Interpretation of Pin-1 and VEGF-C expression in breast infiltrating duct carcinoma

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Abstract. Pin-1 has been shown to regulate several phases of the cell cycle and is strikingly overexpressed in many human cancers. Vascular endothelial growth factor (VEGF)-C is a potent lymphangiogenic factor produced by tumor and stromal cells. However, little is known about the roles of Pin-1 and VEGF-C in breast carcinoma. p53 protein and cyclin D1 overexpressions have been shown to play a role as prognostic factors in many human cancers. To better understand the roles of Pin-1 and VEGF-C in breast carcinoma, we evaluated the immunohistochemical expression of Pin-1 and VEGF-C in relationship with p53 protein or cyclin D1 overexpression and clinicopathological parameters in 128 mammary infiltrating duct carcinomas. There was a positive expression in 100% of Pin-1, 88% of VEGF-C, 35% of p53 protein, and 66% of cyclin D1 in the breast carcinoma. Correlation of the positive expression of Pin-1 with tumor grade ($p<0.01$) and lymph node metastasis or cyclin D1 overexpression ($p<0.05$, respectively) was statistically significant. Significant correlation was observed between VEGF-C and tumor grade, lymph node metastasis or clinical stage ($p<0.01$, respectively). These results indicate that elevated Pin-1 or VEGF-C expression is more common in infiltrating duct carcinomas with poor prognostic characteristics and is partly associated with an unfavorable outcome. Given the role of cyclin D1 overexpression in oncogenesis of breast, these results suggest that overexpression of Pin-1 and VEGF-C may promote tumor progression and metastasis.

Introduction

Recently characterized peptidyl-prolyl *cis/trans* isomerase (PPIase) Pin-1 is involved in the control of cell cycle by phosphorylation through the epidermal growth factor, migrating from the nucleus to the cytoplasm, and inducing cell death. However, as Pin-1 has been revealed to be involved in oncogenesis in various organs, attention has been paid to its accurate action mechanism. Pin-1 accelerates the *cis/trans* isomerization of specific proteins, and consequent structural changes may exert a great effect on numerous Pin-1 substrates (1-4). Among them, the important oncogenic proteins ß-catenin and cyclin D1 have been characterized to be controlled by Pin-1 in such a manner (5,6). The overexpression of Pin-1 has been reported to be associated with the development of various cancers, and proportional to tumor grades. Pin-1 binds to phosphorylated c-Jun and increases cyclin D1 mRNA and protein in cells, whereas the suppression of endogenous Pin-1 decreases the transcriptional activity of phosphorylated c-Jun (6).

Lymphatic ducts are important in the metastasis of cancer, and lymph node metastasis is the most important prognostic factor determining the poor prognosis of various cancers. Among numerous studies on cancer metastasis, studies on lymphangiogenesis are ongoing actively, which is due to the discovery of the important lymphatic duct growth factors VEGF-C and -D, nonetheless, their precise role has not been characterized yet (7,8). VEGF-C, a member of the VEGF family, is a ligand of VEGF receptor-3 (VEGFR-3, Flt-4) that is known to be a specific marker of lymphatic endothelial cells and induces the proliferation of lymphatic ducts (9,10). VEGF-C has been known to not only control physiological angiogenesis and the development as well as progression of diverse angiogenic diseases but also to accelerate tumor lymhangiogenesis, and thus to induce dissemination of cancer cells and lymph node metastasis (11,12).

In Korea, the incidence of breast cancer is on the rise, it is the leading cancer among females despite the improvement of diagnosis and therapeutic methods, it is the leading cause of the cancer mortarlity in women (13). Among factors influencing the prognosis of breast cancer, tumor size and the presence or absence of lymph node metastasis have been reported to be the most important independent prognostic factors (14,15), and as supplement prognostic factors that allow to distinguish the high risk group with recurrence potential. Studies on the role of DNA ploidy, proliferation index (16), various receptors (17,18), oncogenes such as *Her-2/neu* and p53 protein, and tumor suppressor genes (19,20) have been conducted.

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Pin-1 and VEGF-C are known to play an important role in the formation, progression and metastasis of various tumors, nonetheless, such reports in breast cancer are scarce. Therefore, in infiltrating duct carcinoma patients, by immunohistochemical staining, we compared and analyzed based on several clinicopathological data including clinical stage, the survival length of patients, the age of patients, tumor size, histological grade, the number of lymph node metastasis, and examined the relationship of cyclin D1 with p53 protein. In addition, their effect on the progression of tumor and the prognosis of patient were statistically analyzed.

Materials and methods

Patients. Among the women who underwent mastectomy for infiltrating duct carcinoma at Chosun University Hospital (Gwangju, Korea) from January 1990 to June 1996, the present study was done in 128 patients whose paraffin embedded tissues were relatively well preserved. Informed consent was obtained from all patients, and research protocols were approved by the Ethics Committee of Chosun University Hospital. The relationship with survival was investigated in 123 patients, for 5 patients the follow-up was not possible. Patients who underwent chemotherapy or radio-therapy were excluded from the study. Patient age, tumor size, number of lymph node metastasis, and clinical stage were confirmed by reviewing patient charts and pathology files. Patient survival was confirmed through phone calls and mail. The range of follow-up after the first diagnosis was between 1-116 months (less than 40 months, 22 cases; 40-59 months, 52 cases; 60-79 months, 24 cases; 80-99 months, 24 cases; longer than 99 months, 6 cases).

Histological assessment. Histological grade of tumor cells used for the study was the modified version of Bloom-Richardson grading system used in the Nottingham/Tenovus Breast Cancer Study (21). Tumor size was divided into those <2 cm (T1), between 2-5 cm (T2), and >5 cm (T3) according to the TNM classification of the American Joint Committee on Cancer (22). Metastasis to axillary lymph node was divided into no metastasis (N0), 1-3 lymph node metastasis (N1), and 4 or more than 4 lymph node metastasis (N2) according to the criteria by Fisher *et al* (23). Clinical stage was divided according to the staging system set by the American Joint Committee on Cancer Staging (22).

Immunohistochemical staining. All tumors investigated in the study were tested for Pin-1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:400), VEGF-C goat polyclonal antibody (N-19, Santa Cruz Biotechnology; dilution 1:50), p53 mouse monoclonal antibody (DO-7, Dako, Glostrup, Denmark; dilution 1:200) and cyclin D1 rabbit polyclonal antibody (H-295, Santa Cruz Biotechnology; dilution 1:100). Immunolocalization for Pin-1, p53 protein and cyclin D1 was performed using a Histostain-Plus kits, broad spectrum (Zymed, San Franscisco, CA, USA) and immunolocalization for VEGF-C was performed using a goat ImmunoCruz™ staining system (Santa Cruz Biotechnology), according to the supplier's protocol. Briefly, 4-μm thick sections obtained after formalin fixation and paraffin embedding were deparaffinized in xylene and rehydrated with distilled water through graded concentrations of ethanol. Then the sections were placed in a glass jar with 10 mM citrate buffer (pH 6.0) and irradiated in a microwave oven for 15 min, and cooled down in the jar at room temperature for 20 min. Then, the slides were rinsed with Tris-buffered saline (TBS). After quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 10 min, blocking reagent was added for 10 min. The slides were then washed as before, and were subsequently subjected to the primary antibody reaction. Each primary antibody for Pin-1 and p53 protein was applied 1 h in a moist chamber at 37˚C. VEGF-C and cyclin D1 was applied in a moist chamber overnight at 4˚C. After washing with TBS, the biotinylated link antibody was applied for 10 min, followed by horseradish peroxidase (HRP) bound streptavidin for an additional 10 min. After washing with TBS, the localization of anti-bodies was visualized by incubating the sections for 15 min in HRP substrate and counterstaining with Mayer's hematoxylin. An isotype matched control antibody was also used. Positive control for VEGF-C was early placenta tissue, those for PIN-1 and p53 were colonic adenocarcinoma with strong nuclear staining in another study, and that for cyclin D1 was mantle cell lymphoma. Instead of the primary antibody, normal goat serum was used in negative control.

Analysis and interpretation of staining. In the staining for Pin-1, cases showing reaction within the nucleus was considered to be positive reaction, and depending on the reaction intensity, they were classified as strongly positive (3+), moderately positive (2+), weakly positive (1+), and negative (0, no staining) (24).

In the staining for VEGF-C, cases showing staining reaction within the cytoplasm was determined to be positive reaction, and the entire tumor cells were evaluated based on the specimen performed immunostaining, and cases without staining was determined to be negative (-), cases showing positive cells focally in <5% of tumor were 1+, cases positive in 5-20 % were 2+, and cases positive in >20% were 3+, and 2+ and 3+ were re-classified as the high expression group and 1+ as the low expression group (8).

Staining for cyclin D1 and p53 was determined positive when nuclear protein was stained red brown under optical microscope, was negative when nuclear staining was present in <5% of the area of tumor cells, 1+ when 5-10% of tumor cell nuclei was stained positive, 2+ when 11-50% was stained positive, and 3+ when >50% was stained positive (25).

Statistical analysis. SPSS (statistical package for the social sciences), Windows version 12 (SPSS, Korea) was used for statistical analysis. χ^2 test was used to determine the correlation between clinical stage, patient age, histological tumor grade, tumor size, and lymph node metastasis and expression patterns of Pin-1, VEGF-C, p53 and cyclin D1; correlation in the expression patterns among Pin-1, VEGF-C, p53 and cyclin D1; and correlation between the expression patterns of Pin-1, VEGF-C, p53 and cyclin D1 and survival. Wilcoxon rank

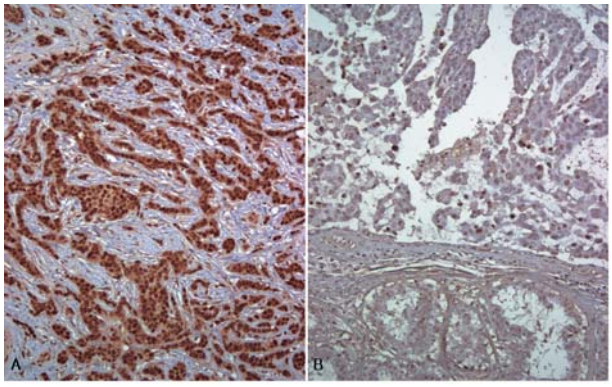

Figure 1. Immunohistochemical staining for Pin-1 in mammary infiltrating duct carcinoma. High grade carcinoma demonstrated strong positive nuclear staining (A) but, lower grade carcinoma demonstrated weak positive nuclear staining (B).

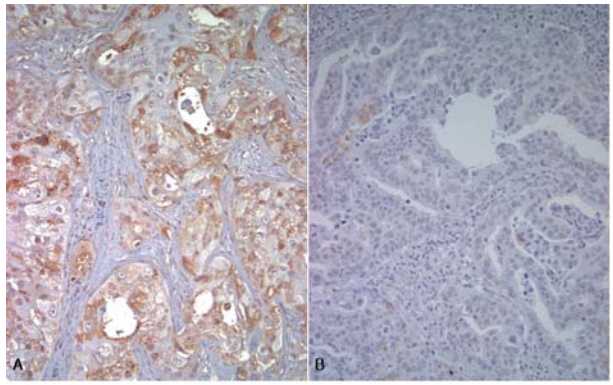

Figure 2. Immunohistochemical staining for VEGF in mammary infiltrating duct carcinoma. High grade carcinoma demonstrated strong positive cytoplasmic staining (A) but, lower grade carcinoma demonstrated weak positive cytoplasmic staining (B).

test was used for the analysis of Kaplan-Meier survival. Statistical significance was determined at $p<0.05$.

Results

Clinical data. The range of patient age was 25-79 years (average: 49 years). Age distribution according to each age group showed that 24 patients (19%) were under 40 years of age, 46 (36%) were between 40-49 years of age, 37 (29%) were between 50-59, and 21 (16%) were 60 or older than 60 years of age. The range of tumor size was between 0.5-8.5 cm (average: 2.7 cm) in which 56 patients (44%) showed tumors <2 cm, 62 (48%) had tumors between 2.0 and 5.0 cm, and 10 (8%) had tumors >5.0 cm.

Histological assessment. When tumors were divided according to the histological grading system used in the Nottingham/ Tenovus Breast Cancer Study, 31 cases (24%) were grade 1, 61 (48%) were grade 2, and 36 (28%) were grade 3. Lymph node metastasis was not present in 71 cases (56%), lymph node metastasis to 1-3 nodes was observed in 23 (18%), and to 4 or >4 nodes in 34 (27%). Clinical stage of tumor was stage 1 in 34 cases (27%), stage 2 in 77 (60%), and stage 3 in 17 (13%).

Immunohistochemical expression patterns of Pin-1, VEGF-C, p53 protein and cyclin D1. The weak staining (1+) of Pin-1 was detected even in the nucleus of normal breast tissues in the vicinity of tumor. In infiltrative lymphatic cancer cases, in most cases, moderately or strongly positive (2+ or 3+) staining was observed, and in cases with ductal carcinoma *in situ* (DCIS) in the vicinity, weak positive results (1+) were shown in most cases. In infiltrating duct carcinoma cases, among 128 cases of study subjects, none showed negative results, 14 cases were weak positive, 32 cases were moderately positive, and 82 cases (64%) showed strongly positive results (Fig. 1).

VEGF-C was observed in the cytoplasm of tumor cells, but it was not detected in normal breast tissues. Among 128 study sujects, 16 cases were negative, and 112 cases (87.5%) were positive, and among them, the distribution that 34 cases were 1+, 50 cases were 2+, 28 cases were 3+ (strongly positive group, 78 cases; weakly positive group, 34 cases) was shown. In regard to the histological tumor grade distribution of VEGF-C positive cases, grade I was 21 cases (67.7%), grade II was 55 cases (90.2%), and grade III was 36 cases (100%) (Fig. 2).

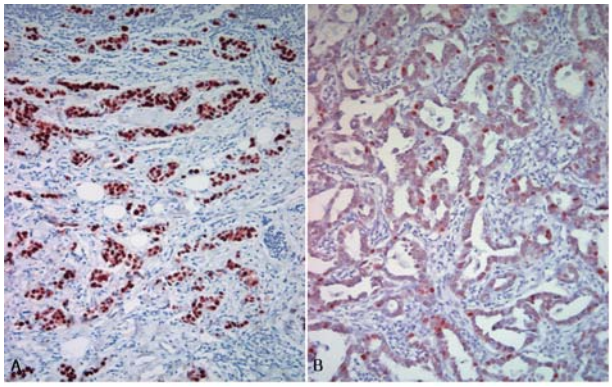

Figure 3. Immunohistochemical staining for p53 protein in mammary infiltrating duct carcinoma. High grade carcinoma demonstrated strong positive nuclear staining (A) but, lower grade carcinoma demonstrated weak positive nuclear staining (B).

Concerning p53 protein, positive results were detected only in the nucleus of tumor cells and not in normal tissues in the vicinity. Among the 128 cases, 45 cases (35.2%) were shown to be positive, 6 cases were 1+, 13 cases were 2+, and 26 cases were shown to be 3+. Examining the distribution of the histological tumor grade of p53 positive cases, grade I was 2 cases (6.5%), grade II was 27 cases (44.3%), and grade III was 16 cases (44.4%) (Fig. 3).

In addition, cyclin D1 was detected only in the nucleus of tumor cells, and it was not observed in normal tissues in the vicinity. Among 128 research subjects, 85 cases (66%) showed overexpression, and among them, weak overexpression in 28 cases (22%), moderate overexpression in 22 cases (17%), and strong overexpression in 35 cases (27%). In regard to the distribution of tumor grade of cases showing cyclin D1 overexpression, grade I was 9 cases (29.0%), grade

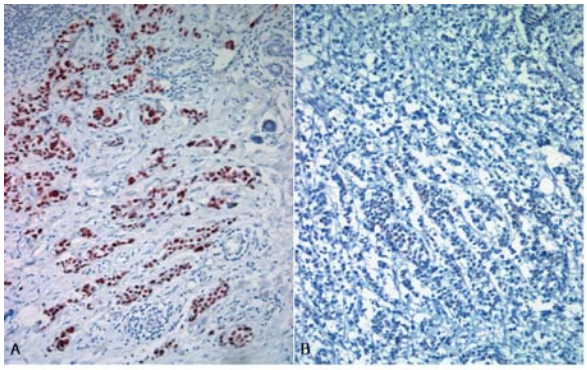

Figure 4. Immunohistochemical staining for cyclin D1 in mammary infiltrating duct carcinoma. High grade carcinoma demonstrated strongly positive nuclear staining (A) but, some high grade carcinoma demonstrated weakly positive nuclear staining (B).

II was 46 cases (75.4%), and grade III was 30 cases (83.3%) (Fig. 4).

Correlation between Pin-1 expression and clinicopathological parameters. The expression of Pin-1 and histological grade as well as the level of lymph node metastasis were statistically significant ($p<0.01$ and $p<0.05$, respectively), nonetheless, it was not significantly associated with the age of patients, tumor size, and clinical stage. In other words, in cases showing the positive expression of Pin-1, particlualry, in cases showing strong positive, the histological grade of tumor was elevated, and the result of the increase of lymph node metastasis was shown (Table I).

Correlation between VEGF-C expression and clinicopathological parameters. The expression of VEGF-C and histological grade, the level of lymph node metastasis and clinical stage ($p<0.01$ each) were statistically significant, but it did not significantly correlate to the age of patient and tumor size. In other words, cases showing the positive expression of VEGF-C, particularly cases showing strong positive, the result that the histological grade was increased, lymph node metastasis was increased, and the clinical stage of tumor was increased was shown (Table I).

Correlation between p53 protein expression and clinicopathological parameters. The expression of p53 protein and histological grade were statistically significantly correlated

Table I. The clinicopathological data according to the expression pattern of Pin-1, VEGF-C, p53 and cyclin D1 in mammary infiltrating duct carcinoma.

	Pin-1[a,b]				VEGF-C[a,c,d]				p53 protein[e]				Cyclin D1[b,e]			
	3+ (n=82)	2+ (n=32)	1+ (n=14)	- (n=0)	3+ (n=28)	2+ (n=50)	1+ (n=34)	- (n=16)	3+ (n=26)	2+ (n=13)	1+ (n=6)	- (n=83)	3+ (n=35)	2+ (n=22)	1+ (n=28)	- (n=43)
Age (years)																
≤39 (n=24)	17	6	1	0	5	11	7	1	5	2	1	16	7	5	6	6
40-49 (n=46)	28	11	7	0	9	18	12	7	9	5	2	30	14	9	11	12
50-59 (n=37)	22	10	5	0	10	12	10	5	8	5	0	24	9	6	7	15
≥60 (n=21)	15	5	1	0	4	9	5	3	4	1	3	13	5	2	4	10
Grade																
I (n=31)	8	8	15	0	1	9	11	10	1	0	1	29	3	2	4	22
II (n=61)	41	21	0	0	9	25	21	6	14	9	4	34	16	18	12	15
III (n=36)	33	3	0	0	18	16	2	0	11	4	1	20	16	2	12	6
Positive nodes																
0 (n=71)	36	22	13	0	2	27	27	15	11	6	3	51	20	8	17	26
1-3 (n=23)	15	7	1	0	6	14	3	0	7	3	2	11	1	9	4	9
≥4 (n=34)	31	3	0	0	20	9	4	1	8	4	1	21	14	5	7	8
Tumor size (cm)																
<2.0 (n=56)	36	11	9	0	11	20	15	10	10	4	3	39	15	12	12	17
2.0-5.0 (n=62)	39	19	4	0	14	25	18	5	13	5	1	43	18	9	14	21
>5.0 (n=10)	7	2	1	0	3	5	1	1	3	4	2	1	2	1	2	5
Stage																
I (n=34)	22	9	3	0	2	14	8	10	7	3	2	22	11	3	8	12
II (n=77)	47	19	11	0	17	32	23	5	15	7	3	52	19	18	16	24
III (n=17)	13	4	0	0	9	4	3	1	4	3	1	9	5	1	4	7

[a]Statistically significant p-value <0.01 in grade; [b]statistically significant p-value <0.05 in positive nodes; [c]statistically significant p-value <0.01 in positive nodes; [d]statistically significant p-value <0.01 in stage; [e]statistically significant p-value <0.05 in grade.

Table II. Interrelation between the Pin-1 and cyclin D1 immunoexpression in mammary infiltrating duct carcinoma (%).

	Pin-1				
	3+ (n=82)	2+ (n=32)	1+ (n=14)	- (n=0)	p-value
Cyclin D1					
3+ (n=35)	33 (94.3)	2 (5.7)	0	0	
2+ (n=22)	19 (86.4)	5 (22.7)	1 (4.6)	0	<0.05
1+ (n=28)	16 (57.1)	8 (28.6)	4 (14.3)	0	
- (n=43)	14 (32.6)	17 (39.5)	12 (27.9)	0	

Table III. Multivariate analysis on disease-related survival (Cox proportional hazard model) according to the clinicopathological variables in mammary infiltrating duct carcinoma.

Variable	Hazard ratio	95% confidence intervals
Age	0.991	0.961-1.023
Tumor grade	1.223	0.727-2.057
Positive nodes	2.041	1.260-3.305
Tumor size	2.107	1.279-3.472
Stage	0.689	0.308-1.542

(p<0.05), and it did not significantly correlate to the age of patient, the level of lymph node metastasis, tumor size or clinical disease stage. In other words, as tumor grade increased, strong increase of the expression of p53 protein was observed (Table I).

Correlation between cyclin D1 expression and clinicopathological parameters. The expression of cyclin D1 and histological grade as well as the level of lymph node metastasis correlated significantly (p<0.05 each), but it did not correlate to the age of patient, tumor size or clinical stage (Table I).

Correlation among the expression of Pin-1, VEGF-C, p53 protein and cyclin D1. When Pin-1 was strongly positive, cyclin D1 was strongly positive in 33 cases, moderately positive in 19 cases, weak positive in 16 cases, and negative in 14 cases. In cases where Pin-1 was moderately positive, cyclin D1 was strongly positive in 2 cases, moderately positive in 5 cases, weakly positive in 8 cases, and negative in 17 cases. When Pin-1 was weakly positive, cyclin D1 was strongly positive in 0 case, moderately positive in 1 case, weakly positive in 4 cases, and negative in 12 cases. In such a manner, when Pin-1 was expressed strongly, the expression of cyclin D1 was elevated significantly (p<0.05). However, among the expression of Pin-1, VEGF-C, and p53, and among the expression of VEGF-C, p53, and cyclin D1, a statistical significance was not observed (Table II).

Correlation between the expression of Pin-1, VEGF-C, p53 protein or cyclin D1 and patient survival or survival length. Depending on the staining intensity of Pin-1, VEGF-C and p53 protein, survival rates and the survival length showed a slight difference. Positive expression, particularly in cases showing strong positivity, a trend for decrease of survival rates and the survival length was shown, nonetheless, it was not statistically significant. The rate of survival depending on the overexpression of cyclin D1 was statistically significant (p<0.05), and in cases without expressing cyclin D1, 76% cases survived during the follow- up period, and in cases exhibiting overexpression, 63% survived, which shows that the expression of cyclin D1 correlates significantly to survival rates. However, the degree of cyclin D1 expression and the survival length were not significantly different.

Correlation between clinicopathological parameters and survival length. The 5-year survival rate of the patients in the follow-up examination was 72%. The correlation of the survival length, the age of patient, tumor size, tumor grade, the presence or absence of lymph node metastasis, the level of lymph node metastasis and clinical stage was examined, and it was found that tumor size as well as the presence or absence of lymph node metastasis mediated statistically significant effects on survival rates and the survival length, and induced the shortening of the survival length. However, the age of patient, tumor grade, the level of lymph node metastasis and clinical stage did not show a significant correlation to the survival length (Table III). In regard to the 5-year survival rate and the mean survival length according to age, the group younger than 39 years was 59% and 71 months, the 40-49 years group was 70% and 77 months, the 50-59 years group was 83% and 87 months, the group older than 60 years was 57% and 74 months, and the survival rate and the survival length according to age was not statistically significant (p>0.05). Concerning the 5-year survival rate and the mean survival length, the grade I was 87% and 89 months, the grade II was 66% and 77 months, the grade III was 71% and 80 months, and the 5-year survival rate and the survival length according to histological grade were not statistically significant (p>0.05). Regarding the 5-year survival rate and the average survival period according to tumor size, the group with tumor size <2 cm was 86% and 91 months, the 2-5 cm group was 74% and 84 months, the group >5 cm was 32% and 49 months, and the 5-year survival rate and the survival length according to tumor size were statistically significant (p<0.05), and as tumor size becomes larger, the survival rate and the survival length were significantly decreased, and in cases with tumor size >5 cm,

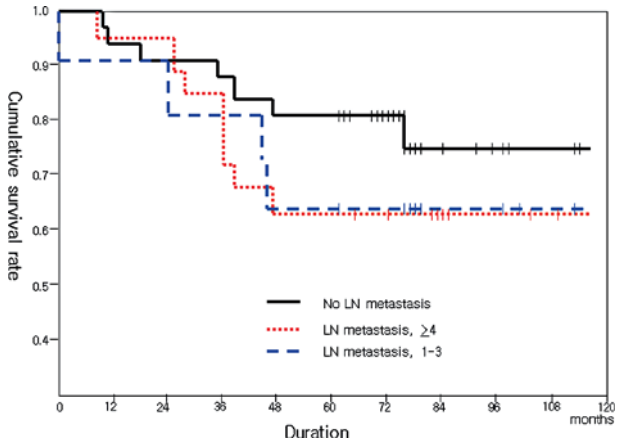

Figure 5. Cumulative survival curve by lymph node metastasis.

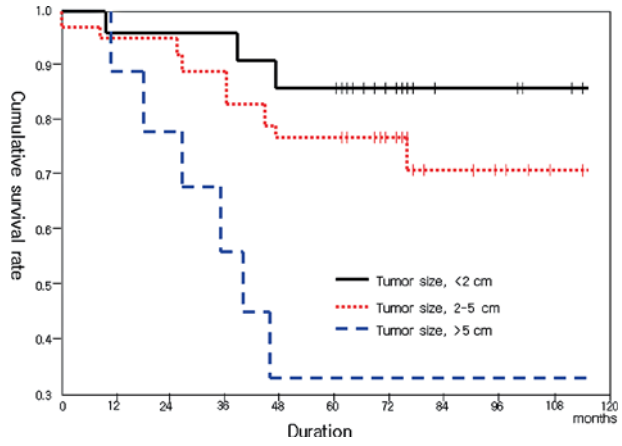

Figure 6. Cumulative survival curve by tumor size.

a noticeable difference was shown (Fig. 5), nevertheless, in the comparison depending on the number of metastatic lymph node, statistical significance was not detected ($p>0.05$). However, according to the result of Cox regression model that is a multivariate analysis method of the age of patient, tumor size, tumor grade, the presence or absence of lymph node metastasis, the level of lymph node metastasis, clinical stage, and the survival length, only the presence or absence of lymph node metastasis was significant, and in comparison with the group absent lymph node metastasis, the group with metastasis in 1-3 nodes was 2.2 times, the group developed metastasis in >4 nodes was ~6.2 times, and the statistically significant increase of mortality was shown (confidence level, 1.16-33.17) (Fig. 6).

Discussion

Pin-1 has been known to control not only DNA replication checkpoints but also several cell cycle points such as G_1/S and G_2/M (1,5,6,26-28), its expression is noticeably increased in many cancers, and it has been reported to be involved in the stability of certain phosphoproteins (1,5,6). Cells lacking Pin-1 develop serious defects in cell cycle checkpoints induced by DNA damages. In addition, DNA damage induces the stabilization and accumulation of p53 that plays a central role in the transcriptional activation of p21 and cell cycle arrest. DNA damage strengthens the interaction of Pin-1 and p53, which is determined by the WW domain of Pin-1 and the Ser/Pro motifs of p53. Furthermore, Pin-1 controls p53 stability and the transcriptional activation to p21 promoter, and thus in Pin-1 knock-out cells or Pin-1 defect tumor cells, even after DNA damage, the increase of p53 or p21 is hardly induced (29). The suppression of Pin-1 induces apoptosis, and the over-expression of Pin-1 increases cyclin D1 protein, and activates its promoter. Furthermore, Pin-1 binds to phosphorylated c-Jun with Ser67/73-Pro motifs induced by activated JNK or oncogenic Ras, Pin-1, in collaboration with activated Ras or JNK, augments transcriptional activity of c-Jun toward cyclin D1 promoter. Therefore, it is determined that the over-expression of Pin-1 in breast cancer augments cyclin D1 through the action of Ras and c-Jun and thus plays a central role in oncogenesis and tumor growth (6). In our study, the overexpression of Pin-1 was statistically significant with tumor grade as well as the level of lymph node metastasis. Nonetheless, it did not significantly correlate to tumor size or clinical stage. In addition, the correlation of Pin-1 and cyclin D1 was examined, and it was found that when Pin-1 was expressed strongly, the expression of cyclin D1 was also significantly increased.

On the other hand, even in normal breast tissues in the vicinity of tumors, weak staining of Pin-1 (1+) was detected in the nucleus, however, in infiltrating duct carcinoma cases, moderately or strongly positive expression (2+ or 3+) was observed in most cases, and thus the overexpression of Pin-1 was determined to be closely associated with tumors, and DCIS cases present in the vicinity of tumors was weakly positive (1+) in most cases, and thus it is speculated to play an important role in the tumor progression process rather than the neoplastic transformation stage.

The critical size that a mass could grow by the diffusion of oxygen and nutrition simply without angiogenesis is 1-2 mm in diameter, and it could not grow more than 3-5 mm^3 (30,31). Therefore, for tumors to grow further, to form new colonies in the vicinity, and to induce metastasis in lymph nodes or other tissues, new blood vessels should be formed within tumors as well as in the vicinity of tumors. The vascular wall of neovasculatures formed in such a manner is weaker than previous normal blood vessels, and thus cancer cells could infiltrate readily, and it also provides the passage for lymph node metastasis. In addition, for the growth of metastasized tumors, angiogenesis is a prerequisite. Therefore, numerous studies and attempts were made to precisely understand the mechanism of the angiogenesis of tumors, to predict prognosis based on this, and to apply the knowledge to treatments, nevertheless, it is not elucidated yet.

Several factors stimulating the formation of blood vessels are known, such as acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), transforming growth factor-ß (TGF-ß), VEGF, platelet derived endothelial cell growth factor (PD-ECGF), interleukin-8, hepatocyte growth factor, and proliferin (32-35), and among them, VEGF is a substance inducing strong mitosis by acting on vascular

endothelial cells selectively, and it has been reported to be the most important and potent factor for the angiogenesis of tumors. Endothelial cell growth factors with the structural homology to VEGF have been continuously characterized, and VEGF-C belonging to the VEGF family is a ligand of VEGF receptor-3 (VEGFR-3, Flt-4), it is known to be a specific marker of lymphatic duct endothelial cells, and to induce proliferation of lymphatic ducts (9,10). VEGF-C has been reported not only to control physiological angiogenesis, the development and progression of various angiogenetic diseases but also to stimulate tumor lymphangiogenesis and thus to induce the dissemination of various tumor cells and lymph node metastasis (11,12). In cases with transitional cell carcinoma in the bladder, the expression of VEGF-C has been revealed to be a useful factor allowing to predict lymph node metastasis as well as poor prognosis (36), and in breast cancer, the strong expression of VEGF-C or COX-2 induces statistically significant recurrence rate and mortality, and the simultaneous expression of VEGF-C and COX-2 is associated with lymphangiogenesis, determining that COX-2 stimulates lymph node metastasis through the lymphangiogenesis pathway by augmenting the expression of VEGF-C (8). In addition, it has been shown that COX-2 was significantly associated with the expression of VEGF or cyclin D1, it exerts significant effects on tumor grade, lymph node metastasis and tumor size, and significant coexpression of COX-2 and VEGF was shown (37). It has been revealed that in glioblastoma, VEGF secretion was accelerated by tumor cells under hypoxic condition and thus induced the proliferation of new blood vessels (38), and subsequently, it has been also revealed in experiments that in tumor cell culture, VEGF plays an important role in the proliferation of vascular endothelial cells (39). The expression of VEGF is elevated primarily by the decrease of oxygen partial pressure, and in addition, its expression is controlled by cytokines such as EGF, TGF-ß, keratinocyte growth factor, and *p53* and other tumor suppressor genes (40-42). However, studies examined their effect on VEGF-C are still rare.

In our study, the expression of VEGF-C was statistically significantly associated with tumor grade, lymph node metastasis, and clinical stage, nevertheless, it did not significantly correlate to the expression of Pin-1, p53 protein and cyclin D1. In addition, concerning the expression of VEGF-C, depending on the staining intensity, mortality and the survival length showed slight differences. In positive expression, particularly in cases showing strong positive expression, a tendency for decreased mortality and the survival length was shown, however, it was not statistically significant.

The tumor suppressor gene *p53* is present within the nucleus in cells and involved in the control of cell cycle, and when stress is delivered to cells or DNA damage is caused, it repairs DNA while arresting cell cycle at G1 stage, and it plays a role of suppressing cell proliferation and transformation by activating several other tumor suppressor genes. However, when this gene is mutated, cell cycle enters the S phase before the repair of damaged DNA, induces the rearrangement of chromosomes or the amplification of genes resulting in the formation of tumors (43,44). *p53* gene is the most frequent target of genetic mutation occurring in diverse cancers (45,46). It has been shown that the accumulation of mutant p53 protein occurs in diverse malignant tumors developing in the breast, lung, and digestive tract (20,47-49). In breast cancer cases, the accumulation of mutant p53 protein is observed in 10-40%, and it has been revealed to be increased in estrogen receptor-negative cases, epithelial growth factor receptor-positive cases, and in higher nuclear grade of tumors, and thus it is considered to be of help in the determination of biological characteristics of tumors as well as the prognosis of patient (20,44).

In our study, the expression of p53 protein was observed in 35%, and as the tumor grade became higher, its expression was increased, and thus it is thought to be associated with poor prognosis, however, it did not significantly correlate to the survival rate of patients. According to previous studies, it correlated significantly to tumor grade, however, it did not correlate significantly to lymph node metastasis, and thus it was inferred that it might not be an independent prognostic factor, on the other hand, contradictory results that the presence or absence of lymph node metastasis correlated to the expression of p53 protein or the survival length of patient have been reported (20,50,51).

In the control of the cell cycle, a series of activation and inactivation processes due to the balance of the cyclin-cyclin dependent kinase (cdk) complex and cdk suppression protein is very important for the formation of tumors, and among the cell cycle progression processes, the G1/S transition stage is the most important. The essential complex of this stage is cyclin D1-cdk4 (52,53). Cyclin D1 is located in chromosome 11q13, and the overexpression by the rearrangement as well as amplification of this gene has been observed in several tumor types. Among the cyclin series, cyclin D1 that has been reported to be related most to the formation of tumor is expressed primarily in the G1 phase, it suppresses the function of retinoblastoma (Rb) protein or other tumor suppressor genes by Rb protein and it could induce the transformation of cells (54). Cyclin D1 plays a central role in the development of various cancers, particularly breast cancer, and the overexpression of cyclin D1 induces the transformation of cells, however, the suppression of cyclin D1 expression causes the growth arrest of tumor cells (55,56). Furthermore, in murine mammary gland, the transgenic overexpression of cyclin D1 induces mammary hyperplasia and ultimately carcinoma (57). More importantly, in mice, when *cyclin D1* gene was destroyed, the ability of Ha-*ras* or c-*Neu*/*HER2* inducing the development of tumors in the mammary gland was completely suppressed (58). Such results suggest that cyclin D1 is an essential downstream target in the process of the formation of breast cancer induced by Ha-*ras* or c-*Neu*, and the major mechanism of the tumor formation process is the phosphorylation process of pSer/Thr-Pro motifs. In protein, pSer/Thr-Pro motifs are present as two types of completely different *cis* and *trans* structure, and their conversion is induced by the catalytic action of prolyl isomerase Pin-1 (2,26,59,60). After the induction of phoshorylation, the conformational change of protein caused by Pin-1 mediates a great influence on their catalytic activity, dephosphorylation, and protein-protein interaction (1,3,4,61). Therefore, phosphorylation-dependent prolyl isomerization is the

decisive control mechanism of the phosphorylation signal transduction system (27).

In our study also, when Pin-1 was expressed strongly, the expression of cyclin D1 was increased significantly, concurring well with previous research.

The expression of cyclin D1 showed different results depending on organs and investigators, and in soft tissue sarcoma cases, the overexpression was associated with poor prognosis (62), in breast cancer cases, it has been reported to be associated with good prognosis (63,64) and it did not correlate contrarily (65,66), and in head and neck squamous cell carcinoma, it has been reported to be associated with poor prognosis (67). In 10-20% of breast cancer, the amplification of cyclin D1 gene was detected, nevertheless, the overexpression of cyclin D1 protein was shown to be approximately 35-80%, and thus it varies widely depending on investigators (68-70). The overexpression of cyclin D1 protein was higher than the frequency of gene amplification, which implies that the expression of cyclin D1 protein could be induced by mechanisms other than gene amplification, it may be induced by mutation or translocation, and it may be due to the enhancement of the sensitivity to hormone, particularly, estrogen (65). In experiments using a breast cancer cell line, cyclin D1 could be induced by estrogen, and thus even without the amplification of the gene, the expression of cyclin D1 could be induced in estrogen receptor-positive cells (71), and in estrogen receptor-positive cells, more cyclin D1 could be synthesized (72).

In our cases, the overexpression of cyclin D1 was observed in 85 cases (66%) and a significant correlation of the expression of cyclin D1 and the expression of Pin-1 was detected, however, it did not significantly correlate to the expression of VEGF-C and p53. In addition, the correlation of cyclin D1 to various clinicopathological markers was examined, and it did not correlate to patient age, tumor size, clinical stage, however, the expression intensity of cyclin D1 significantly correlated to the presence or absence of lymph node metastasis as well as tumor grade. In regard to the rate of survival and the survival length according to the non-expression of cyclin D1 and its overexpression, in cases with the overexpression of cyclin D1, the survival rate as well as the survival length was statistically significantly increased, however, the survival length according to the degree of cyclin D1 overexpression was not significant, and thus it is determined that whether cyclin D1 is expressed or not may be valuable as a predictive factor mediating effects on the survival rate and the survival length.

In addition, the relationship of various clinicopathological markers with the survival length was statistically analyzed, and tumor size as well as the presence or absence of metastasis mediated statistically significant effects on the survival rate as well as the survival length, and thus induce the shortening of the survival length. However, the age of patient, tumor grade, the degree of lymph node metastasis, and clinical stage did not correlate significantly to the survival rate or the survival length.

Based on these findings, Pin-1 and VEGF-C were confirmed to be involved in the progression of breast cancer as well as metastasis, they induce poor prognosis, and as shown previously, p53 protein and cyclin D1 also were confirmed to be negative prognostic factors. In addition, although more studies on Pin-1 as well as VEGF-C should be accumulated, the expression of Pin-1 and cyclin D1 was closely associated, and the expression of VEGF-C and tumor grade, the level of lymph node metastasis and clinical stage were statistically significant, and thus it is determined that it could be used widely as basic information to establish a strategy for chemotherapy.

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