

PAI-1 Expression Levels in Esophageal and Colorectal Cancers are Closely Correlated to those in Corresponding Normal Tissues

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Abstract. To investigate the mechanism of PAI-1 overexpression in esophageal and colorectal cancers, PAI-1 expression levels in these cancers were compared to those in corresponding normal tissues. Quantitative RT-PCR was performed for the PAI-1 gene in esophageal and colorectal cancer tissues and in the corresponding normal tissues and the association between PAI-1 expression levels in these tissues was evaluated. There was a significant correlation between esophageal and colorectal cancer and the corresponding normal PAI-1 expressions with a Spearman's rank correlation coefficient of 0.77 ($p<0.0001$) and 0.81 ($p<0.0001$), respectively. In previous studies, PAI-1 overexpression was found to be significantly associated with the malignancy of esophageal and colorectal cancers. Taken together, PAI-1 overexpression in esophageal and colorectal cancers might originate from higher PAI-1 expression in corresponding normal tissues and result in a malignant phenotype of these cancers.

Accumulating evidence has proven that a series of genetic changes, which activate dominant oncogenes, such as K-ras, and inactivate tumor suppressor genes, such as *p53* and *APC*, are involved in the pathogenesis of human digestive tract cancers (1-3). Recently, plasminogen activator inhibitor-1 (PAI-1) was found by our group to play an important role during the malignant change of esophageal and colorectal carcinomas (4, 5). PAI-1 is a glycoprotein, actively involved in regulating the fibrinolytic system and increased PAI-1 activity in the plasma may promote thrombosis formation by inhibiting production of fibrinolytic enzyme plasmin. PAI-1 is mainly synthesized in the vascular

endothelial cells and regulates fibrinolytic activity in the vasculature by controlling the urokinase-type plasminogen activator (uPA) activity.

PAI-1 plays a role in several biological processes dependent on plasminogen activator or plasmin activity (6). High PAI-1 levels are potential risk factors for cardiovascular disease, etc. (7). In our previous studies, quantitative RT-PCR for the PAI-1 gene was performed and the possible relationship between PAI-1 gene expression levels and clinicopathological findings in esophageal and colorectal cancers was evaluated. It was found that PAI-1 might serve as a new parameter for the prediction of malignancy in esophageal and colorectal cancers. However, the mechanism of PAI-1 overexpression in these forms of cancer remains unknown.

As a first step in assessing the mechanism, PAI-1 expression levels in esophageal and colorectal cancer tissues were compared to those in corresponding normal tissues. Quantitative RT-PCR for the PAI-1 gene was carried out in esophageal and colorectal cancer tissues, as well as in corresponding normal tissues and the association between the PAI-1 expression levels in these tissues was evaluated.

Materials and Methods

Patients and tissue specimens. The study group consisted of 49 esophageal squamous cell cancer patients (mean age 63.3 years; range 50~77 years) and 64 colorectal cancer patients (mean age 64.5 years; range 41~85 years) who underwent surgical operations at the Gastroenterological Surgery of Nagoya University Graduate School of Medicine, Japan, from 1994 to 2003. Written informed consent, as specified by the institutional review board, was obtained from each patient. All patients were followed-up at our hospital with periodic examinations. The median follow-up of the 2 groups was 22 and 33 months, respectively. To date, a total of 25 and 20 patients were lost at follow-up, respectively. All cancer and corresponding normal tissues were collected at surgical resection and stored at -80°C. Samples of normal tissues were obtained from resected specimens at the primary resection, avoiding any anastomotic or scar tissue. The tissue samples were obtained as distinct as possible from the underlying pathological process in order to obtain unaffected tissue for subsequent analysis. These samples were confirmed as histologically normal and were graded according to the tumor-node-metastasis stage of the disease (UICC) as follows: Esophageal cancer: stage I, 4; stage II, 18; stage

Abbreviations: PAI-1, Plasminogen activator inhibitor-1; RT-PCR, reverse transcription-PCR.

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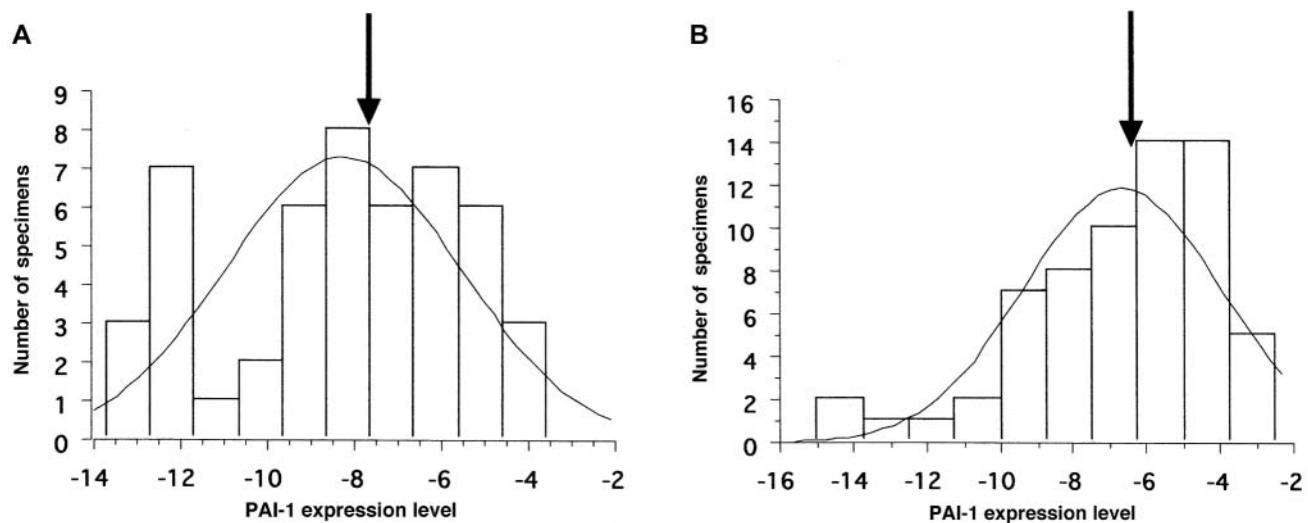


Figure 1. Histogram of PAI-1 expression level at log value in corresponding normal tissues of esophageal cancers (A). Arrow, median expression levels (i.e., -7.7 of the log value). Histogram of PAI-1 expression level at log value in corresponding normal tissues of colorectal cancers (B). Arrow, median expression level (i.e., -6.1 of the log value).

III, 22; and stage IV, 5. Colorectal cancer: stage I, 12; stage II, 15; stage III, 26; stage IV, 11. The patients were classified into two groups according to age, gender, histology, tumor size, tumor site, depth of tumor invasion, lymph node metastasis and distant metastasis.

RNA preparation and reverse transcription (RT). Total RNA was extracted from esophageal and colorectal cancer tissues and corresponding normal tissues with guanidium thiocyanate, as described previously (8). The amount of RNA was measured spectrophotometrically by absorbance at 260 nm. First-strand cDNA was generated from RNA, as described previously (9).

Quantitative RT-PCR. Quantitative RT-PCR was performed in an ABI sequence detection system 7000 using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), as described previously (4). Thermocycling was done in a final volume of 50 μ l containing 2.0 μ l of the cDNA sample, 1.0 μ l each of the PAI-1 primers (forward and reverse) and 25 μ l of Mix SYBR Green I/Enzyme (including Taq DNA polymerase, reaction buffer and deoxynucleotide triphosphate mixture). The PAI-1 primers for quantitative PCR were as described previously (10). PCR amplification consisted of 50 cycles (95°C for 15 sec, 60°C for 60 sec and 72°C for 18 sec) after an initial denaturation step (95°C for 10 min). To correct for differences in both quality and quantity between samples, GAPDH was used as an internal control. GAPDH primers were purchased from Applied Biosystems. PAI-1 and GAPDH mRNA variability were determined from triplicate samples. The quantity of all triplicate samples was in error by less than 10%. An average quantity of triplicated samples was applied. The targets were obtained from the same mRNA preparations.

Quantification of PAI-1 expression. The relative amounts of tumor (T) and corresponding normal tissue (N) mRNA that were

normalized to an internal control GAPDH mRNA were calculated. The logarithmic scale was applied to better understand it, as described previously (11, 12). The PAI-1 expression level was defined as follows: PAI-1 expression level = log e (the amount of tumor (T) or corresponding normal tissue (N) mRNA / the amount of internal control GAPDH mRNA).

Statistical analysis. Data were expressed as means \pm s.e. Analyses were performed treating PAI-1 expression in tumor and corresponding normal tissues as log-transformed continuous variables. The association between expression levels of tumor and corresponding normal tissues was analyzed by computing Spearman's correlation coefficient. Associations between PAI-1 expression levels of normal tissues and clinicopathological characteristics were examined by means of Fisher's exact test. $P < 0.05$ (two-tailed) was considered significant.

Results

The PAI-1 expression was analyzed in 113 digestive tract cancer samples (49 esophageal carcinomas and 64 colorectal cancer specimens) using a quantitative RT-PCR. To search for the factors that determine PAI-1 expression levels in esophageal and colorectal cancers, PAI-1 expression levels were also examined in corresponding normal tissues. The logarithmic distribution of the PAI-1 expression levels in corresponding normal tissues is shown in Figure 1. The average was -8.3 ± 0.39 and -6.7 ± 0.34 in corresponding esophageal and colorectal normal tissues, respectively. In all cases, the median expression levels (i.e., -7.7 and -6.1 of the log value) were chosen as the threshold values to be used for further analysis (Tables I, II).

Table I. Clinicopathological features and PAI-1 expression in corresponding normal tissues of esophageal cancer specimens.

Clinico-pathological feature	Variable	No. of cases	PAI-1 expression ^a		P-value ^b
			High	Low	
Age	<65	29	14	15	0.77
	65≤	20	11	9	
Gender	male	41	18	23	0.14
	female	8	6	2	
Pathological type	well, moderate ^c	40	15	25	0.0006
	poor ^d	9	9	0	
Depth of tumor invasion	≤mt ^e	14	5	9	0.35
Lymph node metastasis	-	35	19	16	
	+	30	18	12	
Distant metastasis	-	44	19	25	0.022
	+	5	5	0	
Total		49	24	25	

^aHigh, PAI-1 expression level >-7.7; low, PAI-1 expression level <-7.7; ^bFisher's exact test; ^cwell- and moderately-differentiated squamous cell cancer; ^dpoorly-differentiated squamous cell cancer; ^emuscular tunic.

In previous studies, quantitative RT-PCR for the PAI-1 gene was performed in esophageal and colorectal cancers (4, 5). In this study, we attempted to examine the association between the PAI-1 expression levels in these cancer specimens and those in corresponding normal tissues. The scattered plot of PAI-1 expression levels in tumor and corresponding normal tissues is shown in Figure 2. A significant correlation was found between esophageal and colorectal cancer and the corresponding normal PAI-1 expressions with a Spearman's rank correlation coefficient of 0.77 ($p<0.0001$; Figure 2a) and 0.81 ($p<0.0001$; Figure 2b), respectively. In previous studies, PAI-1 overexpression was significantly associated with malignancy of esophageal and colorectal cancer specimens. Taken together, PAI-1 overexpression in esophageal and colorectal cancers might originate from higher PAI-1 expression in the corresponding normal tissues and may result in a malignant phenotype of these cancers.

According to the above results, the PAI-1 expression levels in corresponding normal tissues might also predict the malignancy of esophageal and colorectal cancers. As expected, a significant increase in PAI-1 expression was observed in corresponding normal tissues with distant metastasis-positive esophageal and colorectal cancers compared to those with distant metastasis-negative cancer ($p=0.022$ and $p=0.0048$, respectively). These results are summarized in Tables I and II.

Table II. Clinicopathological features and PAI-1 expression in corresponding normal tissues of colorectal cancer specimens.

Clinico-pathological feature	Variable	No. of cases	PAI-1 expression ^a		P-value ^b
			High	Low	
Age	<70	42	24	18	0.19
	70≤	22	8	14	
Gender	male	39	17	22	0.31
	female	25	15	10	
Pathological type	tub ^c	51	24	27	0.54
	poor, muc, sig ^d	13	8	5	
Tumor size (mm)	<50	30	12	18	0.21
	50≤	34	20	14	
Tumor site	C, A, T ^e	17	7	10	0.57
	D, S, R ^f	47	25	22	
Depth of tumor invasion	≤mt ^g	43	21	22	>0.99
Lymph node metastasis	-	21	11	10	
	+	27	13	14	>0.99
Distant metastasis	-	37	19	18	
	+	56	24	32	0.0048
Total		64	32	32	

^aHigh, PAI-1 expression level >-6.1; low, PAI-1 expression level <-6.1; ^bFisher's exact test; ^ctub, tubular adenocarcinoma; ^dpor, poorly-differentiated adenocarcinoma; muc, mucinous adenocarcinoma; sig, signet-cell adenocarcinoma; ^eC, cecum; A, ascending colon; T, transverse colon; ^fD, descending colon; S, sigmoid colon; R, rectum; ^gmt, muscular tunic.

Discussion

Plasminogen activator inhibitor-1 (PAI-1), a 45-kDa serine proteinase inhibitor with a reactive peptide bond, Arg³⁴⁵-Met³⁴⁶, is a multifaceted proteolytic inhibitor that not only functions as a fibrinolytic inhibitor, but also plays an important role in signal transduction, cell adherence and cell migration (13-17). Studies with transgenic mice revealed a functional role for PAI-1 in wound healing, atherosclerosis, metabolic disturbances, such as obesity and insulin resistance, tumor angiogenesis (18-20), chronic stress, bone remodeling, asthma, rheumatoid arthritis, fibrosis, glomerulonephritis and sepsis.

In previous studies, many researchers emphasized the importance of PAI-1 produced by tumor cells that should be a key factor for the malignant change in several human cancers (21-25). We also demonstrated that PAI-1 overexpression was significantly correlated with tumor progression and a poor prognosis in esophageal and colorectal cancer (4, 5). However, little was hitherto known about the mechanisms of the PAI-1 overexpression in these types of cancer.

In the present study, we demonstrated the possibility that PAI-1 overexpression in esophageal and colorectal cancer

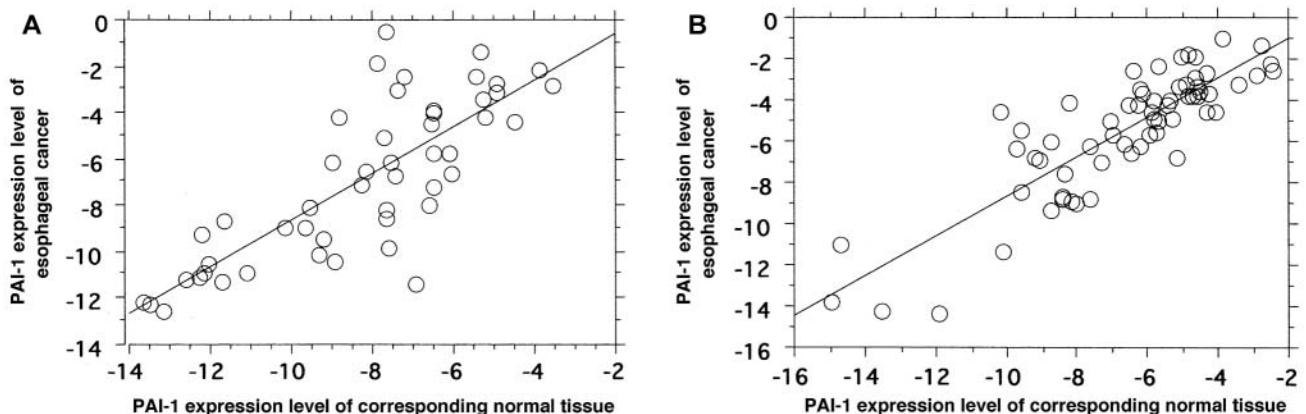


Figure 2. Scattered plot analysis of PAI-1 expression levels of tumor and corresponding normal tissues. A) Significant correlation between esophageal cancer and corresponding normal PAI-1 expressions, with a Spearman's rank correlation coefficient of 0.77 ($p<0.0001$). B) Significant correlation between colorectal cancer and corresponding normal PAI-1 expressions, with a Spearman's rank correlation coefficient of 0.81 ($p<0.0001$).

might originate from higher PAI-1 expression in the corresponding normal tissues and result in a malignant phenotype. This result suggested that higher PAI-1 expression in normal tissues might be a factor promoting more malignant cancer derived from those tissues, while carcinogenesis is a different matter. With regard to the relationship between normal tissues and carcinogenesis, Cui *et al.* reported that loss of imprinting (LOI) of the IGF2 gene was preferentially revealed in the normal colonic tissues of colorectal cancer patients compared to those of healthy individuals (26). The LOI of IGF2 can be assayed with a DNA-based blood test and it may be a predictive marker of an individual's risk for colorectal cancer. In the same way, PAI-1 expression in esophageal and colorectal tissues might be a predictive marker of the malignancy of cancer, if cancer arises from these tissues.

Although the precise mechanism by which PAI-1 overexpression in tumor cells promotes malignancy remains to be elucidated, it is suggested that PAI-1 expression in esophageal and colorectal cancers could be inherited from the corresponding normal tissues. The present study, thus, provides a solid basis for additional studies on the molecular mechanism of PAI-1 expression in esophageal and colorectal cancer and their corresponding normal tissues.

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