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Expression of pancreatic secretory trypsin inhibitor gene in neoplastic tissues

Naohiro Tomita+*, Akira Horii+*, Tatsuo Yamamoto+*, Michio Ogawa*, Takesada Mori* and Kenichi Matsubara+

+*Institute for Molecular and Cellular Biology, Osaka University, Yamada-oka, Suita 565,* and **Second Department of Surgery, Osaka University Medical School, Fukushima-ku, Osaka 553, Japan*

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Expression of the human pancreatic secretory trypsin inhibitor (PSTI) gene was examined in 24 cases of neoplastic tissues by Northern blot analyses. In three cases of lung adenocarcinoma and one case of sigmoid colon polyp, we detected transcripts which hybridized to the human *PSTI* cDNA probe. cDNA libraries were constructed using mRNAs of the two PSTI-positive tumor tissues. Two *PSTI* cDNA clones were obtained from each sample. Sequencing analyses showed that they were completely identical with that of pancreatic *PSTI* cDNA which had been reported [(1985) Biochem. Biophys. Res. Commun. 132, 605–612]. Southern blot analyses showed that the elevated expression of *PSTI* in neoplastic tissues was accompanied by neither *PSTI* gene amplification nor rearrangements.

Pancreatic secretory trypsin inhibitor; Gene expression; cDNA; Neoplastic tissue

1. INTRODUCTION

The pancreatic secretory trypsin inhibitor (PSTI) is a single chain polypeptide which is secreted by mammalian pancreatic acinar cells into pancreatic juice. Its physiological role has been considered to be to prevent premature trypsin-catalyzed activation of zymogens within the pancreas and the pancreatic duct. Material(s) crossreacting with anti-PSTI antibody is (are) produced and secreted into blood vessels. The level of such an antigen increases upon severe injury or surgery [1,2] and under inflammatory conditions [3]. The serum PSTI level remains within the normal range even after total pancreatectomy [4]. These findings strongly suggest that PSTI is produced in extrapancreatic tissues, although its function has not been understood. Interestingly, some malignant diseases have also been associated with high PSTI levels [5]. Huhtala et al. [6] found such material in the urine of a patient with ovarian cancer, and Ogawa et al. [7] detected a similar material in various cancer cells. These findings suggest that some tumor cells may produce PSTI, although no direct evidence is available. If this is the case, the question remains of whether the 'PSTI' produced in tumor cells is identical with that produced in the pancreas or is (a) different material(s) that is (are) related to PSTI. We initiated studies to clarify these points by isolating *'PSTI'* cDNA clones from neoplastic tissues and characterizing its sequence. The results show that this material is identical with that produced in the pancreas, which was described by Yamamoto et al. [8].

Correspondence address: N. Tomita, Institute for Molecular and Cellular Biology, Osaka University, Yamada-oka, Suita 565, Japan

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2. MATERIALS AND METHODS

2.1. *Preparation of mRNA*

The neoplastic tissues are listed in table 1. They were removed in surgical operations, immediately frozen in liquid nitrogen and stored at −70°C. Total cellular RNA was isolated essentially as described [9]. mRNA was purified from the total RNA by repeated passages through an oligo(dT) cellulose (type 7, Pharmacia Co., Sweden) column.

2.2. *Preparation of* PSTI *cDNA probe*

A 303 bp *Xho*I-*Sal*I fragment of pYIAM82 [10] was used as a probe. This DNA covers the entire amino acid coding region of the *PSTI* cDNA [8]. A ^{32}P-labeled cDNA probe (spec. act. 4–8 × 10^8 cpm/μg) was prepared by the random priming method [11] using [α-^{32}P]dCTP (Amersham, 3000 Ci/mmol; 1 Ci = 37 GBq).

2.3. *Construction of cDNA libraries and screening for* PSTI *clones*

cDNA libraries were constructed from two mRNA samples, as prepared from a lung adenocarcinoma (LC 19), and a sigmoid colon polyp (CC 5). Double-stranded cDNAs were prepared and then ligated to λgt10 with *Eco*RI linkers as described in [12]. To screen for *PSTI* cDNA clone, plaque hybridization using a nylon filter (Gene Screen Plus, NEN, USA) was performed as described in [13].

2.4. *cDNA sequencing analysis*

Nucleotide sequence analysis was performed by the chain termination method [14] using bacteriophage M13mp19.

2.5. *Southern and Northern blot hybridizations*

10 μg high molecular mass DNA prepared from tumor tissues or from a non-tumorous portion of LC 19 was digested twice with restriction enzymes (Takara Shuzo, Kyoto, Japan), electrophoresed in 0.7% agarose gel and transferred to a nylon filter [15]. mRNA was denatured by heating at 65°C for 15 min in 50% (v/v) formamide, electrophoresed in a 1% agarose/2.2 M formaldehyde gel as described [16], and then transferred to a nylon filter. After baking at 80°C for 2 h, the filter was prehybridized at 65°C for several hours in a sealed plastic bag containing 6 × SSC (1 × SSC is 0.15 M NaCl, 15 mM Na citrate), 1% SDS. Hybridization was carried out at 65°C overnight in 6 × SSC, 1% SDS, 20 μg/ml of heat denatured herring sperm DNA, containing the *PSTI* cDNA probe (1–2 × 10^6 cpm/ml) described above. After the hybridization, the filter was rinsed at room temperature in 2 × SSC, 1% SDS, washed twice at 65°C in the above solution for 30 min and then rinsed at room temperature in 0.1 × SSC.

2.6. *Primer extension analysis*

A 17-nucleotide single-stranded fragment (fig.2B; position 30–46) was synthesized as a primer by a β-cyanoethyl phosphoramidite method [17], and the 5′-end was labeled with ^{32}P. After heating at 65°C for 5 min, hybridization was done in a 30 μl reaction mixture containing 40 mM Pipes (pH 6.8), 0.4 M NaCl, 1 mM EDTA, 2 mM Na, 0.2% SDS, 10 μg of mRNA, and 0.5 pmol of 5′-end labeled primer at 37°C for 5 h. Next, reverse transcription was carried out in a 100 μl reaction mixture containing 50 mM Tris (pH 8.0), 140 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 2400 U/ml of RNase inhibitor, 300 U/ml of reverse transcriptase (RAV-2, Takara Shuzo, Kyoto, Japan) at 42°C for 1 h.

Following phenol extraction and ethanol precipitation, the primer extension products were electrophoresed in a thin 6% polyacrylamide-8 M urea gel.

3. RESULTS AND DISCUSSION

We first examined the expression of *PSTI* in various neoplastic tissues by Northern blottings. The results are shown in table 1. In three lung cancer cases (LC 2, LC 7, LC 19), all of which were adenocarcinomas, and one case of sigmoid colon polyp (CC 5), we detected the transcripts which hybridized to the *PSTI* cDNA probe. Among the ten cases of lung adenocarcinomas, five were the well differentiated type and the rest were the moderately differentiated type. Interestingly, all the PSTI-positive adenocarcinomas were the well differentiated type. Representative Northern blot profiles of the lung cancers are shown in fig.1. In lane 4 (adenocarcinoma, LC 19) a hybridizing band of about 530 nucleotides was detected. This size coincides with that of *PSTI* mRNA in the pancreas (lane 1). The relative content of this

Table 1

Summary of Northern blot analyses for *PSTI* expression in various neoplastic tissues

Organ	Histology	No. of cases	No. of PSTI-positive cases
Lung	adenocarcinoma	10	3
	squamous cell carcinoma	3	0
	large cell carcinoma	1	0
	small cell carcinoma	2	0
	carcinoid	1	0
Colon	adenocarcinoma	4	0
	villous polyp	1	1
Stomach	adenocarcinoma	2	0

Northern blot hybridization was done using *PSTI* cDNA pYIAM82 as a probe. For details, see section 2. PSTI-positive cases, viz. three lung cancers and one colon polyp were designated as LC 2, LC 7, LC 19, and CC 5, respectively

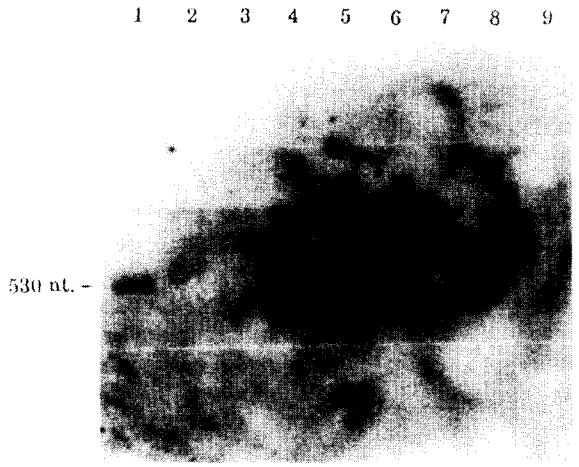

Fig.1. Northern blot profiles of mRNAs from lung cancer tissues using *PSTI* cDNA as a probe. In lane 1, 0.02 μg of pancreatic mRNA was used as a control. In lanes 2–8, mRNAs (5 μg) prepared from tumor tissues of LC 13 (adenoca.), LC 17 (adenoca.), LC 19 (adenoca.), LC 34 (squamous cell ca.), LC 35 (squamous cell ca.), LC 20 (large cell ca.) and LC 23 (small cell ca.) were employed. In lane 9, 5 μg of mRNA from normal lung tissue was used. The mRNAs were denatured, electrophoresed and subjected to Northern blotting as described in section 2. Autoradiography was performed for 5 days at −70°C with an intensifying screen.

transcript was estimated to be about 1/50 of *PSTI* mRNA in the pancreas. In three other PSTI-positive cases, the size and the level of the transcripts were essentially the same as those of this example (not shown). Other lanes, including lane 9 where 5 μg normal lung mRNA was used, showed no detectable bands.

Two cDNA libraries were constructed with λgt10 from two tumor tissue mRNAs obtained from a lung adenocarcinoma (LC 19) and a sigmoid colon polyp (CC 5). Out of 10000 plaques from each library, we obtained two clones each that showed positive signals with *PSTI* cDNA probe. They were designated λTIL-1, λTIL-3 (from LC 19), and λTIC-1, λTIC-3 (from CC 5), and the inserts were found to be 384, 374, 233 and 295 nucleotides, respectively, including poly(A) (fig.2A).

Before comparing the sequences of these inserts with that of pancreatic *PSTI* cDNA, we reexamined the nucleotide sequence of the published *PSTI* cDNA data [8], and found that some errors needed to be corrected. The corrected nucleotide sequence of the *PSTI* cDNA prepared from pancreas mRNA is shown in fig.2B. The *PSTI* cDNA consists of a 237-nucleotide amino acid coding region, a 60-nucleotide 5′-non-coding region and a 81-nucleotide 3′-non-coding region. The insert of λTIC-1, which is the longest among the four clones obtained, covered the entire *PSTI* cDNA and was shown to be completely identical to the pancreatic PSTI by sequence analysis (fig.2B). Although the other three clones, λTIC-3, λTIL-1, λTIL-3, had only partial *PSTI* cDNA inserts, their nucleotide sequences also coincided completely with that of the pancreatic *PSTI* cDNA.

To compare the transcription start points of the *PSTI* gene in the pancreas and the neoplastic tissues, primer extension analyses were done using a synthetic 17-nucleotide (fig.2B) as a primer on mRNA templates obtained from the pancreas. The results shown in fig.3 demonstrate that the *PSTI* gene has one major transcription start point (−60) and three (−94, −70, −39) or more minor transcription start points. S_1 nuclease mapping results agreed well with these results (not shown). Recently, we characterized the human *PSTI* gene and showed that it has no sequence typical for the promoter in the 5′-flanking region (Horii et al., in

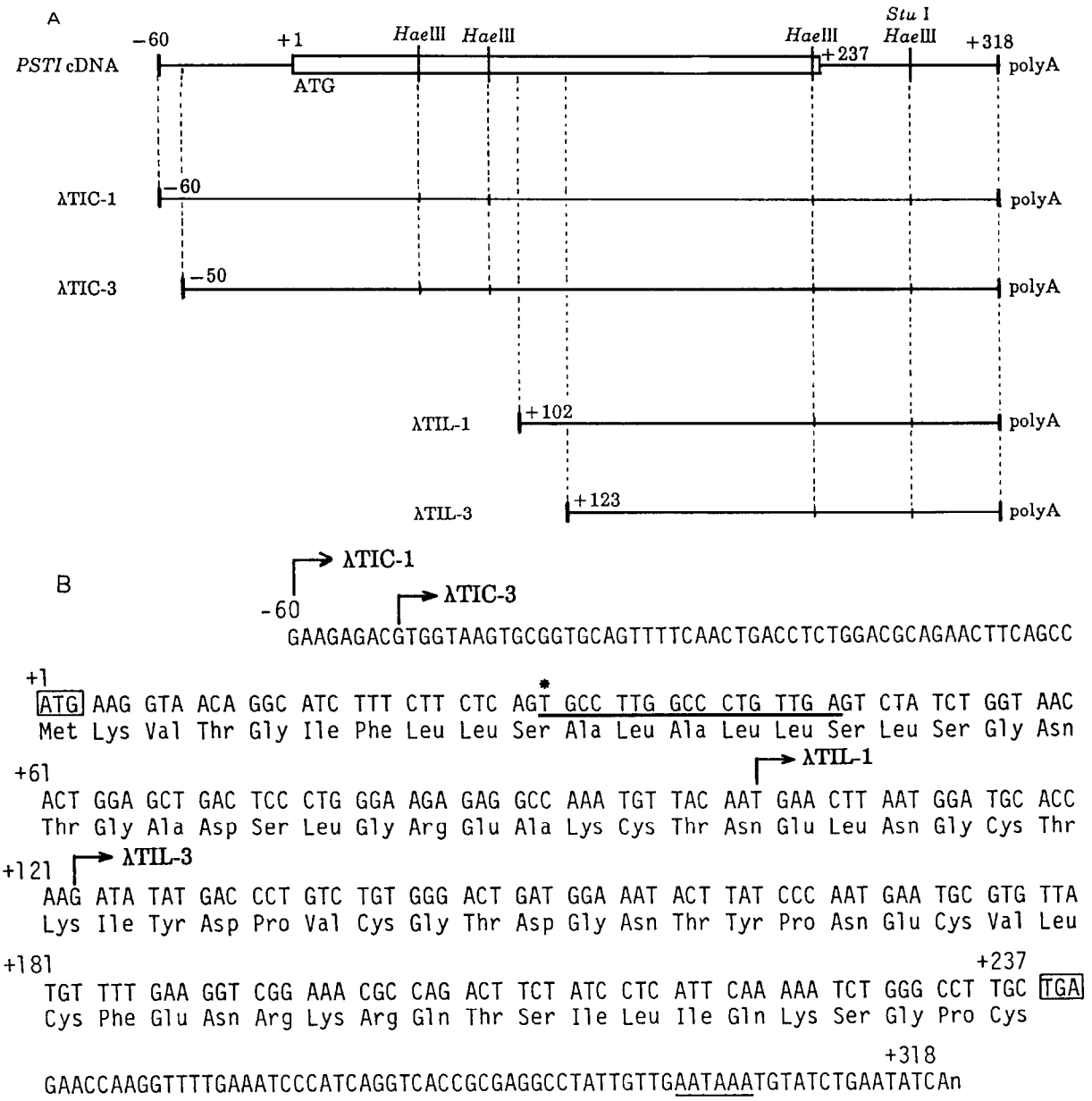

Fig.2. (A) Restriction map of *PSTI* cDNA clones isolated from cDNA libraries prepared from mRNAs of neoplastic tissues. λTIC-1 and λTIC-3 are clones isolated from the tumor tissue cDNA library of sigmoid colon polyp (CC 5), and λTIL-1 and λTIL-3 are those from lung adenocarcinoma (LC 19). Cleavage sites by some restriction enzymes are shown. The white horizontal bar represents the amino acid-coding region. (B) Corrected nucleotide sequence of the *PSTI* cDNA and deduced amino acid sequence. The 5′-ends of the *PSTI* cDNA clones prepared from two different neoplastic tissues are also shown. A few errors in the nucleotide sequence of the *PSTI* cDNA previously published in [8] have been corrected. The translation initiation codon (ATG) and termination codon (TGA) are boxed. AATAAA represents the poly(A) additional signal and An represents the poly(A) stretch. The numbers start from the first base of ATG. *PSTI* cDNA clones obtained from neoplastic tissues cover the regions between the horizontal arrows and poly(A). The underlined section marked with an asterisk represents the region corresponding to the synthesized 17-nucleotide primer used for primer extension analyses.

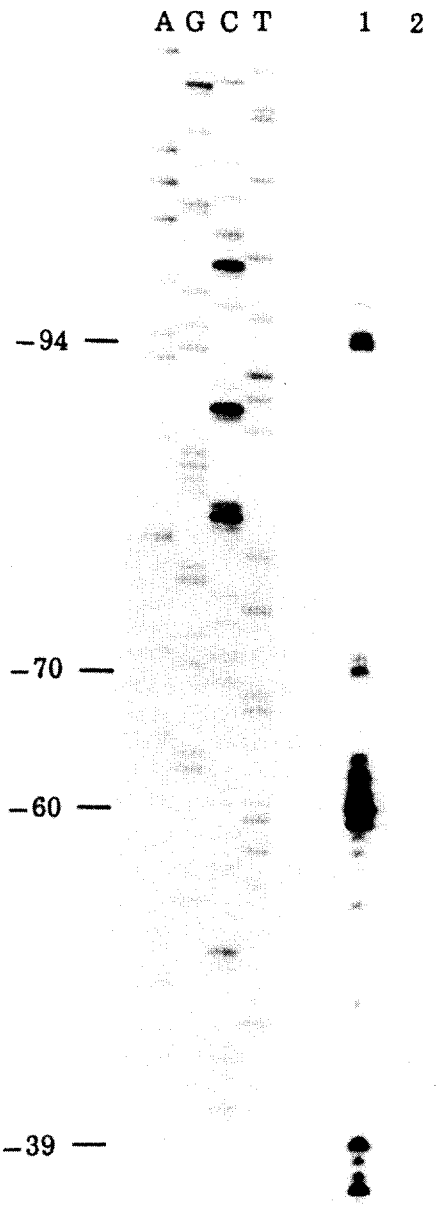

Fig.3. Primer extension analysis for defining transcription start point of *PSTI* mRNA. The ^{32}P-labeled synthetic 17-nucleotide was hybridized to mRNAs and extended with reverse transcriptase as described in section 2. In lanes 1 and 2, 10 μg each of human pancreatic mRNA and rat liver mRNA were used. Lanes AGCT show the dideoxy sequencing ladder as a size marker. Autoradiography was performed at −70°C for 4 h. The numbers indicate the nucleotide number from the first base of the ATG translation initiation codon.

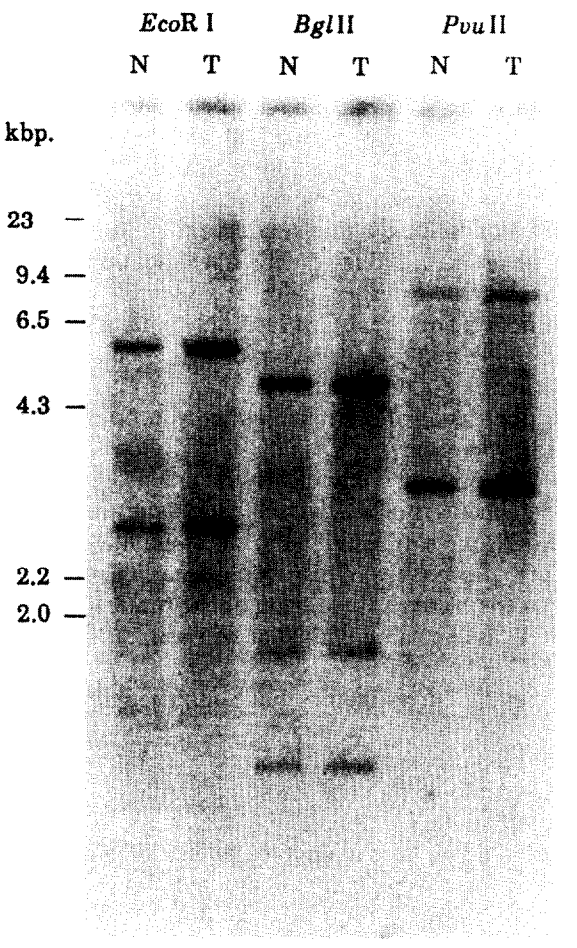

Fig.4. Southern blot analyses of the *PSTI* gene in tumor tissue. N indicates the DNA isolated from the non-cancerous part of lung tissue dissected with lung adenocarcinoma (LC 19), and T indicates the DNA isolated from the tumor tissue (LC 19) that was shown to express *PSTI*. DNAs (10 μg) were digested with restriction enzymes indicated at the top of the figure, electrophoresed, and subjected to Southern blotting analyses using the *PSTI* cDNA as a probe. For details, see section 2. Autoradiography was performed at −70°C for 4 days with an intensifying screen. Size markers (in kb) are the *Hin*dIII digests of λ phage DNA.

preparation). Transcription of the *PSTI* gene may start at multiple points because of this atypical promoter structure. Because of limited tissue supply, primer extension analyses using mRNAs of neoplastic tissues could not be done. However, λTIC-1 carries the insert that covers just from the

−60 to the poly(A) tail. Therefore, the transcription start site in this neoplastic tissue seems to be the same as that acting in the normal pancreas.

In some lung cancers, elevated expression of an oncogene such as c-*myc* [18] or N-*myc* [19], accompanied by gene amplification, has been reported. An intriguing possibility is that PSTI expression in lung cancer might be the result of *PSTI* gene activation due to gene amplification or rearrangement. Southern blot analyses of DNA obtained from the LC 19 sample and its normal counterpart DNA were performed. The results are shown in fig.4. We can see that the *PSTI* gene in tumor tissue underwent neither amplification nor rearrangement.

The present study clearly demonstrated that in some benign or malignant neoplastic tissues, the *PSTI* gene is transcribed in the same manner as in normal pancreas. This does not necessarily mean that the biological function of the tumor-produced PSTI is the same with that of pancreas-produced PSTI.

Several investigators have reported that elevation of proteolytic activity can be seen in some cancerous tissues [20,21], indicating the possibility that tumor cells produce some protease inhibitors in self-defense. Recently, McKeehan et al. [22] isolated two human endothelial cell growth factors from human hepatoma cells, and showed that 25 NH_2-terminal amino acid residues of one of the growth factors were identical with those of human PSTI. In addition, Ogawa et al. [23] observed that human PSTI can stimulate [^{3}H]thymidine incorporation into DNA in human fibroblasts. These results strongly suggest that PSTI may act as a growth factor under some circumstances. One attractive possibility is that the tumor-produced PSTI also acts as a growth factor-like substance in the course of tumor development. In some tumor-bearing patients, the serum PSTI level is high, although the tumor tissue itself has no significant level of immunoreactive PSTI [24]. In another case, patients display high serum PSTI level even after resection of the tumor (unpublished). The high PSTI levels in these cases may be due to production of PSTI as an acute phase reactant from tissues other than the malignant one. Further experiments are needed to clarify these points and to shed light on other possible biological functions associated with PSTI.

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