

# A Tumor-suppressive Role for Trypsin in Human Cancer Progression

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## Abstract

Trypsin is a serine protease family member with a potential role in cancer invasion. We investigated trypsinogen expression at the RNA level in 49 esophageal squamous cell carcinomas (ESCCs) and 72 gastric adenocarcinomas. Almost all primary ESCC tissues (95%) showed reduced expression, and 9 of 13 ESCC cell lines were silenced for trypsinogen expression. Absent expression correlated with promoter hypermethylation of trypsinogen-4 by bisulfite DNA sequence. Moreover, we detected promoter hypermethylation in 50% of primary ESCCs by methylation-specific PCR. A subset of gastric adenocarcinomas (71%) also showed reduced trypsinogen accompanied by reduction in PAR2, a G protein activated by trypsin, and a propensity to penetrate beyond the gastric wall ( $P = 0.001$ ). Our results support the notion that trypsin plays a tumor-suppressive role in human carcinoma.

## Introduction

Trypsin is a member of the serine protease family composed of three trypsinogen genes (*trypsinogen 1, 2, and 4*; Refs. 1 and 2). These family members are highly homologous to each other (>90%) at both the nucleotide and protein levels. Trypsin has potent proteolytic activity, and its inappropriate activation may lead to severe pathological states in various human diseases (3, 4). In fact, trypsin is so potent that it can destroy the tissues in which it resides. Normally, trypsin is detected physiologically in the airway epithelial cells, in which PAR2, a G protein-coupled molecule activated by trypsin, is also localized in the apical glands and has a protective role by lessening cell injury to trypsin exposure (5). In these tissues, trypsin activation and protection from activated trypsin need to be tightly regulated to avoid severe pathological injury.

Trypsin has also been confirmed to be expressed in various carcinomas (6–9). These reports concluded that trypsin plays a positive role, or perhaps even oncogenic role, in carcinoma progression. In this study, we propose a counter-intuitive hypothesis suggesting that trypsin has a tumor-suppressive role in cancer progression. This notion is supported by the observation that trypsin displays marked down-regulation in human cancer tissues tightly associated with promoter hypermethylation. Trypsin is, thus, added to a list of epigenetically silenced genes in human cancers (10, 11).

## Materials and Methods

**Patients and Cell Lines.** Forty-nine esophageal and 72 gastric cancers and paired normal mucosa specimens from patients who underwent surgery at Medical Institute of Bioregulation Hospital, Kyushu University, were assessed for trypsinogen mRNA expression as described previously (11–13). The patients with esophageal carcinoma included 45 males and 4 females. Tumors were located in the upper esophagus ( $n = 3$ ), the middle esophagus ( $n = 35$ ),

and the lower esophagus ( $n = 11$ ). Twelve tumors were well differentiated, 25 were moderately differentiated, and 12 were poorly differentiated squamous cell carcinomas. The depth of invasion included 5 involving the submucosa, 7 into muscularis propria, and 37 to the adventitia and beyond. Cases with lymph node metastases were classified into two groups: a nonmetastatic group ( $n = 8$ ) and a metastatic group ( $n = 41$ ). The patients with gastric carcinoma included 45 males and 27 females (clinicopathological distribution shown in Table 1). ESCC<sup>2</sup> lines TE1, TE3, TE4, TE5, TE13, KYSE30, KYSE70, KYSE110, KYSE140, KYSE150, KYSE200, KYSE410, and KYSE520 were obtained from the Cell Response Center for Biomedical Research Institute of Department, Aging and Cancer, Tohoku University (TE series), and kindly provided by Dr. Y. Shimada (Department of Surgery, Kyoto University, Kyoto, Japan; KYSE series). Cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum.

**5Aza-dC and TSA Treatments of Cells.** Cells were split to low density ( $5 \times 10^5$  per T-25 flask) 12–24 h before treatment. Cells were then treated for 4 or 5 days with 5 or 1  $\mu\text{M}$  5Aza-dC (Sigma), and 0 or 300 nM TSA (Sigma) were added to the media (final 24 h) as described previously (11).

**Total RNA Isolation.** Frozen-tissue specimens were homogenized in guanidinium thiocyanate, and total RNA was obtained by ultracentrifugation through a cesium chloride cushion, as described previously (12, 13).

**cDNA Preparation and RT-PCR.** Eight micrograms of each total RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen Inc., Carlsbad, CA), and 1/100 of the reaction mixture was amplified by PCR and analyzed as reported previously (13). PCR was performed for 1 min at 95°C (denaturation), 1 min at 59°C (annealing), and 1 min at 72°C (elongation) for 24 cycles. Oligonucleotide primer pairs common for trypsinogen-1 and -2 were purchased from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom; sense trypsinogen, 5'-GAAGTCCTG-GAGGGGAATGA-3'; antisense trypsinogen, 5'-TGATTCTGGTGGCCCTGTGG-3'). These primers recognized trypsinogen-1 and -2 (100%) as well as trypsinogen-4 (19 of 20, 95% identity). PCR products were ligated into the pCR 2.1 vector (Invitrogen) and sequenced using the DNA sequencing-Dye terminator kit (Perkin-Elmer Applied Biosystems Inc., Foster City, CA).

A semiquantitative method for trypsinogen expression by RT-PCR was based on the linear increase in signal intensity values in tumor tissue (T value) through 24 cycles (see Fig. 2A). The mRNA expression in tumor and normal tissue in each pair was then estimated based on the counts obtained using control TE13. The T value was the intensity of trypsinogen signal in tumor tissue/intensity of trypsinogen signal in TE13 corrected by GAPDH, as described previously (13).

**Northern Blot Hybridization.** Equal amounts (15  $\mu\text{g}$ ) of total RNA were loaded and analyzed from six primary esophageal carcinoma tissues as described previously (12). The specific activity of the DNA probe for the trypsinogen-4 PCR product subcloned into pCR 2.1 was over  $2 \times 10^8$  cpm per microgram of DNA. The GAPDH probe was used as an internal control as described previously (12).

**Sequence Analysis.** We extracted genomic DNA from Trizol (Invitrogen)-treated samples and performed bisulfite modification of genomic DNA as described (11). Bisulfite-treated DNA was amplified in the 5' region encompassed by the ATG start site (350 bp) using primer sets specific for trypsinogen-4 (Fig. 1D; sense trypsinogen, 5'-GAAGTCCTGAGGGGAATGA-3'; antisense trypsinogen, 5'-TGATTCTGGTGGCCCTGTGG-3'). All of the PCR products were gel extracted (Qiagen) and sequenced on the Applied

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<sup>2</sup> The abbreviations used are: ESCC, esophageal squamous cell carcinoma; 5Aza-dC, 5-aza-2'-deoxycytidine; TSA, trichostatin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSP, methylation-specific PCR; GADC, gastric adenocarcinoma.

Table 1 *Trypsinogen expression and clinicopathology in gastric cancer*

	Trypsinogen expression			P
	Non (n = 38)	Weak (n = 14)	Intense (n = 20)	
Histology				NS <sup>a</sup>
Intestinal	23	7	15	
Diffuse	15	7	5	
Depth of invasion				0.001
Within wall	15	8	18	
Without wall	23	6	2	
Lymph node metastasis				NS
Absent	12	6	12	
Present	26	8	8	
Stage				0.024
I	12	6	8	
II	8	3	9	
III	3	3	2	
IV	15	2	0	

<sup>a</sup> NS, not significant.

Biosystems 3700 DNA analyzer using BD terminator dye (Applied Biosystems) and the forward primer listed above.

**MSP.** Bisulfite-treated DNA was amplified with either a “methylation”-specific or a “nonmethylation”-specific primer set for trypsinogen-4 at 35 cycles: 96°C for 30 s, 61°C for 30 s, and 72°C for 30 s, as described previously (11). The methylation-specific primer sequences for trypsinogen-4 were designed using 5'-CTATACTCGAACTCCTCGCCG-3' as the forward primer and 5'-TTTCGGTTTCGGTTAGCGTGC-3' as the reverse primer. The non-methylation-specific primer sequences were 5'-CAACCTATACTCAAACCTCCTACCA-3' and 5'-TTTTGGTTTTGGTTAGTGTGTGGGG-3'.

**Results and Discussion**

We investigated trypsinogen-4 expression in 13 ESCC cell lines and found that 9 were silenced at the mRNA level (Fig. 1A). We selected three ESCC cell lines (KYSE30, 410, and 520) and tested trypsinogen gene expression after exposure to the demethylating agent 5Aza-dC in combination with TSA (11, 14, 15). Two ESCC cell lines showed reactivation of trypsinogen, as shown in Fig. 1B, confirming that silencing of trypsinogen expression is regulated epigenetically. KYSE410 demonstrated baseline expression and little increase after treatment with 5Aza-dC or 5Aza-dC+TSA. Interestingly, KYSE520 did not reactivate with 5Aza-dC alone but did reactivate with exposure to both reagents, suggesting an important role for hypoacetylated histones in regulation of trypsinogen expression.

We then investigated the status of promoter methylation for trypsinogen-4 and confirmed that all ESCC cell lines were methylated across the entire promoter region (Fig. 1, C and D). As expected, the methylation status of the promoter region was completely consistent with trypsinogen expression (Fig. 1D, *asterisk*). We then developed an MSP assay to confirm promoter methylation in primary ESCC tissues (Fig. 1E). MSP confirmed the promoter methylation status and was consistent with the expression status in the cell lines (see KYSE30 and KYSE410) and several primary tumors (Fig. 1E, cases 1–5).

We then proceeded to test primary tumors for expression of trypsinogen. Trypsinogen mRNA was also down-regulated in primary ESCC tissue specimens when compared with the corresponding

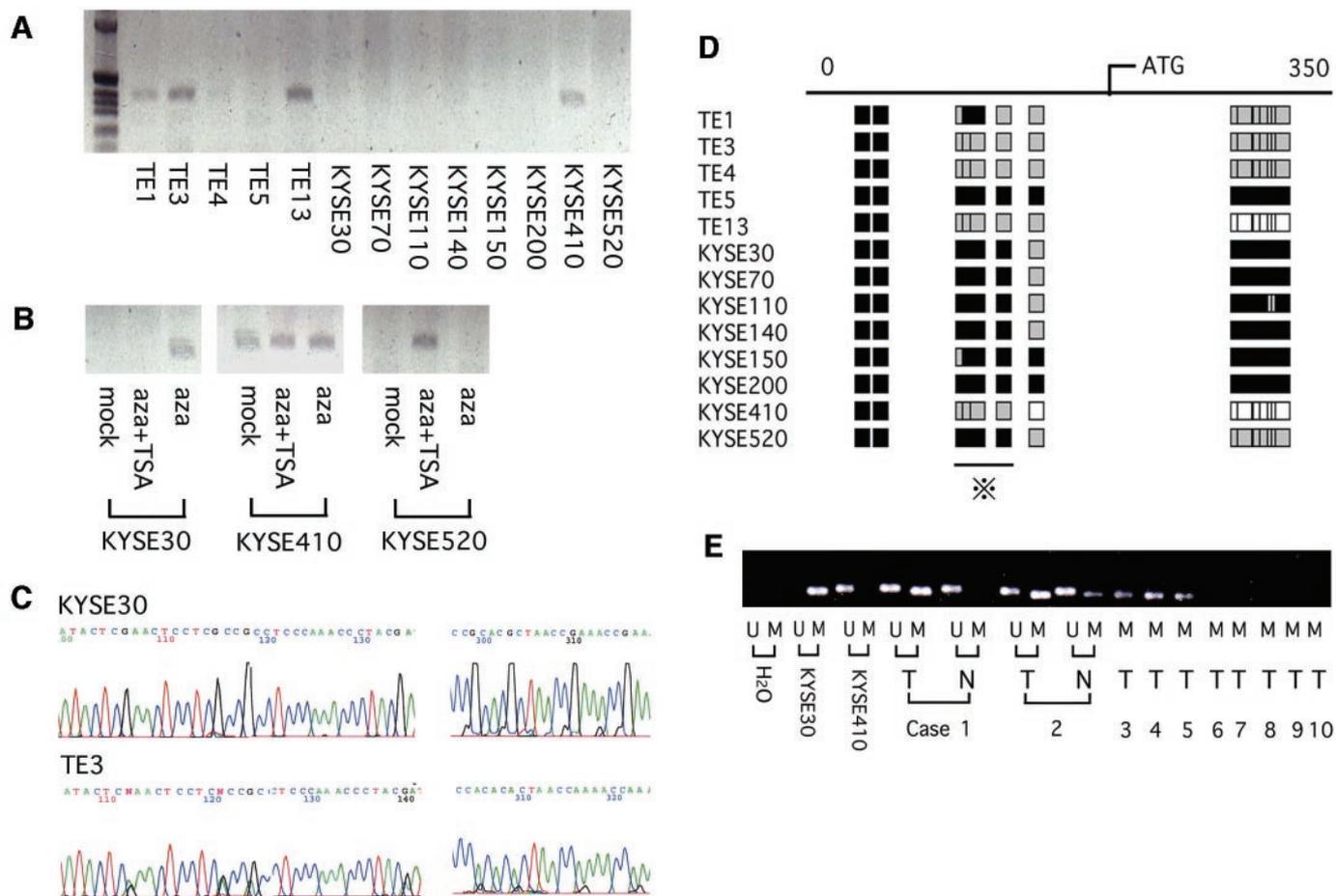


Fig. 1. Promoter hypermethylation and expression of trypsinogen-4 in esophageal carcinoma. A, RT-PCR of trypsinogen mRNA expression in ESCC cell lines. B, 5Aza-dC+TSA treatment reactivated trypsinogen expression for KYSE30 and 520 at the mRNA level. C, trypsinogen promoter hypermethylation is shown for KYSE30 and TE3. KYSE30 is completely methylated in the 5' (left) and flanking (right) regions, whereas TE3 harbored unmethylated alleles in these regions. D, the methylation status of ESCC cell lines. Boxes indicate CpG sites. Black box, complete methylation; gray box, partial no methylation; white box, no methylation. E, MSP analysis of trypsinogen in primary ESCC tissues. KYSE30 and KYSE410 were used as positive controls for methylation or absence of cytosine methylation in the promoter region, respectively.

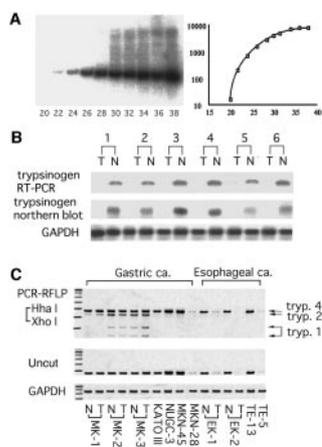


Fig. 2. Trypsinogen expression and isotype analysis in primary tumors. A, radioactive RT-PCR products. Radioactivity increased linearly between 18 and 30 PCR cycles for trypsinogen. B, RT-PCR and Northern blot analysis of trypsinogen mRNA expression in esophageal carcinoma tissues (T) and normal mucosal tissues (N). GAPDH was used as an internal control. C, differential expression of trypsinogen subtypes in esophageal squamous cell carcinoma and gastric carcinoma. PCR, followed by digestion with restriction enzymes, was performed using *HhaI* and *XhoI* in order. Arrows indicate the calculated band for the respective trypsinogen subtypes. Note that ESCC expressed only trypsinogen-4 and GADC expressed trypsinogen-1, -2, and 4 (if not down-regulated).

esophageal normal mucosa tissue at the mRNA level both by semi-quantitative RT-PCR and Northern blot hybridization (Fig. 2B). By semi-quantitative RT-PCR, the mean T value was very low, 0.2 (0–1.2), and only two cases with high expression (tumor:normal,  $\geq 0.7$ ) were identified in 49 patients. Fourteen cases showed moderate expression of trypsinogen ( $0.7 > \text{tumor:normal ratio} > 0.1$ ) in all 49 patients. There was no significant correlation with clinicopathological factors and levels of trypsinogen expression in ESCC. PCR-restriction fragment length polymorphism was performed to identify the correct trypsinogen subtypes by digesting the PCR products with *HhaI* and *XhoI*. We found that ESCC expressed only trypsinogen-4, whereas GADC tissues contained trypsinogen-1, -2, and -4, if not down-regulated (Fig. 2C). These results were also confirmed by sequencing of the PCR products (data not shown). Normal trypsinogen-4 expression was, thus, markedly reduced in most primary ESCCs.

In gastric mucosa tissues, the trypsinogen expression level was more varied, and we, therefore, evaluated the trypsinogen expression level by comparison with the TE13 cell line. The mean intensity of T value corrected for GAPDH was 1.6 (0–3.4). In GADC tissues, we established a cutoff value for T of 0.1 and we designated cases below 0.1 as no expression and 0.7 as high expression (we designated cases from 0.1 to 0.7 as weak expression). Among the 72 tumors, 38 showed no expression, weak expression was seen in 14, and intense expression in 20. Table 1 shows the relation of trypsinogen expression to clinicopathological features in GADC. Interestingly, trypsinogen was almost silenced or markedly reduced in most GADCs that penetrated beyond the wall ( $P = 0.001$ ; Table 1 and Fig. 3A), suggesting that trypsinogen reduction is favored in extension into the peritoneum. Trypsinogen expression was also significantly correlated with increased stage ( $P = 0.024$ ), but this result was weaker than depth of invasion (Table 1). Several cases with submucosal invasion only already silenced trypsinogen, suggesting that a subset of early GADC may have already acquired the potential to advance locally. A recent study suggests that airway epithelial cells express PAR2, a G protein activated by trypsin, for protection against trypsin injury (5). In our tumors (Fig. 3B), PAR2 expression closely correlated with trypsinogen expression in GADC tissues. Cases with intense trypsinogen expression coexpressed intense PAR2, whereas cases with weak or absent trypsinogen expression were associated with low expression of

PAR2 ( $P < 0.0001$ ). These results suggest that PAR2 may also protect tumors from autodigestion by trypsin in GADC tissues.

Our study is the first report to implicate trypsinogen-4 in human disease. Trypsinogen-1 and -2 were described previously as up-regulated in cancer (9, 16). Almost all reports have proposed a positive role for trypsin in cancer because trypsin can activate matrix metalloproteinase family members, which is critical for tumor invasion and metastasis. Several studies showed that high-grade tumors expressed trypsinogen whereas low-grade tumors harbored lower expression (7, 8, 17), proposed to be mediated through activation of type IV collagenase (18). Although matrix metalloproteinase is overexpressed (12, 13), our data suggest that trypsinogen is silenced in human tumor tissues.

Investigators are familiar with the use of trypsin to detach cells in culture. However, long-term exposure of trypsin makes cells weak and sometimes leads to cell death. Cells also become round through superficial shedding of various molecules by trypsin. For example, integrin  $\alpha 5$  is essential for tumorigenesis in various types of cancer, and long-term exposure of cells to trypsin down-regulates superficial integrins (19), to regulate the apoptotic ability of cells. Peritoneal dissemination of GADC may be favored by this apoptotic resistance (20), and trypsin might affect this pathway through integrin modification.

We, thus, present the counter-intuitive hypothesis that trypsin could play a tumor suppressive role in both ESCC and GADC. Moreover, we show that its expression is regulated by promoter methylation. Epigenetic regulation is now a common feature of tumor suppressor genes (11), and we suggest that trypsin is silenced by this pathway. Our findings also have practical implications because trypsin represents a potential therapeutic target. Specifically, inactivation of PAR2

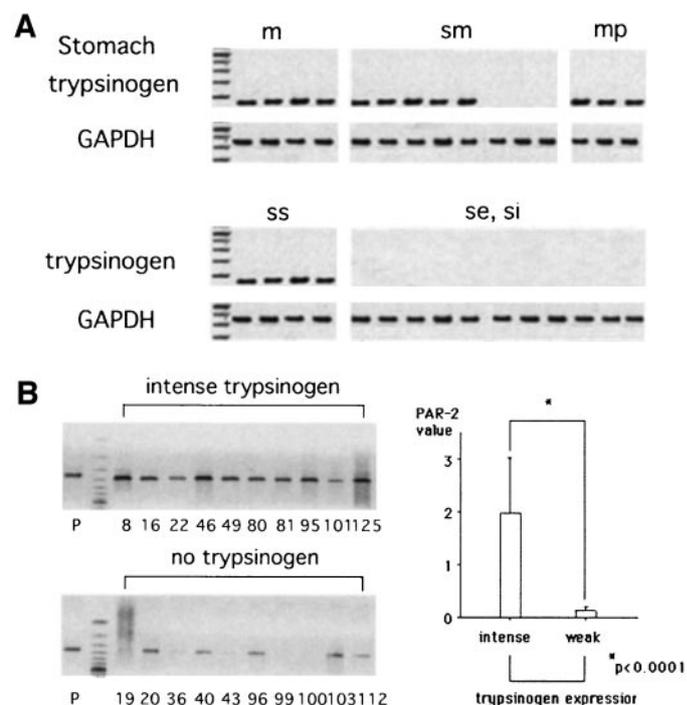


Fig. 3. Relationship between trypsinogen and PAR-2 expression and inverse extension. A, RT-PCR analysis of trypsinogen mRNA expression in GADC tissues. GAPDH was used as an internal control. Trypsinogen expression was retained in cases without gastric wall penetration, whereas expression was markedly reduced in cases with peritoneal extension. A subset of submucosal GADC showed silencing of trypsinogen. B, PAR-2 expression was tightly correlated with trypsinogen expression in GADC tissues ( $P < 0.0001$ ). Cases with high trypsinogen expression showed high expression of PAR-2, whereas those with low trypsinogen expression showed low expression of PAR-2.

could lead to autolysis of primary tumors and, ultimately, to prevention of peritoneal dissemination.

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