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T cell immunoglobulin- and mucin-domain-containing molecule 3 gene polymorphisms and susceptibility to pancreatic cancer

Danian Tong · Yujia Zhou · Wei Chen · Yang Deng · Lei Li · Zhenyi Jia · Dachuan Qi

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Abstract T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3) is a novel transmembrane protein that is involved in the regulation of T-helper 1 cell-mediated immunity. Studies have shown that polymorphisms in TIM-3 gene can be associated with various diseases. Here, we investigated the correlation of TIM-3 polymorphisms with susceptibility to pancreatic cancer in the Chinese population. Three polymorphisms in TIM-3 gene (−1516G/T, −574G/T, and +4259T/G) were identified by polymerase chain reaction–restriction fragment length polymorphism in 306 pancreatic patients and 408 healthy controls. Results showed that the prevalence of +4259TG genotype and +4259G allele were significantly increased in the pancreatic cancer cases than in controls [odds ratio (OR) = 2.82, 95 % confidence interval (CI), 1.45–5.48, $p = 0.0015$, and OR = 2.74, 95 % CI, 1.42–2.94, $p = 0.0017$]. In addition, when analyzing the TIM-3 polymorphisms with different clinical parameters in pancreatic cancer patients, the cases with vascular infiltration had higher numbers of +4259T/G polymorphism than those without vascular infiltration (OR = 3.07, 95 % CI, 1.41–6.68, $p = 0.003$). These results suggested polymorphisms in TIM-3 gene could be new risk factors for the development of pancreatic cancer.

Keywords T cell immunoglobulin- and mucin-domain-containing molecule 3 · Polymorphisms · Pancreatic cancer

Introduction

Pancreatic cancer remains one of the most lethal human cancers with an exceedingly dismal prognosis on account of the late occurrence of symptoms and high metastatic potential [1, 2]. Its aggressive behavior and the lack of sensitivity to most treatment options render this tumor a major cause of cancer-related death, with a mortality rate virtually equal to its incidence [1, 2]. Pancreatic cancer remains multifactorial in etiology and heterogeneous in its development and disease progression [2]. Improving our understanding of the molecular biology of pancreatic cancer will aid to find a better way to cure this disease.

T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3), which was identified as a specific cell surface marker of T-helper 1 (Th1) CD4+ T cells and was preferentially expressed on fully differentiated Th1 lymphocytes, but not on Th2 cells, was one of the Ig superfamily members [3–5]. In this process, TIM-3 was expressed at a late stage, suggesting that TIM-3 might not contribute to the T cell differentiation, but might perform a critical function in the Th1 cells transportation [3–5]. The inhibitor activity of TIM-3 was first described in a series of autoimmune diseases. In the autoimmune disease model, the administration of TIM-3 antibody would result in the activation and expansion of macrophage population, and then the more severe clinical symptom would be occurred

Danian Tong and Yujia Zhou contributed equally to this study.

D. Tong · W. Chen · Z. Jia · D. Qi (✉)
Department of Surgery, The Sixth People's Hospital, Shanghai Jiao Tong University, 600 Yishan Road, Shanghai 200233, China
e-mail: dachuanqi@126.com

Y. Zhou
School of Occupational Therapy, Western University, London, ON N6G 1H1, Canada

Y. Deng · L. Li
Department of Surgery, Ruijin Hospital, Shanghai Jiao Tong University, Shanghai 200025, China

[5]. Another research showed that the administration o TIM-3–Ig in vivo would result in T cell hyperproliferation and would abrogate the induction of tolerance during the development of immune response [6]. When TIM-3 was interacted with its ligand, galectin-9, the Th1 responses would be blockade by promoting the death of IFN gamma inducing Th1 cells [7]. In the previous functional study, experimental data demonstrated that the soluble form of TIM-3 would reduce the antigen-specific T cell responses and down-regulate the anti-tumor immunity in vivo by inhibiting the Th1 responses [8]. Despite TIM-3 had been usually thought to express in T cells, it was not limited. In a series of normal tissue cells and malignant epithelial tissues, the TIM-3 expression was detected and had been proved to accelerate the tumor cell proliferation by inhabiting the production of IL-2 and IFN-gamma which were the pivotal cytokines to induce the CTL and NK cells differentiation [9]. Moreover, the agonism of TIM-3 would significantly exacerbate Th1-mediated pathology, suggesting that it possessed a potential negative regulatory role for the endogenous TIM-3–TIM-3 ligand in vivo [8]. These data suggested that the TIM-3–TIM-3 ligand pathway would contribute to the attenuation of Th1-mediated anti-tumor responses and would be in charge of the development of cancer. In this study, we investigated the three single-nucleotide polymorphisms (SNPs) (−1516G/T, −574G/T, and +4259T/G) of TIM-3 gene to assess whether the genetic variants would be involved in the pancreatic cancer susceptibility in the Chinese population.

Materials and methods

Study population

The study population consisted of 306 pancreatic cancer patients (36–84 years of age) and 422 healthy controls (32–80 years of age), recruited from Shanghai No. 6 People's hospital and Ruijin Hospital from December 2006 to December 2010. The eligibility criteria for patient selection were being diagnosed with a pathologically confirmed pancreatic ductal adenocarcinoma and having an available DNA sample. The median age of the patient group was 61.8 years old. There is no age bias in recruitment in this study and no referral is needed in this hospital. During the recruitment of the pancreatic cancer patient group, we found one patient had hereditary breast ovarian cancer syndrome and another patient had hereditary nonpolyposis colorectal cancer by questionnaire. Since these familial cancer syndromes might be associated with pancreatic cancer and therefore might cause bias on the correlation between TIM-3 polymorphism and this disease, we excluded these two patients from our study population. All subjects were unrelated ethnic Han Chinese. Individuals that smoked more than once a day for over 1 year were defined as ever smokers. Written informed consent was obtained from each participant. The study was performed upon the approval of the Review Boards of Shanghai No.6 People's hospital and Ruijin Hospital. Written informed consent was obtained from each participant.

DNA extraction and genotyping

Genomic DNA was extracted from 5 ml frozen whole blood using the DNA Extraction Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's protocol. The three polymorphisms within the TIM-3 gene promoter and encoding regions were identified by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) assay. The primers and PCR conditions are listed in Table 1. PCR was performed in a total reaction volume of 20 μl containing 2 μl of 10X PCR buffer (Qiagen Inc., Hilden, Germany), 1.5 mM $MgCl_2$, 0.5 μM of each primer (shown in Table 1), 0.2 mM dNTP, 1.2 U Taq polymerase (Qiagen Inc., Hilden, Germany) and 200 ng of genomic DNA. After an initial denaturation at 95 °C for 5 min, the DNA was amplified for 35 cycles at 94 °C for 30 s, 55–60 °C for 40 s (detailed annealing temperatures shown

Table 1 Primers, amplicon conditions and restriction enzymes used in this study

Primers	Annealing (°C)	Amplicon size (bp)	Restriction enzyme
TIM-3 (−1516G/T)			
F: 5′- GCCTTGACCAAGTTCATGCT-3′	60.0	404	*Bsl*I
R: 5′- ACCACCCCGGATAATTTTGT-3′		338 + 66	
TIM-3 (−574G/T)			
F: 5′- AGAAGAAGGATGAGAGTGAGGCTTATGCTGGGAGTTTC-3′	60.0	169	*Taq*I
R: 5′- ACTCAAATCAGTCCCTTCATC-3′		37 + 132	
TIM-3 (+4259T/G)			
F: 5′- CACTCTCAACGTAGGTCTGCAGGCAG-3′	60.0	196 + 21	*Pst*I
R: 5′- GCATCCTTGGAAAGGCAGCAG-3′		160 + 36 + 21	

in Table 1), and 72 °C for 45 s, with a final elongation at 72 °C for 10 min on the Gene-Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA). PCR products containing the three polymorphic sites were then digested with the restriction enzymes *Bsl*I, *Taq*I, and *Pst*I (New England Biolabs, Beverly, MA, USA), respectively, by using the conditions recommended in the manufacturer's instructions. The digested PCR products were fractionated on 2 % agarose Tris–borate–EDTA gel (Agarose 1000; Gibco BRL, Rockville, MD, USA) and stained with ethidium bromide (product size after digestion shown in Table 1). To confirm the genotyping results, more than 15 % of PCR-amplified DNA samples were examined by DNA sequencing. Results between PCR and DNA sequencing analysis were 100 % concordant.

Statistical analysis

The SPSS statistical software package ver.13.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. Demographic data between the study groups were compared by the χ^2 test and by the Student t test. The polymorphisms were tested for deviation from Hardy–Weinberg equilibrium by comparing the observed and expected genotype frequencies using the χ^2 test. For SNP analysis, genotype and allele frequencies of TIM-3 were compared between groups using the χ^2 test, and odds ratios (OR) and 95 % confidence intervals (CIs) were calculated using unconditional logistic regression. p values less than 0.05 were considered significant. Haplotypes were reconstructed from genotype data and were statistically analyzed by the SHEsis program (http://analysis.bio-x.cn/myAnalysis.php).

Results

A total number of 306 pancreatic cancer cases and 422 controls were recruited for the present study. All subjects were ethnic Chinese. Demographic and other selected characteristics of the cases and controls are presented (Table 2). Cases and controls did not show statistically significant differences with regard to age and sex.

The three polymorphisms in TIM-3 gene (−1516G/T, −574G/T, and +4259T/G) in pancreatic cancer patients and healthy controls are summarized in Table 3. The SNPs genotyped were in HWE ($p > 0.05$). We did not observe TT genotype for TIM-3 −1516G/T or TT genotype for −574G/T polymorphism. There were no differences of TIM-3 −1516G/T or −574G/T polymorphism identified between pancreatic cancer cases and controls ($p > 0.05$). The prevalence of +4259TG genotype and +4259G allele were significantly increased in the pancreatic cancer cases than in controls (OR = 2.82, 95 % CI, 1.45–5.48, $p = 0.0015$, and OR = 2.74, 95 % CI, 1.42–2.94, $p = 0.0017$). The −1516G/T and −574G/T polymorphism did not show any significant difference between cases and controls. Also, we analyzed the linkage disequilibrium (LD) and the possible haplotypes constructed by −1516G/T, −574G/T, and +4259T/G polymorphisms. The TIM-3 −574G/T and +4259T/G SNPs were in strong LD with each other ($D' > 0.80$), whereas the −1516G/T and −574G/T, or −1516G/T and +4259T/G were not in LD ($D' < 0.01$ or $D' < 0.008$). There were seven haplotypes observed. The three most common haplotypes are shown (Table 3). We did not find any correlations between these haplotypes and pancreatic cancers. These data suggested that TIM-3 +4259T/G polymorphism was associated with increased susceptibility to pancreatic cancer in the Chinese population.

Table 2 General characteristics of the subjects

Variables	Cancer cases ($N = 306$) (%)	Controls ($N = 422$) (%)	p value
Age (years)	61.8 ± 11.2	61.3 ± 10.6	>0.05
Sex no. (%)			>0.05
Male	195 (63.7)	261 (61.8)	
Female	111 (36.3)	161 (38.2)	
Smoking status			<0.001
Never	116 (37.9)	271 (66.7)	
Ever	190 (62.1)	151 (33.3)	
Drinking status			<0.001
Never	106 (34.6)	245 (58.1)	
Ever	200 (65.4)	177 (41.9)	
Diabetes status			<0.001
No	240 (78.4)	399 (94.5)	
Yes	66 (21.6)	23 (5.5)	
Tumor site			
Head	221 (72.2)		
Non-head	85 (27.8)		
Tumor size (cm)			
≤2 cm	76 (24.8)		
>2 cm	230 (75.2)		
Peri-vascular infiltration			
No	223 (72.9)		
Yes	83 (27.1)		
Clinical stage			
I	82 (26.8)		
II	89 (29.1)		
III	70 (22.9)		
IV	65 (21.2)		
Therapy			
Whipple section	109 (35.6)		
Palliative surgery	121 (39.5)		
Chemotherapy	76 (24.9)		

Table 3 The genotype and allele frequencies of TIM-3 SNPs between pancreatic cancer patients and controls

Polymorphisms	Cases ($N = 306$) (%)	Controls ($N = 422$) (%)	OR (95 % CI)	p value
TIM-3 −1516G/T				
Genotype				
GG	256 (83.7)	360 (85.3)	Ref.	
GT	50 (16.3)	62 (14.7)	1.13 (0.76–1.70)	0.543
Allele				
G	562 (91.8)	782 (92.7)	Ref.	
T	50 (8.2)	62 (7.3)	1.12 (0.76–1.65)	0.560
TIM-3 −574G/T				
Genotype				
GG	290 (94.8)	407 (96.4)	Ref.	
GT	16 (5.2)	15 (3.6)	1.50 (0.73–3.08)	0.269
Allele				
G	596 (97.4)	829 (98.2)	Ref.	
T	16 (2.6)	15 (1.8)	1.48 (0.73–3.03)	0.275
TIM-3 +4259T/G				
Genotype				
TT	279 (91.2)	408 (96.7)	Ref.	
TG	27 (8.8)	14 (3.3)	2.82 (1.45–5.48)	0.0015[a]
Allele				
T	585 (95.6)	830 (98.3)	Ref.	
G	27 (4.4)	14 (1.7)	2.74 (1.42–5.26)	0.0017[a]
Haplotypes				
GGT	549 (89.7)	769 (91.1)	Ref.	
TGT	32 (5.2)	51 (6.0)	0.88 (0.56–1.39)	0.578
TTG	7 (2.3)	9 (1.1)	1.09 (0.40–2.94)	0.866

[a] p value <0.05

We further evaluated the association of the three TIM-3 polymorphisms with clinical-pathological factors in pancreatic cancer patients. The stratification analysis included diabetes, clinical stage and vascular infiltration (Table 4). The pancreatic cancer cases with vascular infiltration showed higher numbers of +4259T/G polymorphism than those without vascular infiltration (OR = 3.07, 95 % CI, 1.41–6.68, $p = 0.003$). These data suggested that TIM-3 +4259T/G SNP may be correlated with the prognosis of pancreatic cancer.

Discussion

In the current study, we showed that TIM-3 polymorphisms were associated with increased susceptibility to pancreatic cancer in Chinese population. It is the first report demonstrating that polymorphisms in TIM-3 gene could be risk factors to affect the development of pancreatic cancer.

Three TIM genes have been identified on the human chromosome 5q33.2, referred as TIM-1, TIM-3 and TIM-4 [10]. TIM-3 is a molecule expressed on terminally differentiated Th1 cells but not on Th2 cells, which negatively regulate Th1 immunity [10, 11], and it is also a phosphatidylserine receptor to mediate phagocytosis of apoptotic cells [12–14]. The polymorphism studies suggested that gene of TIM-3 was associated with both the prevalence of rheumatoid arthritis (RA) in Korean population [15, 16] and other immune-mediated diseases and cancers, including gastric cancer [17], atopic disease [16] and diabetes [18]. The −574T/G and +4259T/G polymorphic sites of human TIM-3 gene have been demonstrated to be associated with susceptibility to RA in Korean population [15]. Our study showed that +4259T/G SNP was associated with increased risk of pancreatic cancer. Further, we observed that the frequency of +4259G/T SNP was significantly higher in pancreatic cancer with peri-vascular infiltration (Table 4). The mechanism remains unclear yet. A recently published paper also showed that TIM-3 +4259G/T SNP was correlated with distant metastasis of gastric cancer [17]. These data indicated that +4259T/G SNP of TIM-3 might play important roles in affecting the metastasis of cancers.

The effect of TIM-3 on cancer is poorly understood. TIM-3 protein was expressed on the surface of activated Th1 cells, dendritic cells, or malignant epithelial tissues,

Table 4 Distribution of TIM-3 polymorphisms in pancreatic cancer cases with different parameters

Allele frequency				
Characteristics	*n* (%)	*n* (%)	OR (95 % CI)	*p* value
Diabetes	Yes (132)	No (480)		
TIM-3 −1516G/T				
G	123 (93.2)	439 (91.5)	1.00	
T	9 (6.8)	41 (8.5)	0.78 (0.37–1.66)	0.522
TIM-3 −574G/T				
G	130 (98.5)	466 (97.1)	1.00	
T	2 (1.5)	14 (2.9)	0.51 (0.11–2.28)	0.372
TIM-3 +4259T/G				
T	129 (97.7)	456 (95.0)	1.00	
G	3 (2.3)	24 (5.0)	0.44 (0.13–1.49)	0.177
Clinical stage	I/II (342)	III/IV (270)		
TIM-3 −1516G/T				
G	313 (91.5)	249 (92.2)	1.00	
T	29 (8.5)	21 (7.8)	1.10 (0.61–1.97)	0.753
TIM-3 −574G/T				
G	334 (97.7)	262 (97.0)	1.00	
T	8 (2.3)	8 (3.0)	0.78 (0.29–2.12)	0.631
TIM-3 +4259T/G				
T	326 (95.3)	259 (95.9)	1.00	
G	16 (4.7)	11 (4.1)	1.16 (0.53–2.53)	0.718
Vascular infiltration	Yes (166)	No (446)		
TIM-3 −1516G/T				
G	157 (94.6)	405 (90.8)	1.00	
T	9 (5.4)	41 (9.2)	0.57 (0.27–1.19)	0.130
TIM-3 −574G/T				
G	161 (97.0)	435 (97.5)	1.00	
T	5 (3.0)	11 (2.5)	1.23 (0.42–3.59)	0.707
TIM-3 +4259T/G				
T	152 (91.6)	433 (97.1)	1.00	
G	14 (8.4)	13 (2.9)	3.07 (1.41–6.68)	0.003[a]

[a] *p* value <0.05

and could trigger the apoptosis pathway of Th1 cells by binding galectin-9 to the TIM-3 extracellular domain [19]. The higher TIM-3 level might result in an elevated CD80 expression of cell, and then the CD80 would preferentially interact with the inhibitory molecule cytotoxic T lymphocyte-associated antigen-4. This would eventually lead to a local immunosuppression [14, 20]. The elevated expression of TIM-3 in cancer cells would reduce its adhering capacity and would contribute to the development of cancer. Therefore, TIM-3 probably functioned as a pivotal immunoregulatory molecule in carcinogenesis. As soluble TIM-3 had been shown to bind to TIM-3 ligand on CD4+ T cells and could inhibit the anti-tumor immunity, the increased numbers of full-length TIM-3 molecule on mast cells could also exert a similar effect [21]. It has been reported that pancreatic patients have increased number of regulatory T (Treg) cells, which plays important roles in suppressing anti-tumor activity [21–23]. Galectin 9, the ligand of TIM-3, expresses on the surface of T cells [21–23] and has been found to promote the induction of T cells [23, 24]. Moreover, galectin 9 has been reported to induce apoptosis of TIM-3 expressing cells in vitro and in vivo [23]. These results suggested that TIM-3 might affect the development of pancreatic cancer through multiple pathways.

As a case-control study, this research has some limitations. The TIM-3 +4259T/G polymorphism was identified to be correlated with pancreatic cancer, especially cases with peri-vascular infiltration. However, we could not reveal the mechanism. Also, we conducted this study in the Han Chinese population. It would be important to perform similar studies in other ethic populations.

In conclusion, this case-control study demonstrates for the first time that the TIM-3 SNPs and haplotype are associated with increased risk of pancreatic cancer in

Chinese population. Our results provide important insights for understanding the genetics of pancreatic cancer and would be helpful for the development of TIM-3 as a possible therapeutic approach to this disease.

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