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ORIGINAL ARTICLE

PRSS1 mutations and the proteinase/antiproteinase imbalance in the pathogenesis of pancreatic cancer

Qiang Yi[1] · Feng Dong[2] · Liqing Lin[3] · Qicai Liu[1] · Shu Chen[3] · Feng Gao[4] · Qingliang He[5]

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Abstract This study aimed to investigate the mutations in the serine protease 1 gene (*PRSS1*) and the imbalance between trypsin and α1-antitrypsin in patients with pancreatic cancer. Polymerase chain reaction (PCR) was performed to amplify the sequences of *PRSS1* from 65 patients with pancreatic cancer and 260 healthy controls, direct sequencing was performed, and the clinical features were analyzed. In addition, enzyme-linked immunosorbent assay (ELISA) was employed to detect serum trypsin and α1-antitrypsin in pancreatic cancer patients and healthy controls in the same period. Mutations were found at the promoter and exon 3 of the *PRSS1* in patients with pancreatic cancer. That is, five patients had c.410 C>T mutation causing p.Thr 137 Met, and three patients had c. −338 T>G mutation at the promoter of the *PRSS1*. In patients with *PRSS1* mutations, serum trypsin was 34.5± 18.3 ng/mL, which was significantly higher than that in normal controls (10.65±6.03 ng/mL) and other pancreatic cancer (28.61±8.96 ng/mL). What is more, in pancreatic cancer patients, serum α1-antitrypsin was 1.69±0.86 g/L, which was comparable to that in normal controls (1.55±0.53 g/L), while the ratio of serum trypsin to α1-antitrypsin was 1.46-fold to normal controls. The results presented here have provided a greater insight into the *PRSS1* mutations and proteinase-inhibitor interactions occurring in pancreatic cancer.

Keywords *PRSS1* mutations · α1-antitrypsin · Pancreatic cancer · Enzyme kinetics · Serine proteinase

Introduction

Pancreatic cancer has been one of the most life-threatening malignancies worldwide. The 5-year survival rate of pancreatic cancer is about 6 % [1–3], and it has been one of the ten leading malignancies in China. Most patients present in an advanced stage and thus are not appropriate surgical candidates. This observation is largely ascribed to the lack of markers for early diagnosis [4].

As our knowledge, the role of multiple factors in the disturbance of pancreatic cancer microenvironment has not been investigated [5, 6]. The pancreas is an important endocrine organ in humans, and it can produce large amounts of digestive enzymes. Abnormalities in zymogens and the imbalance between enzymes and antienzymes are the important mechanisms underlying the pathogenesis of pancreatic diseases and should be studied in depth. The abnormal activation of enzymes including trypsinogen may block the activation of other enzymes or cause over-activation of other enzymes, then causing an imbalance between enzymes and antienzymes, which may result in inflammatory injury and leading to damage to

Qiang Yi and Feng Dong contributed equally to this work.

✉ Feng Gao
fengfang77777@163.com

✉ Qingliang He
Heqingliang89@sina.com

[1] Department of Laboratory Medicine, the 1st Affiliated Hospital, Fujian Medical University, Fuzhou, China

[2] Department of Radiation Oncology, the 1st Affiliated Hospital, Fujian Medical University, Fuzhou, China

[3] Department of Pharmaceutical Analysis, Fujian Medical University, Fuzhou, China

[4] Department of Pathology, the 1st Affiliated Hospital, Fujian Medical University, 20 Chazhong Road, Fuzhou 350005, China

[5] Department of Surgery, the first Affiliated Hospital, Fujian Medical University, 20 Chazhong Road, Fuzhou 350005, Fujian Province, China

the microenvironment of the pancreas. The subsequent mutation of genes (such as *KRAS*) may result in the formation of cancer cells [4, 6, 7]. Then, trypsin may serve as a signaling factor to promote the proliferation of cancer cells and disruption of the matrix, leading to the migration of cancer cells. In addition, trypsin may also act as a stimulator of lymphocytes [8], and play an important role in the immune tolerance of mutated cells in conjunction with α1-antitrypsin (α1-AT). This process also provides a selective advantage to these cancer cells [5–8], which grow rapidly via clone selection, and present malignant phenotypes. Thus, investigation of the imbalance between serum trypsin and antitrypsin may be helpful to identify markers for early diagnosis of pancreatic cancer.

Materials and methods

Study population and diagnosis criteria

We recruited 65 sporadic cases (pancreatic ductal adenocarcinoma) from the 1st Affiliated Hospital, Fujian Medical University, including 48 males and 17 females, with average age 60.2±13.8 years. There was no history of tobacco smoking or alcohol consumption in these patients. All patients originated from the Han ethnicity in the mainland of China and were pathologically confirmed. Serum samples were collected from the patients with pancreatic cancer before surgery. We randomly selected 260 unrelated, age-matched, and sex-matched normal controls originated from the Han ethnicity in the mainland of China, including 200 males and 60 females, with an age range from 46 to 75 years (average 60.9±16.8). This study was approved by the Fujian Medical University Ethics Committee and the ethics committee supervises the process of the experiment.

Methods

Analysis of gene mutations

Informed consent was obtained from patients and their relatives and controls. Blood was collected and DNA was extracted using a Tiangen Genomic extraction kit (Beijing, China). The full-length of serine protease 1 gene (*PRSS1*) were amplified. Following purification, sequencing was performed.

Detection of trypsin and α1-antitrypsin

Detection of serum trypsin and α1-antitrypsin was done with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). The α1-AT concentration was measured in the serum samples using ELISA kit (Universal Biologicals, Cambridge, UK), according to the manufacturer's instructions.

Immunohistochemistry analysis

The conditions of antigen retrieval, the antibody probing, and the scoring were performed. The sections were incubated with the anti-α1-antitrypsin (1:150) and antitrypsin (1:100). Sections were washed with deionized water, and lightly counterstained with hematoxylin. For each slide, a total of five random images at ×400 magnifications were selected, and examined with an Olympus microscope system (Tokyo, Japan) to score the staining. The ratio of the number of stained cells to the total number of cells was calculated. Total cell ratio for the staining of anti-α1-antitrypsin and antitrypsin was ranked into the following three groups, based on the percentage of positive tumor cells: high (+++, >35 %), medium (++, 18–35 %), and low or negative (±, ≤17 %). All of the slides were independently viewed and scored by two pathologists. All of the antibodies were purchased from ABclonal Ltd.

Statistical analysis

All data were analyzed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Continuous variables are expressed as median (min-max). The Kruskal-Wallis *H* test and Mann-Whitney *U* nonparametric test were used for comparison between and within groups. The Spearman's rank correlation was performed between variables. For all tests, a two-sided *P* value <0.05 was considered to be statistically significant.

Results

Patient's profile and laboratory findings

In patients with *PRSS1* mutations, the serum levels of trypsin, hyaluronic acid, type IV collagen, laminin, and type III procollagen N-terminal peptide were significantly increased (Table 1).

Molecular genetic analysis

In our study, five patients were found to have c.410 C>T mutation at exon 3 of the *PRSS1*, causing p.Thr137Met mutation, which was reported in our previous study in another pancreatic cancer patient who had both p. Thr137Met and p. Thr135Met mutations [9]. Moreover, three patients had c. −338 T>G mutation at the "GCCAT" box of the promoter of the *PRSS1* (Fig. 1). Both of the mutations were not observed in 260 healthy controls.

Molecular modeling analysis of *PRSS1* mutations

To investigate the influence of mutations on the conformation of the PRSS1 protein, Swiss-Model software was employed,

Table 1 The clinical data of the patients with *PRSS1* mutations

Pathological type	c.410 C>T					c. −338 T>G		
	No. 1 PDA	No. 2 PDA	No. 3 PDA	No. 4 PDA	No. 5 PDA	No. 1 PDA	No. 2 PDA	No. 3 PDA
α1-AT (%) (0.90–2.00)	2.02	1.05	1.28	1.06	1.25	1.36	1.62	1.33
Trypsin (2–8) nmol/L	39.26	41.21	46.30	28.12	33.02	32.62	26.33	29.07
Hyaluronic acid (0–120) ng/mL	557.2	226.5	140.6	320.8	205.4	330.2	201.4	385.2
Laminin (0–130) ng/mL	503.6	85.6	63.5	162.3	241.3	220.9	128.5	330.6
Procollagen type III (<12) ng/mL	15.3	20.6	3.9	52.3	30.5	8.9	3.7	26.3
Collagen type IV (0–140) ng/mL	204.1	59.6	52.4	80.2	197.5	36.6	177.3	59.8

PDA pancreatic ductal adenocarcinoma

and the PRSS1 complex model in the protein database was used as a basis, aiming to analyze the spatial conformation of mutants. The results showed that p.137Thr was highly conserved (Fig. 2a), and the p.137Thr mutation changed the spatial structure of autolytic sites (Fig. 2b).

Detection of serum trypsin and α1-antitrypsin

In the patients with c.410 C>T mutation or c. −338 T>G mutation, serum trypsin was 34.5±18.3 ng/mL, which was significantly higher than that in healthy controls (10.65±6.03 ng/mL) and other patients (28.61±8.96 ng/mL). In addition, serum α1-antitrypsin was comparable between pancreatic cancer patients (1.69±0.86 g/L) and healthy controls (1.55±0.53 g/L). It was 1.46 times of the ratio of serum trypsin to α1-antitrypsin in patients with the *PRSS1* gene mutation to that in healthy controls (Fig. 3).

Immunohistochemical expression of trypsin and α1-antitrypsin in pancreatic cancer tissues

α1-antitrypsin has low expression in the pancreatic cancer tissues with *PRSS1* c.410 C>T mutation or c. −338 T>G mutation (Fig. 4a), while they were strongly expressed in the

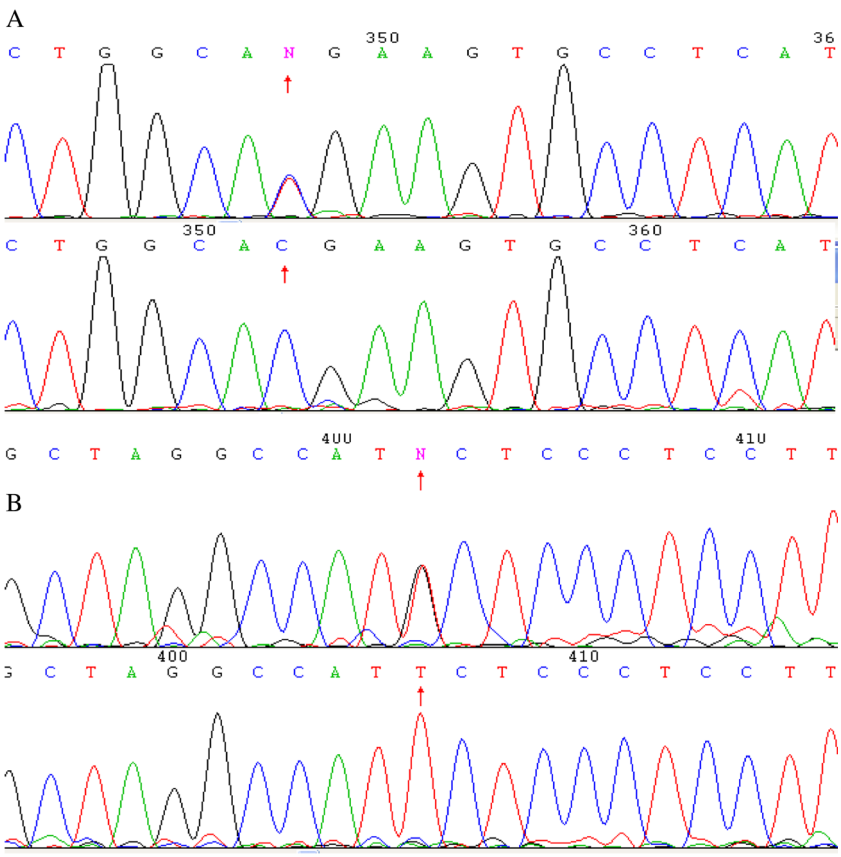

Fig. 1 Mutations of the *PRSS1* gene. **a** c.410 C>T (p.Thr 137 Met); **b** c. −338 T>G

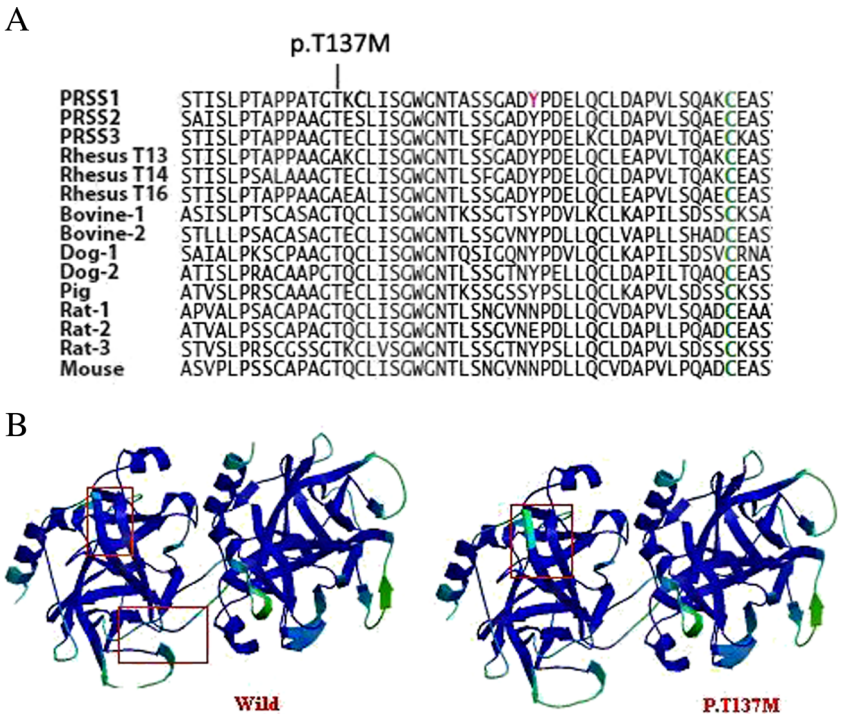

Fig. 2 Species conservation (**a**) and PRSS1-p.Thr 137 Met complex model in the protein database (**b**)

cytoplasm of chronic pancreatitis with wild type of the *PRSS1* gene (Fig. 4b). The rate of trypsin positive (high cytoplasmic expression) was as high as 100 % (8/8) in the pancreatic cancer tissues from *PRSS1* mutations (Fig. 4c), while in normal pancreatic tissues, the positive rate of trypsin was less than 5 % (1/20) (Fig. 1d).

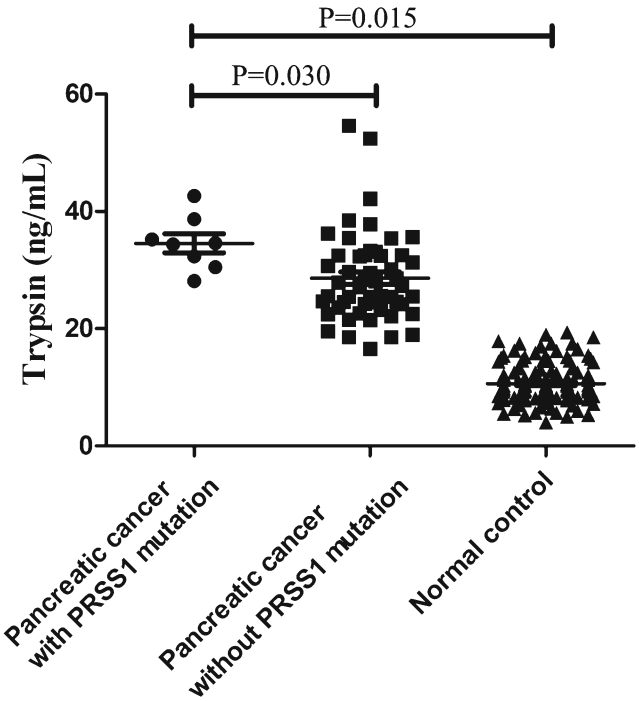

Fig. 3 The difference of serum trypsin concentration between pancreatic cancer and normal control

Discussion

With the progression of studies on the genes related to chronic pancreatitis, some genotypes of the *PRSS1* gene are found to be protective, neutral, or even carcinogenic [5, 9, 10]. Therefore, the *PRSS1* gene mutation can only explain the pathogenesis of pancreatic cancer in a few patients, and it fails to elucidate why a majority of patients who develop pancreatic cancer do not have *PRSS1* mutation. Previous studies have shown that abnormal activation of trypsin may block the activation of other zymogens or may cause over-activation of other zymogens (imbalance between enzymes and antienzymes), which may cause an inflammatory injury, resulting in damage to the microenvironment of pancreatic cells, damaging intracellular molecules, including DNA. The damaged DNA usually results in cell death, but it could also bring carcinogenesis in case of concomitant impaired DNA repair mechanism. In addition, trypsin may serve as a stimulator of lymphocytes, and plays an important role in immune tolerance of mutated cells via cooperation with α1-AT. This process also provides a selective advantage to cells [5, 6].

Although the theory of "central role of trypsin" has been widely accepted [11, 12], many investigators focus on the role of mutations of genes (such as *KRAS* and *PRSS1*) in the pathogenesis of pancreatic cancer [12–15]. However, the

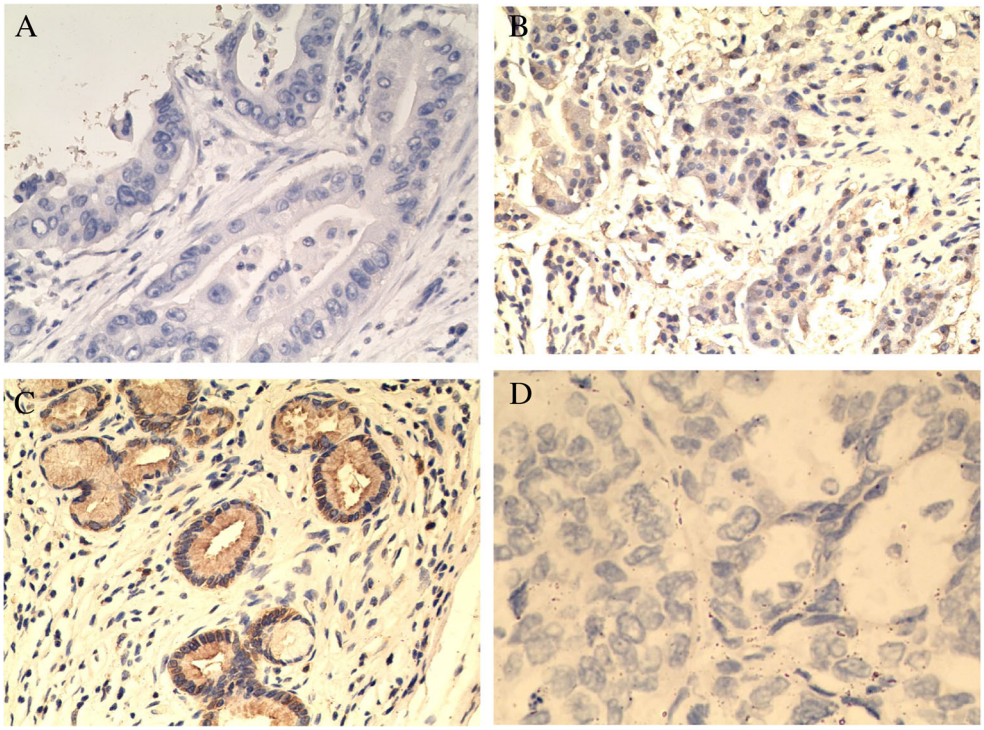

Fig. 4 The difference of trypsin and α1-antitrypsin expression in pancreatic tissue. **a** The cytoplasmic was low expression of α1-antitrypsin in pancreatic cancer tissue with *PRSS1* c.410 C>T mutation. **b** The high level of α1-antitrypsin expressed in chronic pancreatitis with wild-type PRSS1. **c** The high level of trypsin expressed (cytoplasmic expression) in the pancreatic cancer tissues from *PRSS1* c.410 C>T mutations. **d** The low level of trypsin expressed in the cytoplasm. (Original magnification ×400)

importance of imbalance between trypsin and antitrypsin was neglected. Thus, it is difficult to explain the immunological escape and clonal proliferation of pancreatic cancer cells. In the present study, our results demonstrated that the mean serum trypsin in patients with pancreatic cancer was 1.68 times higher than that in healthy controls, and higher serum trypsin concentration was specifically found in pancreatic cancer patients with *PRSS1* mutation. What is more, trypsin was highly expressed in the pancreatic tissues with *PRSS1* mutation. This may be explained by the fact that *PRSS1* mutation may cause the activation of trypsin in situ, leading to the release of trypsin into the circulation. p.137Thr is highly conserved among species, and p.137Met may change the activation sites of calcium or alter pH dependence. Under physiological conditions, the serine protease inhibitor Kazal-type 1 (*SPINK1*; 20 %) and α1-AT (about 80 %) may antagonize trypsin [6, 15, 16].

Trypsin may degrade the extracellular matrix and basement membrane, and thus, be involved in the transformation, invasion, and migration of pancreatic cancer cells [5, 13, 17]. Thus, abnormal increase in serum trypsin may be a risk signal for pancreatic cancer, and malignant transformation may be explained as follows: trypsin activates proteinase-activated receptor-2 (PAR-2) [16–18] and promotes the formation of p-ERKI/2. Then, p-ERK enters the nucleus, and upper-regulated expression of c-fos, which promotes form into the dimerization of c-fos and c-jun, resulting in increase of AP-1. AP-1 can bind to the AP-1 site of the proliferating cell nuclear antigen (PCNA) and upper-regulated expression of PCNA, and resulting in malignant transformation [15, 18–21].

Thus, we speculate that mutation of genes including the *trypsinogen* gene may cause imbalance between trypsin and antitrypsin, which may change the pancreatic microenvironment, cause cell mutation and escape from immune surveillance, and lead to clonal proliferation, resulting in pancreatic cancer. On the basis of this speculation, the balance between trypsin and antitrypsin may become a sensitive marker for early identification and diagnosis of pancreatic cancer.

This study was conducted to investigate imbalance between enzymes and antienzymes, and to elucidate the relationship between immune tolerance and clonal proliferation of pancreatic cancer cells. Our findings may be helpful to elucidate changes in the cancer microenvironment and the immunological mechanism underlying the pathogenesis of pancreatic cancer, to provide sensitive and specific markers for the early identification and diagnosis of pancreatic cancer, and also to present a novel target for immune therapy and gene therapy of pancreatic cancer.

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Conflicts of interest None

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