)	0.012
	Yes	18	2	1	10	5	15 (83.3)	

*Number (%)

Results

Immunohistochemical Analysis of TBX2 Expression in Pancreatic Cancer and in Normal Tissue

Expression of TBX2 protein was studied by immunohistochemistry of 48 pancreatic cancers and 12 normal pancreatic specimens. Results of the Immunohistochemical staining are showed in table 2, including 2 phenotypically identifiable groups: grade I and II (negative or weak immunoreactivity) vs. grade III and IV (moderate or strong immunoreactivity). TBX2 staining was found positive (grade III and IV) in 29 (60.4%) cases of pancreatic cancer specimens, but no positive (grade III and IV) expression of TBX2 was observed in normal specimens. The frequency of higher grade expression (grade III and IV) of TBX2 in pancreatic cancer tissues was significantly ($p < 0.01$) higher than that in nonneoplastic pancreatic tissues.

Analysis of TBX2 Clinicopathological Characteristics in Pancreatic Cancer

Further analysis of the clinicopathological characteristics from 48 pancreatic cancer specimens revealed a positive association of TBX2 staining intensities with the degree of tumor differentiation. With respect to the TNM stage, the frequency of TBX2 high-grade expression was much higher in patients of grade III and IV than those of grade I and II ($p < 0.05$). There was a difference ($p < 0.05$) in the frequency of TBX2 high-grade expression between tumors with metastasis and those without. And there was no correlation between TBX2 staining and sex or age of the patients, diameter or location of tumor (Table 3).

Western Blot Analysis of TBX2 in Pancreatic Cancer and in Normal Tissue

Expression of TBX2 was examined by Western blot in the pancreatic cancers and their adjacent normal tissues taken from 6 patients (Figure 1a). It was not easy to correlate the high-level expression of TBX2 to the clinical outcome due to the small size of the sample.

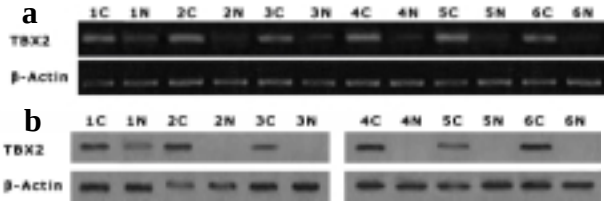

Figure 1 Expression of Tbx2. a) Western blot analysis of Tbx2 protein; b) RT-PCR detection of TBX2 mRNA.
N, normal; C, cancer

Level of TBX2 mRNA in Pancreatic Cancer and in Normal Tissue

To examine the expression of TBX2 transcripts, semiquantitative RT-PCR was performed in the pancreatic cancer and adjacent normal tissues taken from 6 patients. As shown in Figure 1b, TBX2 mRNA expression was found in 6 patients (100%). The semiquantitative RT-PCR products were detected by a scientific electrophoresis gel imaging system and analyzed statistically (1D imaging software, Kodak). The levels of β-actin, as internal controls, were different among pancreatic cancer tissues and the matched adjacent normal tissues ($p < 0.05$). The expression of TBX2 in the pancreatic cancer tissues was up-regulated ($p < 0.05$) compared with that in the matched adjacent normal tissues. These results further support our model that TBX2 might play a role in the tumorigenesis of pancreatic cancer.

Discussion

TBX2 is one of the members of a family of genes encoding developmental transcription factors, characterized by a 200 amino acid DNA binding domain (T-box) (Rowley et al., 2004) .TBX2 has the potential to recognize mitotic chromatin in a DNA dependent fashion, can interact specifically with the histone h3 N terminal tail , a property shared with tbx4 , tbx5 and tbx6 , and can also recognize nucleosomal DNA , with binding to nucleosomes being antagonized by the presence of the histone tails. In vivo tbx2 co localization with pericentric heterochromatin appears to be regulated and ectopic expression of tbx2 leads to severe mitotic defects. Tbx2 are able to target chromatin and may indicate a role for the T box factors in epigenetic reprogramming events (Sinclair et al., 2002) . Because polyploidy frequently precedes aneuploidy, the role of tbx2 in tumorigenesis is associated with high malignancy and poor prognosis (Guo-Chang et al., 2000).

As a potent immortalizing gene, tbx2 acts by downregulating cdkn2a p19arf. Tbx2 represses the cdkn2a p19arf promoter and attenuates e2f1, Myc or hras mediated induction of cdkn2a p19arf(Kim et al., 2004) . Phosphorylation leads to increased tbx2 protein levels, predominant nuclear localization of the protein, and an increase in the ability of tbx2 to repress the p21waf1 cip1 sdii promoter. The ability of tbx2 to repress the p21 gene

is enhanced in response to a stress induced senescence pathway, which leads to a better understanding of the regulation of the anti senescence function of tbx2(Demay et al., 2007) . The expression of tbx2 modulate induction of senescence by expressing kai1 attach to vascular endothelial cells in cancer cells (Davis et al., 2008) . The tbx2 oncogene was amplified in 50 % of pancreatic cancer cell lines, indicating increased involvement toward the q telomere of chromosome 17 (Jacobs et al., 2000) . The activity of endogenous tbx2 is critically required to maintain proliferation and suppress senescence in melanomas (Abrahams et al., 2008). Tbx2 maps to discrete amplicons and functions as an oncogene contributing to tumor progression in breast cancer cells (Bandyopadhyay et al., 2006). Tbx2 is amplified in a subset of primary human breast cancers, indicating that it might contribute to breast cancer development (Kim et al., 2004) . In previous studies, the level of TBX2 increased in BRCA1 and BRCA2 mutant tumors (Mahlamaki et al., 2002), and TBX2 was found to play a role in multiple drug-resistant in cancer cells (Erson et al., 2001; Vance et al., 2005) .

We clearly demonstrated that the expression of TBX2 in pancreatic cancer tissues was increased. There were significant differences in TBX2 expression between primary tumor and adjacent normal tissue samples, which suggested that TBX2 might be involved in malignant behaviors of pancreatic cancer. Further evaluation revealed several significant associations of high-level TBX2 expression with histopathological and other features of the tumors. Firstly, the expression of TBX2 protein was inversely correlated with the degree of tissue differentiation. Well-differentiated tissues consistently contained relatively low TBX2 protein levels, whereas poorly differentiated pancreatic cancer tissues showed stronger TBX2 reactivity. Secondly, TBX2 expression was significantly correlated with distant metastasis or with late TNM clinical stage. These results provided evidence that TBX2 might play a role not only in the onset of pancreatic cancer but also in its progression. To the best of our knowledge, our data provide the first evidence of positive TBX2 expression in pancreatic cancer tissues, although its exact role in pancreatic cancer progression and tumorigenesis remains open to further investigation

In conclusion, our present study revealed that TBX2 was up-regulated during the early phase of carcinogenesis of pancreatic cancer, which might be useful for diagnosis and possibly for early detection. The data also indicated that TBX2 was associated with both progression and invasion of pancreatic cancer. Although these results need to be certified in an independent set of experiments, our findings suggested that TBX2 might serve as a useful molecular marker for pancreatic cancer and an indicator for tumor progression, and might also be a useful therapeutic target to prevent pancreatic cancer or inhibit its malignant progression.

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