Jpn. J. Cancer Res. 87, 480–487, May 1996

Inverse Correlation between mRNA Expression of Plasminogen Activator Inhibitor-2 and Lymph Node Metastasis in Human Breast Cancer

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We examined mRNA expressions of urokinase-type plasminogen activator (u-PA), its specific receptor (u-PR), and plasminogen activator inhibitors (PAI-1 and PAI-2) in 50 human breast cancers by the reverse transcriptase-polymerase chain reaction method. The expressions of the genes are discussed in relation to the clinicopathological findings. In the overall population in breast cancers, a low level of PAI-2 expression was significantly associated with lymph node involvement ($P<0.0001$). The u-PA, u-PR, and PAI-1 expressions tended to be at high levels in such metastatic cancers. Also, positive expression of u-PA, u-PR, and PAI-1 was significantly correlated with negative expression of PAI-2. These results indicate that PAI-2 may play a critical role in the regulation of extracellular matrix degradation during tumor cell invasion and metastasis, and the expression of PAI-2 may be useful as a marker to evaluate the prognosis of breast cancers.

Key words: PAI-2 — Lymph node metastasis — Breast cancer — RT-PCR

The urokinase-type plasminogen activator (u-PA) is a member of the serine protease family. It is important in degradation of the extracellular matrix, which is considered to be an essential step in metastasis.[1)] The u-PA catalyzes the conversion of plasminogen into plasmin, while it is inactivated by several inhibitors such as plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2). It has been reported that over-expressions of u-PA, its specific receptor (u-PR), and PAI-1 correlate with reduction of the disease-free interval better than axillary node status or tumor size in malignant tumors, including lung or colon cancer.[2,3)] The involvement of u-PA, u-PR, and PAI-1 in the invasion and metastasis of breast cancer has been well documented.[4,5)] However, there is little information about the physiological significance of PAI-2 in the micro-environment of cancer cells. Synthesis of u-PA is modulated by a variety of effector molecules, such as phorbol esters, growth factors, hormones, and retinoids.[6)] In particular, basic fibroblast growth factor (bFGF) and transforming growth factor-α (TGF-α) can modulate the extracellular proteolytic activity by increasing the secretion of u-PA.[7,8)] It has been reported that adhesion molecules are also implicated in elevated u-PA production in cancer cells. Pathological examination has shown that reduced E-cadherin, a family of calcium-dependent cell adhesion molecules, is associated with differentiation and metastasis in primary human tumors.[9,10)] When T47D and MCF-7 human differentiated breast carcinoma cells were treated with an E-cadherin antibody, the cells dissociated from each other and lost their epithelioid morphology, in parallel with a rise in the secretion of u-PA into the extracellular milieu.[11)]

In order to evaluate the significance of u-PA and related factors, especially PAI-2, for the malignancy of human breast cancers, we analyzed mRNA expression of the genes by the reverse transcriptase-polymerase chain reaction (RT-PCR) method and the Southern blotting method, and also investigated the relationship among the expressions of genes of the urokinase system and various factors, such as E-cadherin, estrogen receptor, bFGF, and TGF-α.

MATERIALS AND METHODS

Cell culture Human breast cancer cell lines, MCF-7, T47D, BT-20, BT-549, Hs578T, MDA-MB-435, MDA-MB-231 from American Type Culture Collection , and YMB-1-E from the Japan Cancer Research Resources Bank were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum at 37°C, with 5% CO_2.

Clinical specimens Human breast cancer specimens were obtained at surgery in the School of Medicine, Kanazawa University from 1991 to 1994, and after histological examination, a part of the tissue was kindly provided by Dr. M. Earashi and stored under liquid nitrogen cooling until use. The 50 tumors included 6 papillary carcinomas, 43 invasive ductal carcinomas, and one mucinous carcinoma (classified by WHO criteria) and were from women of 37 to 71 years old.

RNA extraction and cDNA synthesis by reverse transcription RT-PCR analysis was done by a modification of the method of Conboy *et al.*[12)] Briefly, total RNAs from cultured breast cancer cells and breast cancer tissues (about 100 mg) were extracted with 1 ml of ISOGEN (Nippon Gene, Tokyo) as described by Chomczynski and Sacchi.[13)] The resulting total RNAs (1 μg)

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were mixed with an antisense primer (50 pmol) (Table I), incubated for 15 min at 68°C and then quickly chilled on ice for 5 min. The RNA samples were reverse-transcribed at 40°C for 90 min with RT solution [50 m*M* Tris-HCl pH 8.3, 40 m*M* KCl, 8 m*M* $MgCl_2$, 0.5 m*M* each dNTPs, 225 μg/ml bovine serum albumin, 5 m*M* dithiothreitol, 20 units of RNasin (Promega, Madison, WI), and 4 units of AMV reverse transcriptase (Life Science, St. Petersburg, FL)] in a total volume of 20 μl and the resulting cDNA was used for PCR.

PCR amplification and Southern blot hybridization The cDNA was amplified by adding 80 μl of PCR mixture [50 m*M* Tris-HCl pH 8.3, 40 m*M* KCl, 8 m*M* $MgCl_2$, 0.5 m*M* each dNTPs, 50 pmol of each sense and anti-sense primer, and 2.5 units of *Taq* polymerase (Takara, Kyoto)]. Oligonucleotides used for the RT-PCR and Southern blot hybridization are shown in Table I. Three cycles of PCR, consisting of denaturing for 1 min at 94°C, annealing for 2 min at 48°C, and extension for 2 min at 72°C per cycle were done, followed by 25 cycles of 40 s at 94°C, 1 min 30 s at 48°C, 1 min 20 s at 72°C on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). PCR products were electrophoresed on a 1% agarose gel with a 100-bp DNA ladder (GIBCO BRL, Grand Island, NY) as a size marker and visualized by ethidium bromide staining. Then the products were

Table I. Oligonucleotides for RT-PCR and Southern Blot Hybridization

Target gene	Primer and probe[a)]	Location	Expected size of PCR product (bp)	Reference No.
u-PA	5′-AGAATTCACCACCATCGAGA-3′	2494–2513		
	5′-ATCAGCTTCACAACAGTCAT-3′	4174–4193	474	26)
	5′-AGGCAGATGGTCTGTATAGT-3′	3707–3726		
u-PR	5′-TTACCTCGAATGCATTTCCT-3′	356–375		
	5′-TTGCACAGCCTCTTACCATA-3′	791–810	455	27)
	5′-TCATCAGACATGAGCTGTGA-3′	380–399		
PAI-1	5′-ATGGGATTCAAGATTGATGA-3′	379–398		
	5′-TCAGTATAGTTGAACTTGTT-3′	811–830	452	28)
	5′-AGAGAGCCAGATTCATCATCAAT-3′	584–606		
PAI-2	5′-TAAGCTGTTTGGTGAGAAGT-3′	9658–9677		
	5′-TACATCATCTGTACAGGTGT-3′	14845–14846	327	29)
	5′-TAGACTTCCTAGAATGTGCA-3′	10254–10273		
E-Cadherin	5′-ACCTCTGTGATGGAGGTC-3′	1165–1182		
	5′-CCACATTCGTCACTGCTACG-3′	1789–1808	546	30)
	5′-AACGTCGTAATCACCACACT-3′	1498–1517		
ER	5′-GAGGGAGAATGTTGAAACACA-3′	1142–1162		
	5′-GCCAGGCTTTGTGGATTTGAC-3′	1450–1470	329	31)
	5′-CTAGGATCTGATTGCATTGCAC-3′	1253–1274		
bFGF	5′-GTGTGTGCTAACCGTTACCT-3′	649–668		
	5′-GCTCTTAGCAGACATTGGAAG-3′	865–885	237	32)
	5′-GGAAGATTACTGGCTTCTATGT-3′	685–708		
TGF-α	5′-CGCCCTGTTCGCTCTGGGTAT-3′	188–208		
	5′-AGGAGGTCCGCATGCTCACAG-3′	593–612	425	33)
	5′-GACAAGCCAGCATGTGTCTGCCA-3′	363–385		
α-Catenin	5′-TATGCACTCAATAACTTTGA-3′	929–948		
	5′-GATTGAGGTTGCCAACTTGG-3′	1381–1400	473	34)
	5′-ACTTGCGTAGACAGCTCCGCA-3′	1221–1241		
β-Catenin	5′-TGTATGAGTGGGAACAGGGA-3′	388–407		
	5′-AGCCTTATTAACCACCACCT-3′	694–713	326	35)
	5′-ACGTGCAATCCCTGAACTGA-3′	649–668		
β-Actin	5′-GAAAATCTGGCACCACACCTT-3′	245–265		
	5′-TTGAAGGTAGTTTCGTGGAT-3′	817–836	592	36)
	5′-ACTGACTACCTCATGAAGAT-3′	656–675		
TFR	5′-ACAGACTCTACATGTAGGAT-3′	1338–1357		
	5′-AAACCTTGAAGTTGCTGGT-3′	1733–1752	415	37)
	5′-TATCCTCTAGCCATTCAGT-3′	1654–1673		

a) The top row is the sense primer, the middle row is the antisense primer, and the bottom row is the probe for Southern blot hybridization. ER, estrogen receptor; TFR, transferrin receptor.

transferred to a nylon membrane filter (Hybond N, Amersham International plc, Buckinghamshire) and hybridized to a ^{32}P-end labeled probe specific for the target cDNA fragment. The membrane was analyzed by autoradiography and the statistical significance of differences between the expression of genes was evaluated by using the χ^2-test. All experiments were repeated 2 to 5 times with similar results.

RESULTS

mRNA expressions of u-PA related factors, E-cadherin, estrogen receptor (ER), α-catenin, β-catenin, bFGF, and TGF-α in 8 human breast cancer cell lines The mRNA expressions of u-PA, u-PR, PAI-1, PAI-2, E-cadherin, ER, α-catenin, β-catenin, bFGF, and TGF-α in 8 human breast cancer cell lines were examined by RT-PCR anal-

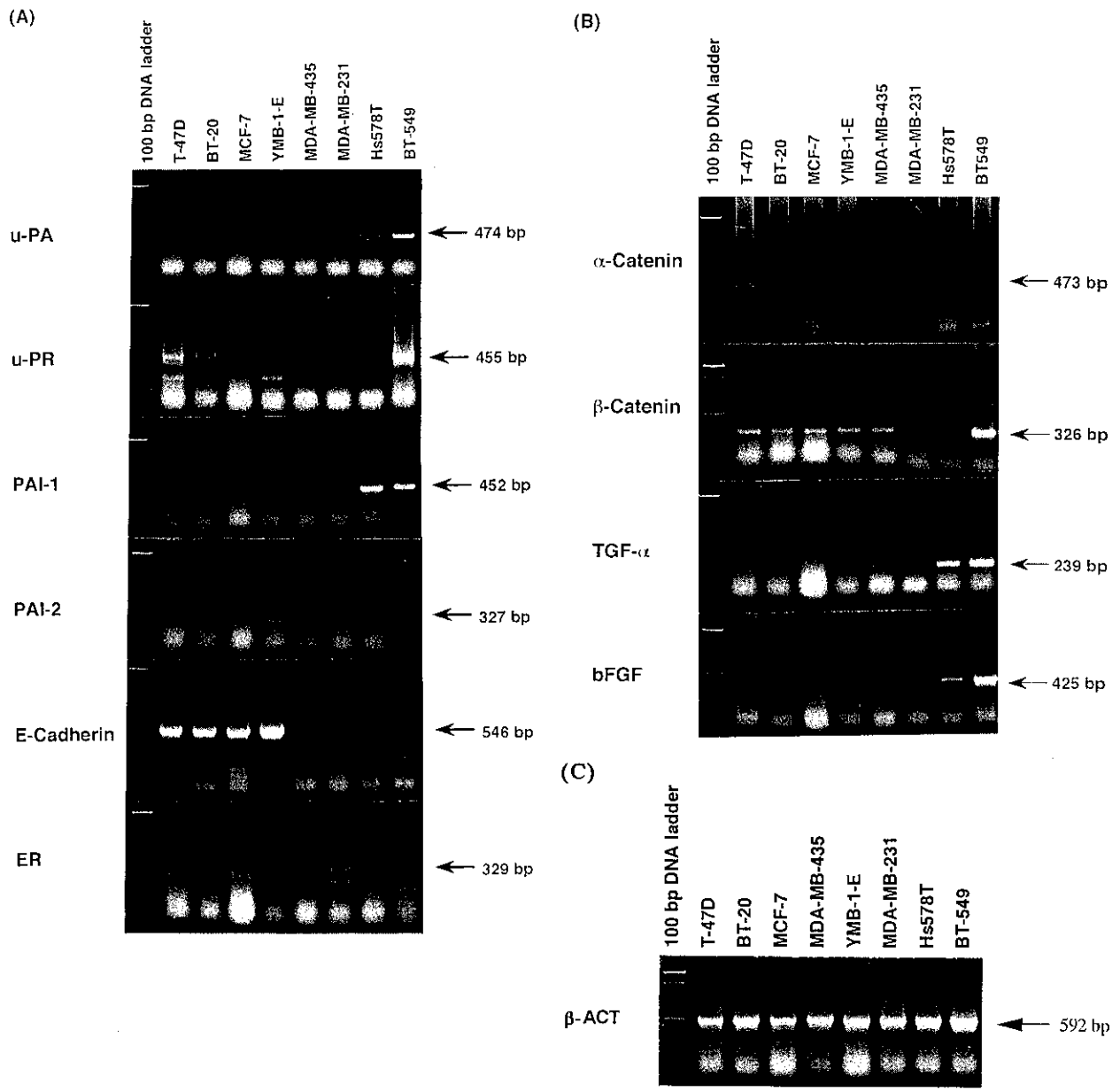

Fig. 1. Gene expressions of various factors analyzed by RT-PCR in 8 human breast cancer cell lines. The products were electrophoresed on 1% agarose gel. The expected sizes (bp) of the RT-PCR products are indicated on the right. Expression of β-actin was evaluated as a control.

ysis. The amplified cDNA fragments from each mRNA were detectable on an agarose gel by ethidium bromide staining, as presented in Fig. 1. The PCR products were detected and confirmed by Southern blot hybridization with a specific probe. Metastatic ability of each cell line has been reported.[14-16] Briefly, 4 cell lines (BT-549, Hs578T, MDA-MB-435 and MDA-MB-231) are highly metastatic and locally invasive *in vivo*. Another 4 cell lines (MCF-7, BT-20, T47D and YMB-1-E) are non-metastatic. The u-PA mRNA was expressed in 7 cell lines (BT-549, Hs578T, MDA-MB-435, MDA-MB-231, MCF-7, BT-20 and YMB-1-E) except T47D, and the PAI-1 mRNA was expressed in 2 cell lines (BT-549 and Hs578T). The levels of u-PA and PAI-1 mRNA expressions tended to be higher in the metastatic cell lines than the non-metastatic cell lines. The u-PR mRNA was expressed in 5 cell lines (BT-549, MDA-MB-435, MCF-7, BT-20 and T47D) and the PAI-2 mRNA was expressed in 3 cell lines (MCF-7, YMB-1-E and Hs578T). On the other hand, E-cadherin was significantly expressed in the non-metastatic cell lines (MCF-7, BT-20, T47D and YMB-1-E), but not in the metastatic cell lines (BT-549, Hs578T, MDA-MB-435 and MDA-MB-231). β-Catenin mRNA was expressed in all of the cell lines, but α-catenin mRNA expression was observed in one metastatic cell line (BT-549) and in 3 non-metastatic cell lines (T47D, MCF-7 and YMB-1-E). The bFGF and TGF-α mRNA expressions tended to be high in the metastatic cell lines. For example, in the highly metastatic cell line BT-549, the u-PA, u-PR, PAI-1, bFGF and TGF-α mRNAs were highly expressed but PAI-2 and E-cadherin mRNAs were not detectable. In the non-metastatic cell line YMB-1-E, the expression of u-PA, PAI-1, bFGF and TGF-α mRNAs was decreased, but PAI-2 and E-cadherin mRNAs were expressed at high levels. There was no significant correlation between mRNA expression of ER and metastatic ability.

u-PA mRNA and related factors in human breast cancer

To evaluate the relationship between the gene expression of the urokinase system and malignancy of breast cancer *in vivo*, we examined the gene expressions of u-PA, u-PR, PAI-1, and PAI-2 in 50 human breast cancer specimens and 6 normal breast tissues by RT-PCR analysis. The results were compared with the clinicopathological findings. Among 22 specimens with lymph node metastasis, positive expression of the u-PA gene was observed in 20 cases (90.9%), that of the u-PR gene in 20 cases (90.9%), and that of PAI-2 in only 3 cases (13.6%). On the other hand, positive expression of PAI-2 was seen in 24 cases (85.7%) of the other 28 specimens without lymph node metastasis, and that of u-PR in 19 cases (53.5%). In cases 1, 6, 10, and 19 with lymph node metastasis, mRNA expression of u-PA, u-PR, and PAI-1 tended to be at high levels but PAI-2 expression was diminished or completely deficient. Cases 23, 28, 32, and 39, which were without lymph node involvement, had low levels of u-PA, u-PR, and PAI-1 expression and high levels of PAI-2 expression, while such tendencies were not observed in normal tissues (Fig. 2, Table II). The relationship between the expression of the genes and

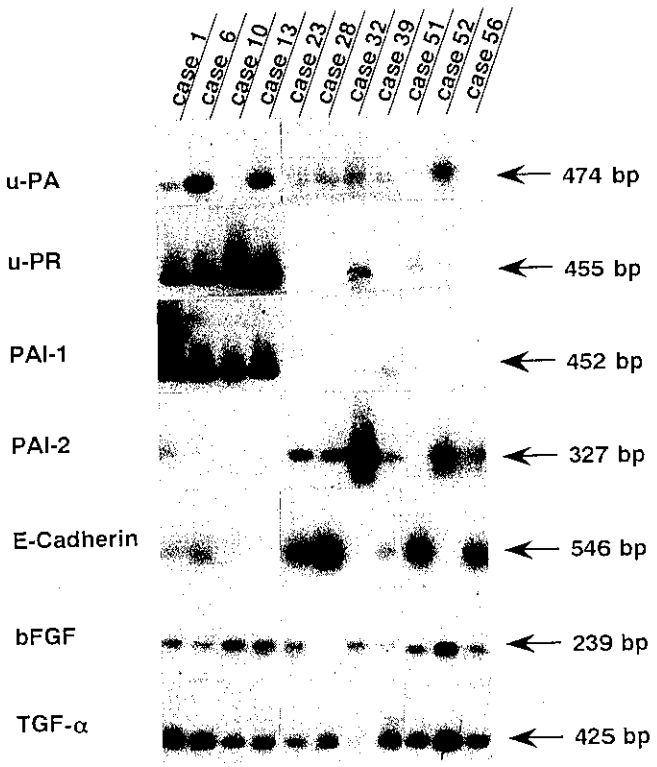

Fig. 2. Gene expression of u-PA and related factors in breast cancer specimens and normal breast tissues. The products were electrophoresed on 1% agarose gel and then hybridized to a ^{32}P-end-labeled probe specific for the target cDNA.

Table II. mRNA Expression of u-PA-Related Genes in Normal Breast Tissue[a)]

Case No.	51	52	53	54	55	56
u-PA	−	+	−	−	−	−
u-PR	+	−	−	−	−	−
PAI-1	−	−	−	−	−	−
PAI-2	−	+	−	−	−	+
E-Cadherin	+	−	+	−	−	+
ER	−	−	−	−	−	−
bFGF	+	−	−	−	−	+
TGF-α	+	+	−	−	−	+

a) Positive expression of genes is represented as +, and negative expression as −.

lymph node metastasis was examined by use of the χ^2-test. The results are presented in Table III, and indicate that diminished expression of PAI-2 mRNA was significantly correlated with lymph node metastasis ($P<0.0001$), while positive mRNA expression of u-PR was related to lymph node metastasis.

Expressions of E-cadherin, ER, bFGF, and TGF-β in human breast cancer We also examined mRNA expressions of E-cadherin, ER, α-catenin, β-catenin, bFGF, and TGF-α in 50 human breast cancers. Decreased E-cadherin expression correlated with lymph node metastasis ($P<0.05$) but no significance or tendency related to lymph node metastasis were seen in the mRNA expression of ER, bFGF, or TGF-α.

Relationship between factors (u-PA, u-PR, PAI-1, PAI-2, E-cadherin, and ER) The interrelation of the factors was analyzed by using the χ^2-test (Table IV). In the urokinase system, the expression of u-PA and u-PR was significantly associated ($P<0.05$), but no correlation was found between the other u-PA related factors. The expressions of E-cadherin and PAI-2 or of E-cadherin and ER were correlated with each other ($P<0.05$). The expression of E-cadherin was also associated with that of u-PA in the cases with lymph node metastasis ($P<0.05$) (data not shown).

Table III. Relationship between Nodal Metastasis and Gene Expressions

		Lymph node metastasis		P value
		Po[a]	N[a]	
u-PA	Po[a]	20	21	0.1461
	N[a]	2	7	
u-PR	Po	20	19	0.0508
	N	2	9	
PAI-1	Po	17	18	0.3199
	N	5	10	
PAI-2	Po	3	24	<0.0001
	N	19	4	
E-Cadherin	Po	9	21	0.0146
	N	13	7	
ER	Po	10	14	0.7495
	N	12	14	

Statistical significance of differences in gene expression was evaluated by the χ^2-test, with a P value less than 0.05 as the criterion of significance.
a) Po, positive; N, negative.

DISCUSSION

Metastasis is a complex phenomenon involving dissociation of cancer cells from a primary site, invasion of stromal tissue and basement membrane, adhesion to a target organ, and cell growth.[17, 18] The urokinase pathway for plasminogen activation, which is called the urokinase system, has been implicated in proteolytic degradation of extracellular matrix by invasive and metastatic cancer cells.[19] It is considered that u-PA is a key enzyme in the activation cascades of extracellular matrix degrading enzymes, because plasmin participates in the activation of precursors of matrix metalloproteinases. However, the practical significance of the relationship between the u-PA system and malignancy is not fully clarified. We have examined the mRNA expression of u-PA, u-PR, PAI-1, and PAI-2 in human breast cancer by the RT-PCR method. The results were compared with the clinicopathological findings to elucidate the relationship between lymph node metastasis and the gene expres-

Table IV. Relationship between Various Factors in Human Breast Cancer[a]

		u-PR		P value	PAI-1		P value	PAI-2		P value	E-cadherin		P value
		Po[b]	N[b]		Po	N		Po	N		Po	N	
u-PA	Po	33	2	<0.0001	24	11	0.409	19	10	0.086	20	15	0.851
	N	6	9		12	3		12	3		9	6	
		u-PR		P value	PAI-1		P value	PAI-2		P value	ER		P value
		Po	N		Po	N		Po	N		Po	N	
E-Cadherin	Po	22	7	0.288	23	6	0.176	22	7	0.014	20	9	0.011
	N	13	8		13	8		9	12		5	16	
		u-PA		P value	u-PR		P value	PAI-1		P value	PAI-2		P value
		Po	N		Po	N		Po	N		Po	N	
ER	Po	19	6	0.355	20	5	0.123	16	9	0.208	13	12	0.145
	N	16	9		15	10		20	5		18	7	

a) Statistical significance of differences in expression of various factors was evaluated by the χ^2-test, with a P value less than 0.05 as the criterion of significance.
b) Positive expression of factors is indicated as Po, and negative expression as N.

sion of the u-PA system. Lymph node metastasis is an important prognostic indicator in human breast cancer. In highly metastatic cell lines the expression levels of u-PA and PAI-1 tended to be high, but the relation of PAI-2 expression to metastatic ability was obscure.

We examined the gene expression of these factors in 50 human breast cancer specimens. The same association between gene expressions and lymph node metastasis was observed. In particular, depressed PAI-2 mRNA expression was significantly correlated with lymph node metastasis, and positive expression of PAI-2 mRNA was conversely correlated to absence of lymph node metastasis ($P<0.0001$). The results support the findings that PAI-2 antigen detected by the immunoenzymic method is correlated with a shortened disease-free survival in breast cancer[20] and that occurrence of lymph node metastasis follows diminished PAI-2 expression in non small cell lung carcinoma.[21] The plasminogen activation system consists of two types of plasminogen activators, u-PA and t-PA, and inhibitors of PAI-1, PAI-2, and PAI-3.[22] From our findings, it is suggested that PAI-2 is a more important inhibitor for u-PA activity than PAI-1, which is continuously expressed in breast cancer tissues. Also, u-PR gene expression was observed in 90.9% of the cases with lymph node metastasis and in 53.5% of the cases without it. Such u-PR expression almost always accompanied positive u-PA expression. The activity of specific receptor binding by u-PA is considered to be important in invasion and metastasis. In breast cancers, its activity is probably important in formation of lymph node metastasis.[5, 23]

E-Cadherin is a family of calcium-dependent cell to cell adhesion molecules, and pathological examination has shown that reduced E-cadherin immunoreactivity is associated with differentiation and metastasis in primary human tumors *in vivo*.[24] In this study, the gene expression of E-cadherin was high in non-metastatic cell lines, but in the metastatic cell lines the expression was completely lost. In breast cancer specimens, decreased E-cadherin expression was significantly correlated with lymph node involvement in breast cancer. It has been demonstrated that tumor cell invasion coincident with u-PA stimulation was triggered by the loss of E-cadherin function.[11] Our data suggest that the positive expression of u-PA is significantly associated with the depression of E-cadherin expression in breast cancer specimens with lymph node metastasis. Also, PAI-2 expression was significantly associated with E-cadherin expression ($P<0.05$). On the other hand, several growth factors influence cell adhesion[25] and modulate the extracellular proteolytic activity by enhancing the secretion of u-PA and PAI-1.[9] We also examined the gene expression of bFGF and TGF-α. Both growth factors were expressed at high levels in metastatic cell lines and at low levels in the non-metastatic cell lines, but no correlation between these growth factors and u-PA, PAI-1, or E-cadherin was observed. It has been reported that a high level of PAI-1 was associated with low estrogen and progesterone receptor levels.[4] In our study, we could not find any association between PAI-1 and ER. However, the expression of E-cadherin was significantly correlated with that of ER and so decreased expression of E-cadherin and ER may be associated with poor prognosis.

Although the five-year survival rate has greatly improved, metastasis remains the leading cause of mortality in breast cancer. Lymph node metastasis is the most important prognostic parameter, and the prognosis for cancer patients is significant for deciding on therapy. Our results suggest that PAI-2 in the u-PA system is available as a prognostic marker for lymph node involvement in breast cancer and that the expressions of the u-PA system components, especially u-PA, u-PR, and PAI-2, are closely associated with E-cadherin and the ER.

ACKNOWLEDGMENTS

We thank Dr. M. Earashi of Kanazawa University for providing human breast cancer specimens. N. I. is deeply indebted to Prof. Y. Watanabe of Kanazawa University for his continued interest and support. This work was supported in part by Grants-in-Aid for Cancer Research and the 2nd Term Comprehensive Ten-Year Strategy for Cancer Control from the Ministry of Health and Welfare, and Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

(Received November 6, 1995/Accepted January 25, 1996)

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