ERβ, which are thought to be important target genes in breast cancer. The five cell lines used were as follows: T-47D, MCF-7, and KPL-1, which are ERα-positive; and MDA-MB231 and SKBR-3, which are ERα-negative. Transfection of YB-1 siRNA decreased the expression of YB-1 mRNA by ≥70% in all five cell lines (Fig. 1*A*). Both EGFR and HER2 mRNA levels were found to be decreased in YB-1 siRNA–treated T-47D and KPL-1 cells but not in MDA-MB231 and SKBR-3 cells (Fig. 1*B*). EGFR and HER2 mRNAs were not detected in MCF-7 cells. It has been reported that the 5′ regulatory region of the ERα gene contains several Y-box–like sequences. Cellular mRNA levels of ERα were reduced by YB-1 siRNA in T-47D, KPL-1, and MCF-7 cells by 74%, 75%, and 40%, respectively, (Fig. 1*B*). CXCR4 and MVP/LRP mRNA levels were also decreased in YB-1 siRNA–treated T-47D and KPL-1 cells but not in MDA-MB231 and SKBR-3 cells (Fig. 1*B*).

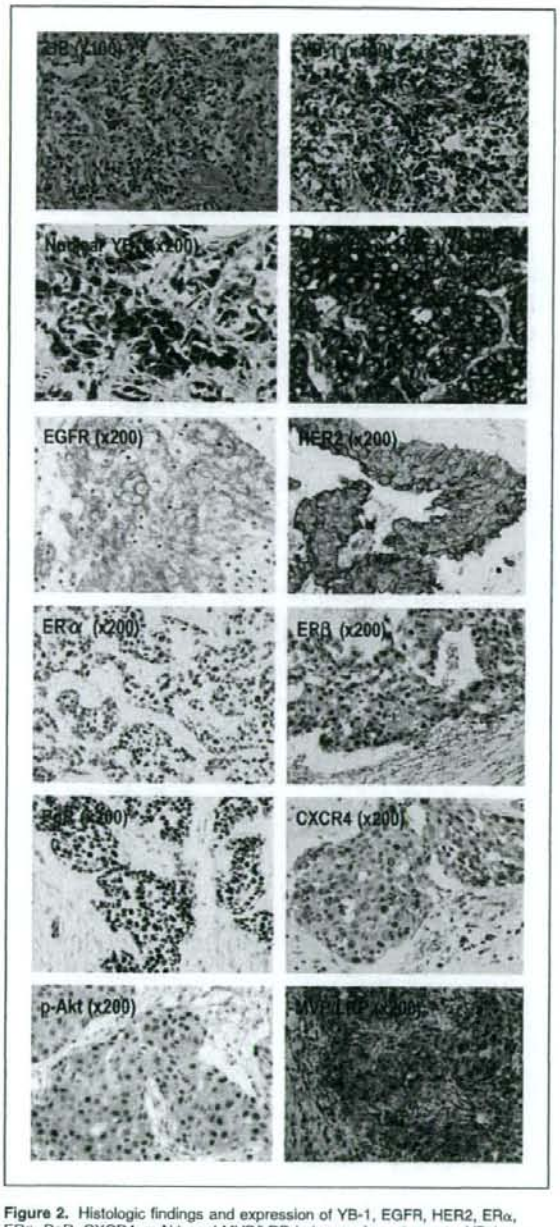

Figure 2. Histologic findings and expression of YB-1, EGFR, HER2, ERα, ERβ, PgR, CXCR4, p-Akt, and MVP/LRP in human breast cancer. YB-1 expression was recognized in two patterns: nuclear positive or negative. Cancer cells showed strong expression of EGFR and HER2 in the membrane. Strong expression of ERα, ERβ, and PgR was found in the nucleus. Moderate-to-strong expressions of CXCR4, p-Akt, and MVP/LRP were found in the cytoplasm.

Western blot analysis showed that siRNA to YB-1 decreased protein levels of EGFR, HER2, and ERα in T-47D cells; however, we did not observe decreased expression of EGFR and HER2 as a result of YB-1 knock-down in MDA-MB231 cells (Fig. 1*C* and *D*). These observations show that YB-1 only reduces the expression of EGFR and HER2 when ERα is present. Moreover, the expression of ERα was also affected by YB-1 knock-down. We next examined the causal relationship between HER2, ERα, and nuclear YB-1 in breast cancer cells in culture. Treatment with HER2-targeting trastuzumab blocked the nuclear localization of YB-1 in both T-47D and MDA-MB231, but the inhibitory effect was less in MDA-MB231 (Supplementary Fig. S2*A*). By contrast, nuclear YB-1 expression was not affected by ERα knock-down in T-47D cells in culture (Supplementary Fig. S2*B*). HER2 might directly modulate the cellular localization of YB-1 in breast cancer cells; however, ERα might not directly affect nuclear YB-1 localization.

Immunostaining of EGFR, HER2, ERα, ERβ, CXCR4, p-Akt, and MVP/LRP in human breast cancers. To examine which genes are specifically associated with nuclear YB-1 localization in human breast cancers, we selected eight molecular markers: EGFR, HER2, ERα, ERβ, PgR, CXCR4, p-Akt, and MVP/LRP. Representative immunohistochemical staining patterns in the presence and absence of nuclear YB-1 are shown in Fig. 2. Expression of nuclear YB-1 was detected in 30 of 73 patients (40%; nuclear YB-1 positive). Clinical and pathologic characteristics at diagnosis of the 73 patients in this study are summarized in Supplementary Table S2. There was no significant correlation between the expression of nuclear YB-1 and age ($P = 0.2562$), histologic grade ($P = 0.1910$), menopausal status ($P = 0.1508$), tumor size ($P = 0.1478$), or lymph node metastasis ($P = 0.0620$).

Figure 2 also shows representative examples of immunohistochemical staining for EGFR, HER2, ERα, ERβ, PgR, CXCR4, p-Akt, and MVP/LRP. There were significant correlations between the expression of nuclear YB-1 and HER2 ($P = 0.0153$), ERα ($P = 0.0122$), and CXCR4 ($P = 0.0166$; Table 1). By contrast, there was no significant correlation between nuclear YB-1 expression and the expression of EGFR ($P = 1.0000$), PgR ($P = 0.0944$), ERβ ($P = 0.0576$), p-Akt ($P = 0.0521$), or MVP/LRP ($P = 0.0577$).

Effects of nuclear YB-1 on survival and other molecular markers. The estimated product-limit survival functions of nuclear YB-1 are shown in Fig. 3*A* (overall survival) and Fig. 3*B* (progression-free survival), as well as the results of log-rank tests. Survival curves for patients with nuclear YB-1 were significantly different from those without nuclear expression ($P = 0.0139$ for overall survival; $P = 0.0280$ for progression-free survival). The results of log-rank tests for other factors are given in Table 2, showing that the tests for lymph node metastasis were significant ($P = 0.0001$ for overall survival; $P < 0.0001$ for progression-free survival).

The first eight principal components were used in the subsequent analysis, as their cumulative coefficients of variance

Table 1. Correlation between nuclear YB-1 expression and expression of eight target genes

Variables	All patients		Nuclear YB-1				*P*
			Negative		Positive		
	No. of patients	%	No. of patients	%	No. of patients	%	
EGFR							
Negative	58	79	34	79	24	80	1.0000
Positive	15	21	9	21	6	20	
HER2							
Negative	59	81	39	91	20	67	0.0153
Positive	14	19	4	9	10	33	
ERα							
Negative	24	33	9	21	15	50	0.0122
Positive	49	67	34	79	15	50	
ERβ							
Negative	18	25	7	16	11	37	0.0576
Positive	55	75	36	84	19	63	
PgR							
Negative	39	53	19	44	20	67	0.0944
Positive	34	47	24	56	10	33	
CXCR4							
Negative	29	40	12	28	17	57	0.0166
Positive	44	60	31	72	13	43	
p-Akt							
Negative	27	37	20	47	7	23	0.0521
Positive	46	63	23	53	23	77	
MVP/LRP							
Negative	41	56	20	47	21	70	0.0577
Positive	32	44	23	53	9	30	

were ~80%. Denoting the i-th principal component by PRINi, the results of Cox regression analysis were as follows. For overall survival, PRIN1 and PRIN7 were statistically significant [HR = 1.52 and $P = 0.0090$ (for PRIN1); HR = 2.06 and $P = 0.0499$ (for PRIN7); Fig. 4*A*]; and for progression-free survival, PRIN1, PRIN6, PRIN7, and PRIN8 were significant [HR = 1.59 and $P = 0.0009$ (for PRIN1); HR = 1.86 and $P = 0.0103$ (for PRIN6); HR = 2.30 and $P = 0.0078$ (for PRIN7); HR = 1.68 and $P = 0.0508$ (for PRIN8); data not shown]. PRIN1 was positively correlated with YB-1 [correlation coefficient (r) = 0.593], HER2 ($r = 0.397$), histologic grade ($r = 0.557$), tumor size ($r = 0.577$), and lymph node metastasis ($r = 0.522$). PRIN1 was negatively correlated with ERα ($r = -0.684$), PgR ($r = -0.453$), CXCR4 ($r = -0.460$), menopausal status ($r = -0.618$), and age ($r = -0.607$). This might indicate that some effect shared by YB-1, HER2, ERα, PgR, and CXCR4 leads to poor survival. PRIN7 was positively correlated with tumor size and negatively correlated with EGFR and p-Akt. Note that PRIN7 was not correlated with YB-1; this points to the existence of different mechanisms that influence survival apart from those involving PRIN1.

Stepwise variable selection was used to select the following molecular markers for graphical modeling: YB-1, HER2, ERα, ERβ, and CXCR4. Figure 4B shows the results of graphical modeling of these markers when two markers were positively correlated; a plus symbol is shown on the path, otherwise a minus symbol is shown. The relationships are indicated between markers; for example, YB-1 is related to CXCR4, ERβ, and HER2 but not directly to ERα. Note that HER2, YB-1, CXCR4, and ERα are correlated with PRIN1, emphasizing their important effects on survival.

Discussion

In this study, we assessed whether the expression of EGFR and ErbB2/HER2 was affected by YB-1 in breast cancers, as this might influence prognosis. We developed two independent approaches to identify which genes are under the control of YB-1 in human breast cancer cells. One approach involved microarray analysis, qRT-PCR, and immunoblotting to determine whether the expression of EGFRs, ERα, and other YB-1–related proteins is controlled by YB-1 in culture. The other approach consisted of immunohistochemical analysis of those protein molecules closely associated with nuclear localization of YB-1 in patients with breast cancer.

The expression of EGFR and HER2 was down-regulated by YB-1 knock-down in ERα-positive, but not ERα-negative, breast cancer cell lines, suggesting that YB-1 siRNA–induced suppression depends upon the presence of ERα. By contrast, immunohistochemical analysis showed that nuclear YB-1 expression was significantly associated with the expression of HER2 but not of EGFR. Janz et al. (6) have also reported a close association of YB-1 nuclear localization with the expression of HER2 in primary breast cancers. Moreover, Wu et al. (20) found that the introduction of a p-Akt–insensitive mutation into YB-1 markedly decreased the expressions of both EGFR and HER2, suggesting a close linkage between YB-1 and EGFR/HER2 expression in breast cancer cells in culture. YB-1 overexpression in human breast epithelial cells did not affect HER2 but caused up-regulation of EGFR, with concomitant EGF-independent phosphorylation of EGFR (18). The effect of YB-1 on EGFR and/or HER2 might depend in part on the particular cell line examined.

Oda et al. (11) found a highly significant association of p-Akt with nuclear YB-1 expression in human ovarian cancers, and both p-Akt and nuclear YB-1 expression were independent prognostic biomarkers; however, we observed no statistically significant association of p-Akt expression with nuclear YB-1 expression in our immunohistochemical analysis (Table 1). Cross-talk between growth factor receptors, such as EGFR, insulin-like growth factor (IGF) receptor, and estrogen signaling cascades occurs at the level of ERα (28, 29); this leads to activation of PI3K/Akt and ultimately to activation of ERα (30, 31). Thus, activation of ERα as well as YB-1 and its translocation to the nucleus seem to be coordinately controlled in breast cancer cells by the PI3K/Akt pathway in response to growth factors such as EGF/transforming growth factor α and IGF. PI3K/Akt activation could therefore be primarily dependent on the active state of ERα, which seems to play a major role in the nuclear translocation of activated YB-1 in ERα-positive breast cancer cells. In relation to a possible association of hormone receptors with nuclear YB-1 localization, we found that the

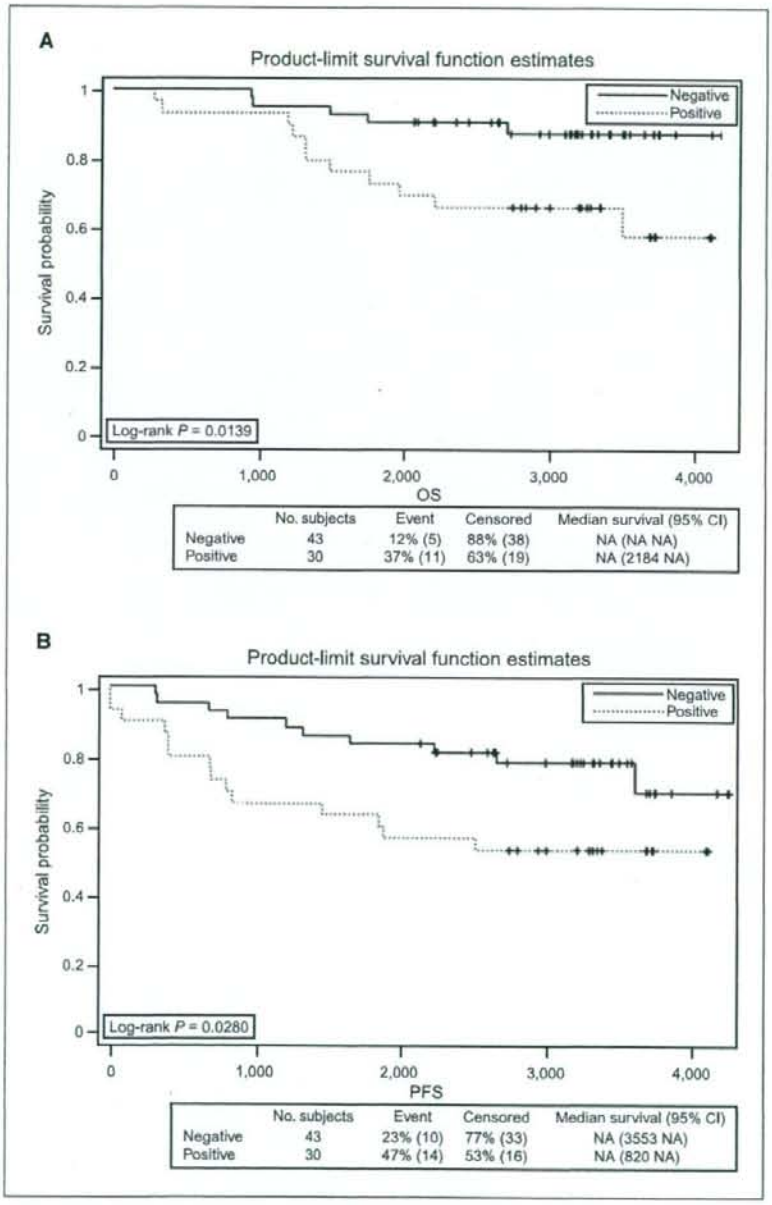

Figure 3. Kaplan-Meier overall survival (*A*) and progression-free survival (*B*) according to nuclear YB-1 expression in 73 patients with breast cancer. Nuclear expression of YB-1 has a significant predictive value for survival.

expression of ERα and ERβ was down-regulated by YB-1 knock-down. Wu and colleagues (20) have reported an inverse relationship between ERα and YB-1 in breast cancer samples. In the present study, ERα expression was inversely correlated with nuclear YB-1 localization, whereas ERβ expression was positively correlated with nuclear YB-1 localization. Like ERα, ERβ expression is closely associated with the PI3K/Akt signaling cascade (32). ERβ has emerged as an important determinant in breast cancer (33) and is a useful biomarker for breast cancer independent of ERα expression (34). The close linkage of nuclear YB-1 localization with ERβ expression points to the presence of a novel signaling pathway that could be a target for anticancer therapy in breast cancer.

We examined two targets of YB-1, MVP/LRP and CXCR4, which were identified by our expression profiling analysis. MVP/LRP expression, which is involved in drug resistance, is promoted by 5-fluorouracil and other anticancer agents in response to transcriptional activation by YB-1, suggesting a direct link between YB-1– and MVP/LRP-mediated drug resistance (35–37). MVP/LRP expression was not affected by YB-1 knock-down in ovarian cancer cells in

Table 2. Univariate analysis of patient characteristics and target gene expression regarding overall survival and progression-free survival

Variables	No. of patients	Overall survival		Progression-free survival	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Nuclear YB-1					
Negative	43	1.00		1.00	
Positive	30	3.48 (1.21–10.02)	0.0139	2.41 (1.07–5.44)	0.0280
EGFR					
Negative	58	1.00		1.00	
Positive	15	0.49 (0.11–2.17)	0.3376	0.46 (0.14–1.56)	0.2021
HER2					
Negative	59	1.00		1.00	
Positive	14	1.54 (0.50–4.77)	0.4528	2.01 (0.83–4.84)	0.1137
ERα					
Negative	24	1.00		1.00	
Positive	49	0.58 (0.21–1.54)	0.2661	0.60 (0.27–1.36)	0.2114
ER β					
Negative	18	1.00		1.00	
Positive	55	0.86 (0.27–2.66)	0.7867	1.14 (0.43–3.06)	0.7909
PgR					
Negative	39	1.00		1.00	
Positive	34	0.47 (0.16–1.36)	0.1535	1.160 (0.21–1.16)	0.0980
CXCR4					
Negative	29	1.00		1.00	
Positive	44	0.63 (0.24–1.68)	0.3509	0.59 (0.26–1.31)	0.1866
p-Akt					
Negative	27	1.00		1.00	
Positive	46	1.88 (0.61–5.83)	0.2669	1.56 (0.65–3.77)	0.3171
MVP/LRP					
Negative	41	1.00		1.00	
Positive	32	0.78 (0.28–2.15)	0.6283	0.76 (0.33–1.74)	0.5109
Age					
<56	38	1.00		1.00	
≥56	35	0.83 (0.31–2.22)	0.7032	0.63 (0.27–1.43)	0.2623
Histologic grade					
I	33	1.00		1.00	
II	20	1.56 (0.48–5.12)		1.05 (0.38–2.90)	
III	20	1.41 (0.43–4.62)	0.7364	1.42 (0.56–3.60)	0.7478
Menopausal status					
Pre	31	1.00		1.00	
Post	42	0.54 (0.20–1.45)	0.2138	0.47 (0.21–1.06)	0.0629
Tumor size					
<2 cm	30	1.00		1.00	
≥2 cm	43	2.26 (0.73–7.01)	0.1476	2.44 (0.97–6.16)	0.0508
Lymph node metastasis					
Absent	39	1.00		1.00	
Present	34	6.33 (1.80–22.29)	0.0010	8.49 (2.88–25.03)	<0.0001

Abbreviation: 95% CI, 95% confidence interval.

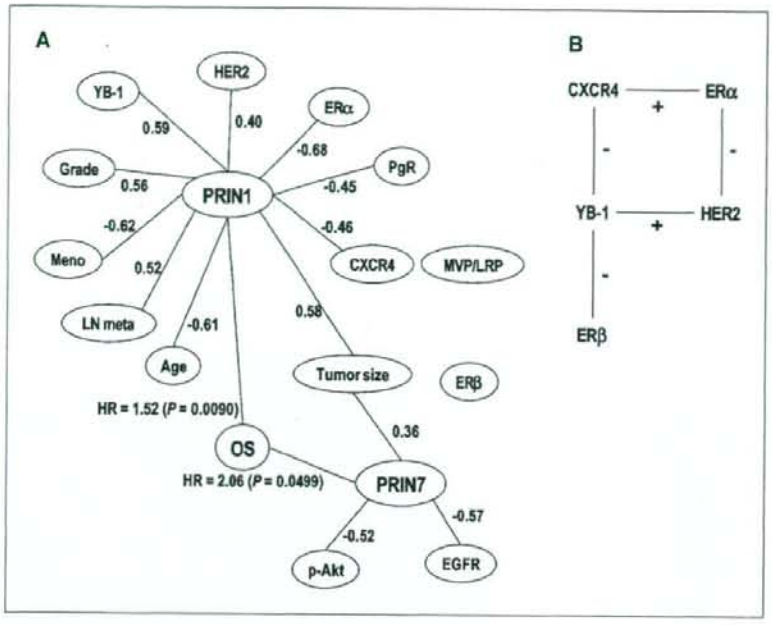

Figure 4. Statistical modeling of nuclear YB-1 localization–based network in human breast cancer. *A*, relationships among principal components, which were found significantly related to overall survival (PRIN1 and PRIN7) and clinicopathologic findings/molecular markers. Principal components and clinicopathologic findings or molecular markers are linked by a line if and only if the absolute value of correlation coefficient among them is >0.3. Each line is labeled by the correlation coefficient. *B*, relationship of molecular markers by graphical modeling incorporating with logistic regressions (+, positive correlation; −, negative correlation).

culture, although nuclear YB-1 expression and MVP/LRP expression are closely associated in patients with ovarian cancer (11, 27). CXCR4 is also known to play a critical role in the growth and metastasis of human breast cancers (38, 39). CXCR4 expression was down-regulated in YB-1 siRNA–treated ovarian cancer cells, and nuclear YB-1 expression was closely associated with CXCR4 expression in clinical samples of human ovarian cancers (11, 27). A significant positive association of nuclear YB-1 location with CXCR4 expression in breast cancer was also shown in the present study.

Nuclear localization of YB-1, in part mediated by Akt activation, thus modulates the expressions of EGFR, HER2, ERα, ERβ, and CXCR4 in breast cancer cells. YB-1–driven cell signaling of growth, survival, and hormone responses might be mainly mediated by transcriptional activation of the above-mentioned genes (1, 2); however, from our biostatistical analysis, YB-1 nuclear expression was positively associated with the expression of HER2, and negatively associated with the expressions of CXCR4 and ERβ (Fig. 4B). Moreover, ERα expression was positively correlated with CXCR4 expression and negatively correlated with HER2 expression. Although there remain inconsistencies between the data for cultured breast cancer cells and actual breast cancers with regard to the relationship between YB-1 nuclear location and the expression of other biomarkers, our biostatistical linkage map should provide important information for the development of strategies for molecular diagnosis and therapy.

In conclusion, nuclear YB-1 expression might be a prognostic marker in breast cancer. Furthermore, YB-1 plays a key role in the network annotation of genes such as HER2, CXCR4, ERα, and ERβ (Fig. 4). In addition to YB-1–mediated acquisition of multidrug resistance, the close association of nuclear YB-1 localization with HER2 expression should be considered part of the underlying mechanism. The determination of the nuclear versus cytoplasmic localization of YB-1 might provide a useful molecular indicator for personalized therapeutics of anticancer drugs targeting HER2 and/or ERα.

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Mutant MCP-1 therapy inhibits tumor angiogenesis and growth of malignant melanoma in mice

Mitsuhisa Koga [a], Hisashi Kai [a,*], Kimiyasu Egami [b], Toyoaki Murohara [d], Ayami Ikeda [a], Suguru Yasuoka [a], Kensuke Egashira [e], Toyojiro Matsuishi [b], Mamiko Kai [f], Yasufumi Kataoka [g], Michihiko Kuwano [c], Tsutomu Imaizumi [a]

[a] Department of Internal Medicine, Division of Cardio-Vascular Medicine and Cardiovascular Research Institute, Kurume University School of Medicine, 67 Asahimachi, Kurume 830-0011, Japan
[b] Department of Pediatrics, Kurume University School of Medicine, Kurume, Japan
[c] Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, Kurume, Japan
[d] Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[e] Department of Cardiovascular Medicine, Kyushu University Graduate School of Medicine, Fukuoka, Japan
[f] Department of Pharmaceutics, Inenaga Hospital, Chikuzen, Japan
[g] Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan

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Abstract

We investigated whether blocking of monocyte chemoattractant-1 (MCP-1) function would inhibit recruitment of tumor-associated macrophages (TAMs) and prevent tumor angiogenesis and tumor growth of human malignant melanoma. B16-F1 melanoma cells were implanted onto the back of C57BL/6 mice (Day 0). At Day 7, a dominant negative MCP-1 mutant (7ND) gene was transfected in the thigh muscle to make overexpressed 7ND protein secreted into systemic circulation. 7ND treatment inhibited TAM recruitment and partially reduced tumor angiogenesis and tumor growth. Also, 7ND treatment attenuated inductions of tumor necrosis factor-α (TNFα), interleukin-1α (IL-1α), and vascular endothelial growth factor (VEGF) in the stroma and tumor. Melanoma cells expressed not only MCP-1 but also its receptor CCR2. Accordingly, it was suggested that MCP-1 would enhance tumor angiogenesis and early tumor growth in the early stages by inducing TNFα, IL-1α, and VEGF through TAM recruitment and probably the direct autocrine/paracrine effects on melanoma cells.
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Keywords: MCP-1; Macrophages; Melanoma; Angiogenesis; Inflammation; Gene therapy; Cytokines; VEGF

There is increasing evidence that recruitment of tumor-associated macrophages (TAMs) in the stroma, and loose connective tissue surrounding the tumor is an important trigger for tumor angiogenesis [1], which promotes tumor growth and hematogeneous metastases [2–5]. The stromal TAM recruitment is related to poor prognosis of human cancers [6–11]. Monocyte chemoattractant protein-1 (MCP-1) is a potent macrophage-recruiting molecule. It was reported that MCP-1 was expressed during the early stages of human malignant melanoma [12,13]. However, it has remained unknown whether MCP-1 production has causal relationships with TAM recruitment, tumor angiogenesis, and early tumor growth of malignant melanoma.

A mutant of MCP-1, which lacks the N-terminal amino acids 2–8 (7ND), has a potent dominant negative activity [14]. We have shown that transduction of 7ND gene in

Abbreviations: MCP-1, monocyte chemoattractant protein-1; 7ND, a dominant negative MCP-1 mutant lacking the N-terminal amino acids 2–8; TAM, tumor-associated macrophage; VEGF, vascular endothelial growth factor; IL-1α, interleukin-1α; TNFα, tumor necrosis factor-α; PCR, recombinant polymerase chain reaction; RT-PCR, reverse-transcribed PCR.

* Corresponding author. Fax: +81 942 33 6509.
E-mail address: naikai@med.kurume-u.ac.jp (H. Kai).

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the thigh muscle as means to make overexpressed 7ND protein secreted into systemic circulation is a useful strategy for blocking MCP-1 activity in the remote target organs, such as atherosclerotic artery and heart [15,16]. Moreover, we have demonstrated that 7ND overexpression inhibits ischemia-induced neovascularization in the mouse hindlimb by preventing macrophage recruitment and vascular endothelial growth factor (VEGF) production [17]. In the present study, we sought to determine blocking of intrinsic MCP-1 by 7ND treatment would inhibit the initiation of tumor angiogenesis and early growth of human malignant melanoma engrafted in mice.

Materials and methods

The present study protocol was reviewed and approved by the Animal Care and Treatment Committee of Kurume University. Male C57BL/6 mice were purchased from Clea Japan Inc. (Tokyo, Japan) and housed under standard conditions of humidity, room temperature, and dark–light cycles in plenty of chow and water.

Expression vector. Human 7ND cDNA was constructed by recombinant polymerase chain reaction (PCR) using a wild type MCP-1 cDNA and cloned into BamHI (5′) and NotI (3′) sites of the pCDNA3 expression plasmid vector (Invitrogen Corp., Tokyo) [15]. The dominant negative activity was verified in vivo as described previously [15].

Tumor-implanted mouse model. We employed B16-F1 melanoma cells (Batch No. 1224725, Flask Passage No. 26; American Type Culture Collection, Manassas, VA), which can grow in the C57BL/6 strain [18] for a mouse model of tumor angiogenesis [18,19]. B16-F1 melanoma cells were cultured in DMEM supplemented with 4 mmol/L of L-glutamine, 2.5 g/L glucose, 10% fetal bovine albumin, and antibiotics and were harvested as described previously [19]. Under anesthesia with intraperitoneal pentobarbital (30 mg/kg), 1×106 B16-F1 melanoma cells were solved in 200 μL of phosphate-buffered saline (PBS) and injected subcutaneously into the back of C57BL/6 mice at 8-week old (Day 0).

7ND gene transfer. Naked cDNA method was used for 7ND gene transfer, as described previously [17,20]. Briefly, at Day 4, bupivacaine (12.5 μg/g body weight; AstraZeneka, Tokyo) was injected into the thigh muscle to improve the efficiency of gene transfer [21]. At Day 7, 7ND plasmid or the blank plasmid (control) solved in 30 μL PBS was injected at the same site as bupivacaine had been injected. We confirmed preliminary that a single 7ND gene transfer elevated serum 7ND protein levels at least for 14 days after gene transfer, and that 200 μg was the minimum dose that induced the maximum serum level elevation and did not cause histological damage of the thigh muscle at the site of gene transfer [17]. Thus, 200 μg of 7ND plasmid was injected throughout the following experiments.

Tumor volume measurement. Tumor size was measured using a caliper at denoted days ($n = 9$/group). Tumor volume was calculated according to the formula: tumor volume (mm^3) $= (L \times W^2) \times 0.52$, where L = length (mm) and W = width (mm) (length is greater than width) [22].

Tumor microangiography. Postmortem tumor microangiography was performed using an X-ray mammography system (Senographe 500T; GE Medical Systems-Europe, Paris) at Day 13 [19].

Immunohistochemical analysis of capillary density and TAM infiltration. At Day 13, mice were euthanized by an overdose pentobarbital. Tumor and the stroma (surrounding subcutaneous tissue approximately 3 mm from the tumor margin) were carefully isolated, fixed in methanol overnight, embedded in paraffin, and sectioned into 5-μm slices [19]. The sections ($n = 5$/group) were subjected to immunohistochemistry using monoclonal antibodies against mouse MCP-1 (SantaCruz Biotechnology, Santa Cruz, CA), mouse CCR2 (SantaCruz Biotechnology), mouse CD31 (PharMingen, San Diego, CA), mouse F4/80 (Serotec, Raleigh, CA), and a commercially available detection kit (Dako, Glosup, Denmark) [23].

The capillary was defined as the luminal structure surrounded by CD31-labeled endothelial cells (ECs) [19]. The number of the capillaries and F4/80-labeled macrophages was counted and averaged in 15 random microscopic fields of the tumor and stroma from 3 independent sections in each animal. Necrotic cores inside the tumor were excluded from the analysis. Capillary density and TAM count were expressed as the number of capillaries and macrophages per high-power field (400×), respectively.

RT-PCR analysis. At Day 13, after mice were euthanized with an overdose of pentobarbital and perfused with ice-cold PBS, the tumor and stroma were excised en bloc ($n = 5$/group). The tumor was carefully resected from the stroma. Total RNA was extracted from the tumor and stroma using TRIzol reagent (Invitrogen) and reverse-transcribed by using Ready-To-Go You-Prime First-Strand Beads (Amersham, NJ, USA) [24]. Equal amount of the resulting cDNA was subjected to PCR with a primer pair and a Taq DNA polymerase kit (Toyobo, Tokyo, Japan), according to the manufacturer's instructions. The nucleotide sequences of primers were as follows: for VEGF (sense) 5′-GCC AGC ACA TAG AGA GAA TGA GC-3′ and (antisense) 5′-CAA GGC TCA CAG TGA TTT TCT GG-3′; for IL-1α (sense) 5′-CGT CAG GCA GAA GTT TGT CA-3′ and (antisense) 5′-GTG CAC CCG ACT TTG TTC TT-3′; and for TNFα (sense) 5′-ACG GCA TGG ATC TCA AAG AC-3′ and (antisense) 5′-CGG ACT CCG CAA AGT CTA AG-3′. Primer pairs for MCP-1, CCR2, and GAPDH were purchased from Proligo (Tokyo, Japan). Cycle numbers of amplification were 32 cycles for MCP-1, CCR2, VEGF, IL-1α, and TNFα and 25 cycles for GAPDH (95 °C for 1 min, 60 °C for 1 min, and 72 °C for 45 s). The PCR products were separated on 1.5% agarose gel stained with ethidium bromide. The signals were scanned and analyzed with digital densitometry. The expression level of target gene was normalized for the GAPDH level in each sample [25].

Statistics. Data are means ± SE. Unpaired Student's t test was performed for statistical comparison. A p value less than 0.05 was considered statistically significant.

Results and discussion

Early tumor growth, TAM infiltration, and tumor angiogenesis

At Day 7, implanted B16-F1 melanoma cells began to be seen as grossly visible tumor (Fig. 1), although F4/80-positive macrophages, TAMs, and tumor-related angiogenesis were not observed in the stroma (data not shown). To determine the effects of MCP-1 function blocking on TAM recruitment and the initiation of tumor angiogenesis, 7ND gene transfer was performed into the thigh muscle at Day 7.

In control mice, tumor enlarged progressively after Day 7 and huge tumor developed at Day 13 (Fig. 1). Postmortem tumor microangiography showed the formation of tumor-feeding vessels (Fig. 2A). Control mice showed a lot of CD31-labeled capillary vessels with various luminal sizes in the stroma, whereas the inside of the tumor was rich in small-sized capillaries (Fig. 2B). At Day 13, diffuse TAM infiltration was observed in the stroma, whereas TAMs were scarcely found inside the tumor (Fig. 2C).

When mice received 7ND treatment, the tumor volume was significantly reduced until Day 13 (Fig. 1). The formation of angiographically visible tumor-related vessels was reduced by 7ND treatment (Fig. 2A). 7ND treatment reduced the tumor-related capillary density in the stroma and tumor by 33% and 23%, respectively (Fig. 2B). Moreover, 7ND treatment reduced TAM recruitment by 50% in the TAM number (Fig. 2C).

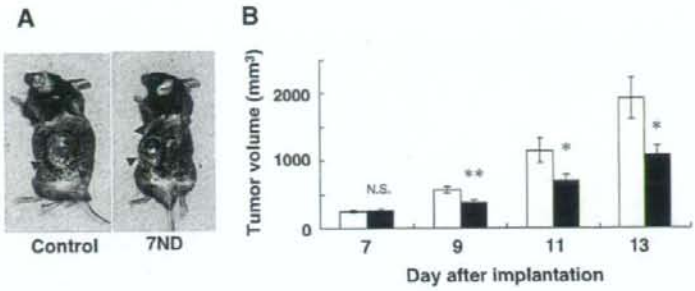

Fig. 1. (A) Representative photographs of engrafted tumors at Day 13 after B16-F1 melanoma cells implantation. (B) Time course of tumor volume. 7ND-treated mice (closed column) received 7ND gene transfer into the thigh muscle at Day 7. Control mice (open column) received the blank plasmid injection. Bar = 1 × SM ($n = 9$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

The present study suggests that MCP-1 not only acts as the TAM-recruiting molecules but also promotes the initiation of tumor angiogenesis and early tumor growth of malignant melanoma. Earlier studies demonstrated that the stromal TAMs trigger tumor angiogenesis [1]. Because nutritional tumor angiogenesis is crucial for tumor growth [2–5], our results suggest that MCP-1-mediated TAM recruitment might enhance the early growth of malignant melanoma by increasing tumor angiogenesis.

MCP-1 and CCR2 expression

In control mice at Day 13, most of malignant melanoma cells expressed immunoreactive MCP-1 (Fig. 3A), consistent with earlier studies [12,13]. It was noteworthy that CCR2, the sole receptor for MCP-1, was expressed in the majority of malignant melanoma cells as well. To our best knowledge, this is the first report that malignant melanoma cells express not only MCP-1 but also its receptor. 7ND treatment reduced the melanoma cells expressing MCP-1 and CCR2 (Fig. 3B). This finding may suggest that MCP-1 creates positive feedback loop for activating melanoma cells in an autocrine/paracrine manner. Also, a part of the stromal TAMs expressed MCP-1 and CCR2 and they were reduced by 7ND treatment.

Angiogenic factor and inflammatory cytokine expressions

VEGF is a major angiogenic factor and is produced by melanoma cells and TAMs in this model [19]. A recent in vitro study has shown that activated macrophages produce IL-1α and TNFα, which in turn induce VEGF production of cultured melanoma cells [1]. Moreover, TAMs have been shown to be prerequisite for neovascularization and tumor growth of Lewis lung cancer cells in the cornea model [26]. Thus, we examined the effects of 7ND treatment on the expressions of IL-1α, TNFα, and VEGF (Fig. 4). It is noteworthy that IL-1α and TNFα inductions were potent in not only the stroma but also the tumor in this model. The most important, novel finding of this study is that 7ND treatment remarkably reduced inductions of the inflammatory cytokines and VEGF in the tumor and stroma. Since TAM number was small and was not changed by 7ND treatment (Fig. 2C), the changes in cytokine and VEGF expressions in the tumor were attributable to melanoma cells. In addition to TAM recruitment, MCP-1 may directly activate VEGF induction and proliferation of malignant melanoma cells via the CCR2 activation in autocrine/paracrine fashion (Fig. 3). Moreover, MCP-1 would enhance tumor growth at least in part through the direct effects of IL-1α and TNFα on malignant melanoma cells. Recent studies have shown that IL-1α promotes tumor growth by directly enhancing melanoma cell proliferation and by activating inflammatory and angiogenic pathway in the stroma [27] and that TNFα prolongs tumor cell survival through the anti-apoptotic effect [28]. Taken together, MCP-1 may induce tumor angiogenesis and early tumor growth of human malignant melanoma by inducing VEGF and inflammatory cytokines through the TAM recruitment and the direct autocrine/paracrine effects on melanoma cells. Recently, it has been demonstrated that inflammatory stimuli from TAMs and tumor cells synergistically promote tumor growth and angiogenesis [29]. From the present study, however, we were not able to determine the precise interaction of these cytokines and VEGF. This issue should be addressed in future study.

There are several limitations in this study. First, the inhibitions of TAM recruitment, tumor angiogenesis, and early tumor growth by 7ND treatment were partial in this study. If higher local concentration of 7ND could be achieved, we might have more potent effects. Second, from this study, we did not know the long-term effects on tumor growth and prognosis. To address these issues, the improvement of the expression efficiency (i.e. the adjunctive treatment for naked DNA method or the use of viral expression vectors) and/or repeated gene transfers would be necessary for sustained transgene expression at higher level for longer periods. Finally, we do not deny the possibility of the involvement of direct angiogenic effect of MCP-1 on the ECs via the CCR2 activation [30].

In conclusion, MCP-1 may play a role in tumor angiogenesis and early tumor growth of human malignant mela-

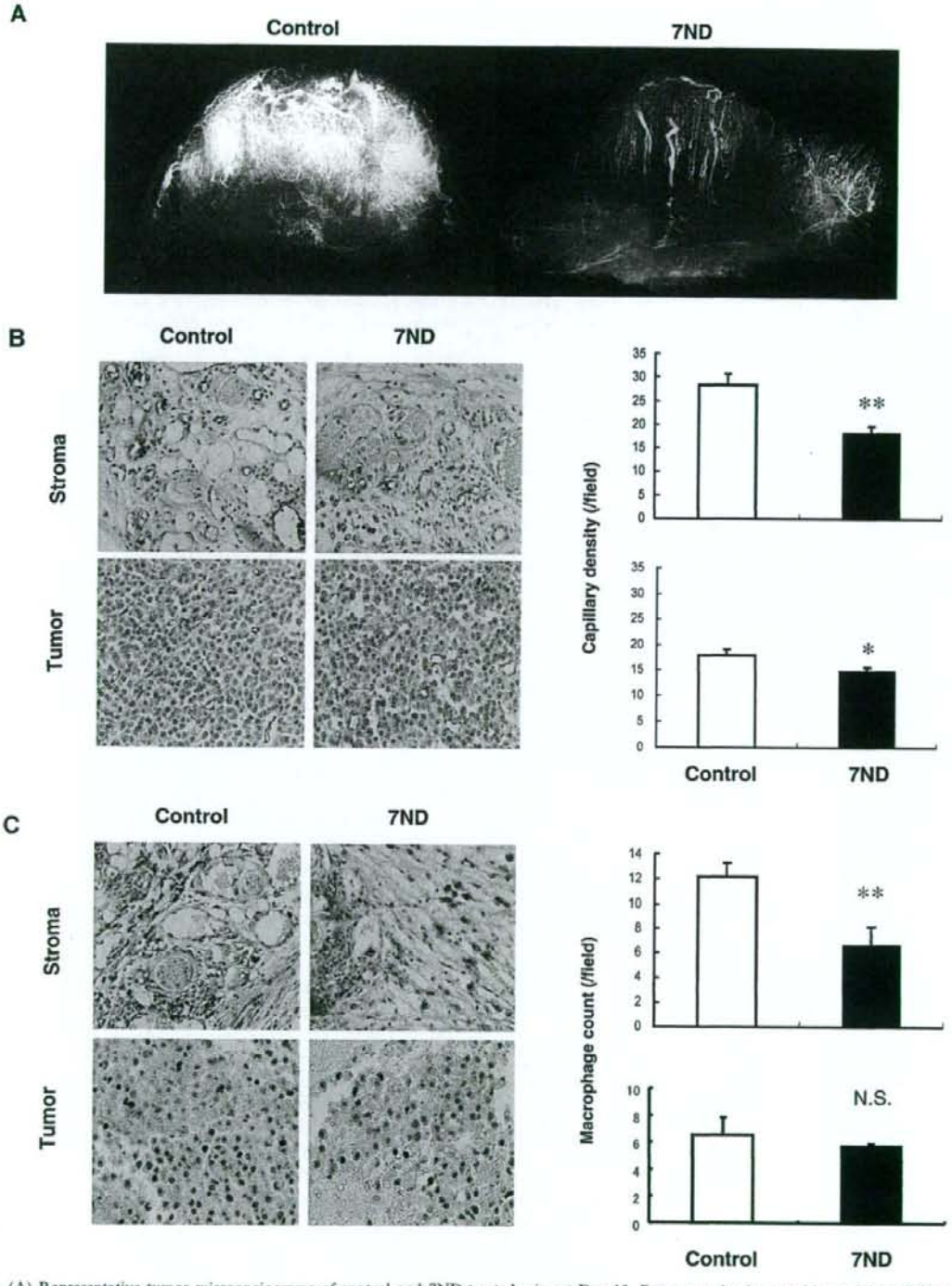

Fig. 2. (A) Representative tumor microangiograms of control and 7ND-treated mice at Day 13. Representative immunohistostainings (left) and the pooled data (right) showing effects of 7ND treatment on the capillary density (B) and TAM infiltration (C) at Day 13. Bar = 1 × SM ($n = 5$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

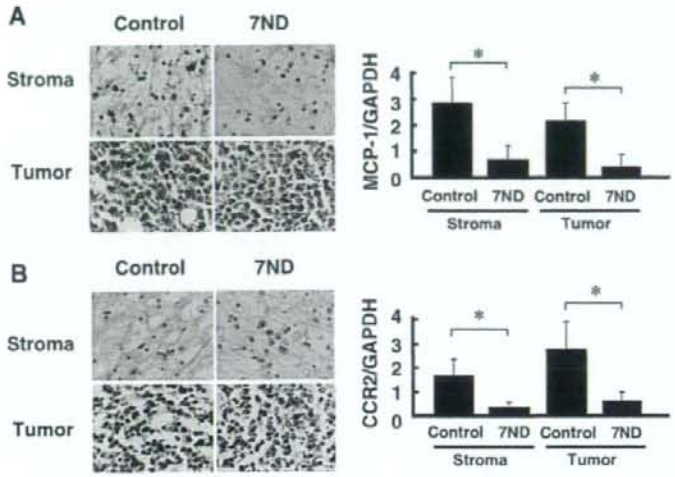

Fig. 3. Representative immunohistostainings (left) and the pooled data (right) of mRNA expression of MCP-1 (A) and CCR2 (B) at Day 13. Bar = 1 × SM ($n = 5$). $^{*}p < 0.05$ vs. control.

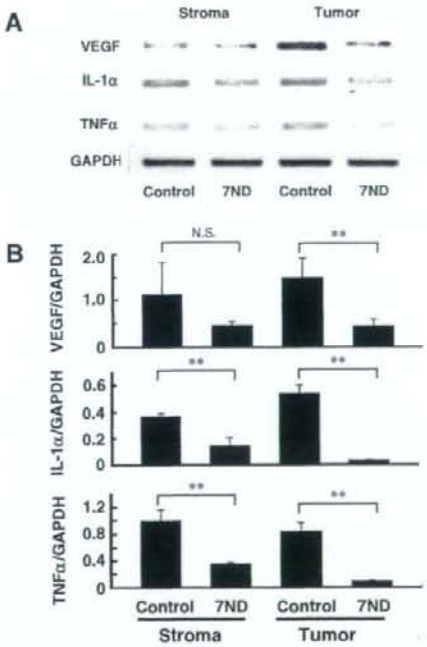

Fig. 4. (A) Representative photographs of the electrophoresis of RT-PCR products of VEGF, IL-1α, and TNFα of control and 7ND-treated mice at Day 13. (B) The pooled data of the effects of 7ND treatment on mRNA expressions. Bar = 1 × SM ($n = 5$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

noma by inducing VEGF and inflammatory cytokines, including IL-1α and TNFα, through the TAM recruitment and the direct autocrine/paracrine effects on melanoma cells. The present study raises the possibility that inhibition of MCP-1 might be an adjunctive therapy for malignant melanoma.

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Cap43/NDRG1/Drg-1 is a molecular target for angiogenesis and a prognostic indicator in cervical adenocarcinoma

Shin Nishio [a,*], Kimio Ushijima [a], Naotake Tsuda [a], Shuji Takemoto [a], Kouichiro Kawano [a], Tomohiko Yamaguchi [b], Naoyo Nishida [a,b], Tatsuyuki Kakuma [c], Hitoshi Tsuda [d], Takahiro Kasamatsu [e], Yuko Sasajima [f], Masayoshi Kage [b], Michihiko Kuwano [g], Toshiharu Kamura [a]

[a] *Department of Obstetrics and Gynecology, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka, 830-0011, Japan*
[b] *Department of Pathology, Kurume University Hospital, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan*
[c] *Biostatistics Center, Kurume University, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan*
[d] *Department of Basic Pathology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama, 359-8513, Japan*
[e] *Division of Gynecologic Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[f] *Division of Diagnostic Pathology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[g] *Research Center of Innovative Cancer Therapy, Kurume University, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan*

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Abstract

Cap43 is a nickel- and calcium-inducible gene that plays important roles in the primary growth of malignant tumors, as well as in invasion and metastasis, most likely through its ability to induce cellular differentiation. This study investigated associations of Cap43 expression with angiogenesis and other clinicopathological factors in cervical adenocarcinoma. The clinical records of 100 women who underwent surgery for cervical adenocarcinoma were reviewed retrospectively. Microvessel density and the expression of Cap43 and VEGF in the surgical specimens were evaluated immunohistochemically. The Cap43 expression level was significantly associated with angiogenesis, tumor diameter, stromal invasion, lymphovascular space invasion, lymph node metastasis, and histopathological differentiation. Kaplan–Meier analysis showed a significant association between the Cap43 expression level and survival: high Cap43 expression was related to poor survival. Our results suggest that increased expression of Cap43 is associated with angiogenesis and may be a poor prognostic indicator in women with cervical adenocarcinoma.
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Keywords: Cap43; Cervical adenocarcinoma; Angiogenesis; Prognosis

1. Introduction

The Cap43 gene is a nickel- and calcium-inducible gene [1], identical to the previously described N-myc downstream-regulated gene 1 (NDRG1). It

* Corresponding author. Tel.: +81 942317573; fax: +81 942350238.
E-mail address: shinshin@med.kurume-u.ac.jp (S. Nishio).

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is one of the four closely related genes (NDRG1-4) whose expression is down-regulated by c-myc or the N-myc/Max complex [2–5]. Cap43 is also identical to the homocysteine-inducible gene, whose expression is reduced in tumor cells (RTP/rit42) [6], and to the differentiation-related gene-1 (Drg-1) [7]. The protein encoded by Cap43 has a molecular weight of 43 kDa. It has three unique 10-amino acid tandem-repeat sequences at its carboxyl terminal and is phosphorylated by protein kinase A [8].

The functions of Cap43 remain poorly understood. Expression of the Cap43 gene has been strongly associated with nickel, cobalt, oxidative stress, hypoxia, phorbol esters, vitamins A and D, steroids, histone deacetylase-targeting drugs, homocysteine, β-mercaptoethanol, tunicamycin, and lysophosphatidylcholine, as well as with oncogenes (N-myc and c-myc) and the products of tumor-suppressor genes (p53 and VHL) [1,2,6,9,10]. Cap43 is expressed in most organs, most prominently in the prostate, ovary, colon, and kidney. Its expression in the kidney, brain, liver, and gut is actively modulated during postnatal development [2,3,11,12], suggesting that Cap43 plays a key role in organ maturation.

Transfection studies by Kurdistani and colleagues demonstrated that Cap43 inhibits the primary growth of human breast, prostate, and bladder cancer cell lines and suppresses the anchorage-independent growth of these cell lines in soft agar [6]. Moreover, the overexpression of Cap43 markedly promotes the growth of human pancreatic cancer xenografts in mice, but not of pancreatic cancer cells in culture [13]. The survival rate of patients with pancreatic cancer whose tumors expressed high levels of Cap43 was found to be significantly higher than that of patients whose tumors expressed low levels of Cap43 [13]. In another study, low tumor Cap43 expression was strongly associated with poor outcomes in women with breast cancer [14]. Chua and colleagues recently reported that Cap43 overexpression significantly correlates with tumor differentiation, vascular invasion, and overall survival in patients with hepatocellular carcinoma, suggesting that increased Cap43 expression may be a useful indicator of tumor aggressiveness and prognosis [15]. Taken together, the above findings suggest that Cap43 may have tissue-of-origin-specific functions in human malignancies [16].

The incidence of invasive cervical cancer has decreased in developed countries, presumably because of intensive national screening programs. This declining incidence is attributed primarily to a decrease in squamous cell carcinoma, whereas the incidence of adenocarcinoma has remained stable or risen slightly [17,18]. The prevalence of adenocarcinoma among women with cervical cancer has increased from 5% to 13% in the 1950s to 20% in the 1990s [19,20]. Recent studies attribute this rise to an increased incidence of cervical adenocarcinoma among young women [21,22]. Cervical adenocarcinoma is associated with unfavorable outcomes, attributed to late detection on Papanicolaou smears, a poorer response to radiotherapy than squamous cell carcinoma, or the inclusion of subtypes with particularly poor outcomes, such as clear cell carcinoma [23]. New tumor markers that can be used to predict outcomes predictors have been identified by numerous studies, including immunohistochemical analyses [24–26].

Angiogenesis is an important pathological aspect of tumor growth and chronic inflammatory diseases [27]. Of the various angiogenesis factors identified to date, vascular endothelial growth factor (VEGF)-A plays a key role in pathological angiogenesis, including that required for the rapid growth of solid tumors. Antiangiogenesis agents targeting VEGF-A and VEGF-receptor 2 have been developed and are currently used clinically [28,29]. In a previous study, we demonstrated that higher tumor Cap43 expression is associated with higher tumor microvessel density (MVD) than lower tumor Cap43 expression in patients with pancreatic cancer [13]. Angiogenesis in cervical carcinoma has been shown to be inversely related to survival [30,31]. Kaku and colleagues [32] demonstrated a significant correlation of MVD with both progression-free survival and overall survival in cervical adenocarcinoma.

In this study we immunohistochemically evaluated the intensity of Cap43 expression in patients with stage I or II cervical adenocarcinoma according to the staging system of the International Federation of Gynecology and Obstetrics (FIGO). We also examined correlations of Cap43 staining intensity with angiogenesis and other clinicopathological factors.

2. Materials and methods

2.1. Patients and treatment

Between 1990 and 2005, a total of 100 patients with stage I or II cervical adenocarcinoma underwent surgery at Kurume University Hospital and National Cancer

Center Hospital. The procedure was radical hysterectomy in 93 patients and simple abdominal hysterectomy in the other seven. Pelvic lymphadenectomy and para-aortic lymph node biopsy were performed in all patients. Patients with deep stromal invasion, lymph node metastasis, or both were considered candidates for postoperative adjuvant therapy. After surgery, 23 patients received postoperative adjuvant radiotherapy, and 5 received adjuvant platinum-containing chemotherapy. For external beam radiotherapy, a dose of 50.4 Gy was delivered to the entire pelvis. Intracavitary brachytherapy was performed if the surgical margin in the vaginal cuff was histologically positive or if the free margin was <1 cm.

2.2. Immunohistochemical staining

All specimens were fixed in 10% formalin and embedded in paraffin wax. Tissue sections 4 μm thick were mounted on slides, deparaffinized, rehydrated, and heated in a microwave oven for 60 min in CCI buffer. Immunohistochemical staining was performed using a Ventana NX automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ, USA) and polyclonal primary antibodies to Cap43 (produced in our laboratory), [10,33] VEGF (upstate, Cosmo Bio Co. Ltd., Lake Placid, NY, USA; dilution 1:50), and CD34 (Nichirei, Tokyo, Japan; dilution 1:1). A preliminary study of Cap43 immunohistochemical staining of cervical adenocarcinoma revealed that only the membrane of tumor cells stained positively; normal glands and the nuclei and cytoplasm of tumor cells stained negatively. Since the staining intensity varied, we considered the staining intensity of the tumor cell membranes to represent the expression intensity of Cap43 in the cervical adenocarcinoma specimens. The intensity of membrane staining was scored as follows: no staining, 0; dotted pattern staining, 1+; weak or moderate circumferential staining in >10% of the tumor cells, 2+; strong circumferential staining in >10% of the tumor cells, 3+ (see Fig. 1A–D). To statistically analyze the patients' survival curves (data not shown), we classified Cap43 expression scores of 0, 1+, and 2+ as low Cap43 expression, and scores of 3+ as high Cap43 expression. VEGF expression in the tumor cells was evaluated according to the following semiquantitative scoring system: no staining at all or staining in <10% of the tumor cells, 0; light staining in >10% of the tumor cells, 1+; moderate staining in >10% of the tumor cells, +2; and dark staining in >10% of the tumor cells, +3. Staining of the tumor stroma was ignored in this assessment (see Fig. 1E–H). All procedures were performed by one gynecological oncologist and two pathologists who were blinded to clinical outcomes in this series of patients. Discordant results among the investigators were re-evaluated. MVD was calculated on the basis of the immunohistochemical expression of CD34. For each sample, the mean number of microvessels was calculated for five vascular hotspots to assess the MVD for each case. Only CD34 staining in tumor areas was reviewed, and endothelial cell clusters of two or more cells were considered a single, countable microvessel (see Fig. 1I and J). All counts were made by three independent observers who had no knowledge of the corresponding clinicopathological data.

2.3. Statistical analysis

Statistical calculations were performed with the SAS version 9.1.3 (SAS Institute, Cary, NC, USA) software package. The Kaplan–Meier method was used to calculate the progression-free survival rate and overall survival rate; prognostic significance was evaluated by the log-rank test. The Mann–Whitney *U*-test was used to compare continuous variables. *P* values for correlations of Cap43 expression with VEGF expression and other clinicopathological factors were calculated with Fisher's exact test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Patient characteristics

The patients' characteristics are shown in Table 1. The median follow-up time was 51.3 months. At the time of the analysis, tumor recurrence had been diagnosed in 30 patients, and 25 patients had died. Table 2 shows the Cap43 expression, VEGF expression, and microvessel density.

3.2. Correlation between Cap43 expression and angiogenesis

High Cap43 expression correlated with high VEGF expression (Table 3). Immunohistochemical staining analysis showed that median MVD was 39.4 in the specimens with high Cap43 expression and 26.1 in the specimens with low Cap43 expression. MVD correlated with the intensity of Cap43 expression ($P < 0.0001$, Fig. 2). These results suggested that high Cap43 expression was closely associated with high angiogenic activity in cervical adenocarcinoma.

3.3. Correlation between Cap43 expression and clinicopathological factors

High Cap43 expression significantly correlated with tumor diameter, stromal invasion, lymphovascular space invasion, lymph node metastasis, and histopathological differentiation, but not with FIGO stage (Table 4).

3.4. Correlation between Cap43 expression and survival time

The median progression-free survival time was 52.4 months in patients with tumors showing low Cap43

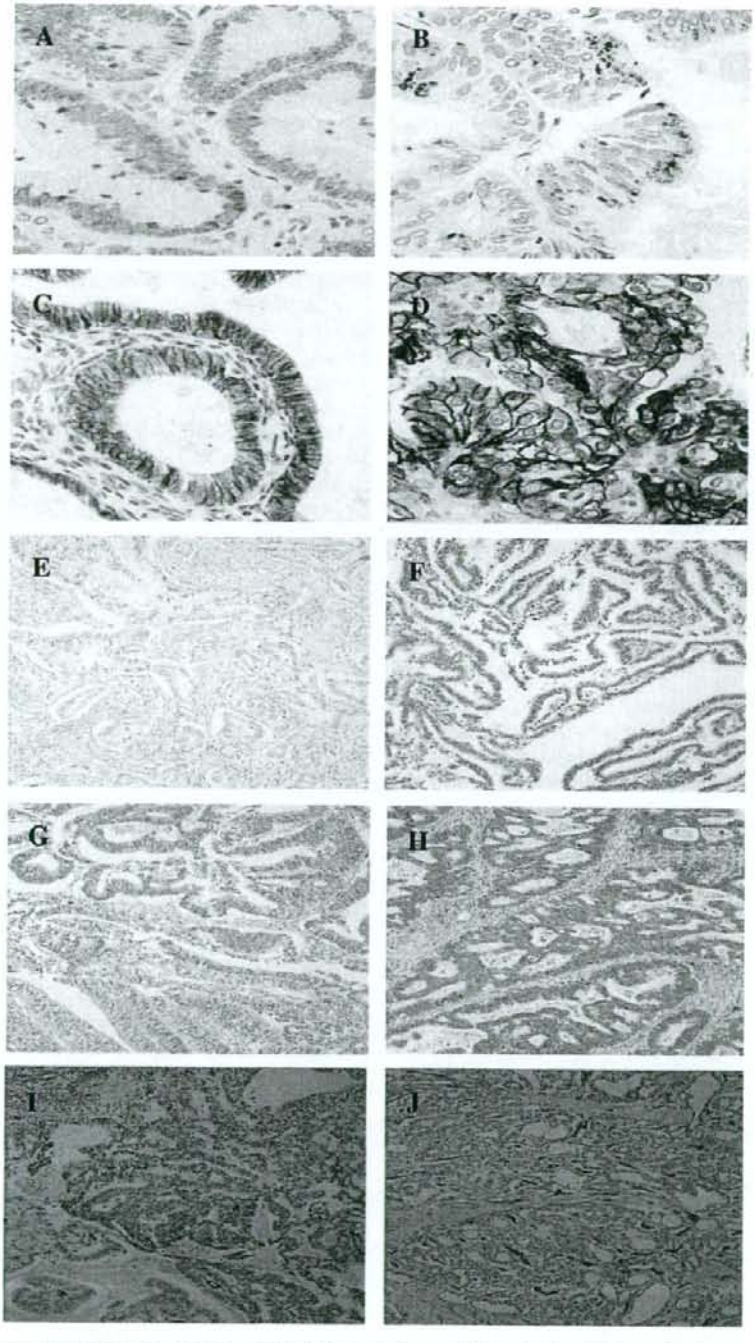

Fig. 1. Representative examples of Cap43 staining in cervical adenocarcinoma. (A) No staining; (B) dotted pattern staining (1+); (C) weak or moderate circumferential staining (2+); (D) strong circumferential staining (3+) (original magnification A–D ×400). Immunohistochemical staining for VEGF expression: (E) no immunostaining (0); (F) light staining (1+); (G) moderate staining (+2); (H) dark staining (+3) (original magnification E–H 200×). Immunohistochemical staining for anti-CD34 antibody. Tumor areas with low vessel density (I) and high vessel density (J) are shown (original magnification I, J 100×).

Table 1
Patients Characteristics ($n = 100$)

Age (years) median (range)	49 (29–74)
<35	10
35–50	45
⩾50	45
FIGO stage	
I	82
II	18
Tumor diameter (mm) median (range)	30 (4–118)
Depth of stromal invasion (mm) median (range)	11 (1–21)
Differentiation	
Well	80
Moderate	11
Poorly	9
Histopathology	
Endocervical type	60
Endometrioid type	33
Intestinal type	2
Serous	3
Clear cell	2

Table 2
Cap43 expression, VEGF expression and MVD

	No. of patients
Immunohistochemical expression of Cap43 score	
0	13
1+	20
2+	35
3+	32
Immunohistochemical expression of VEGF score	
0	13
1+	23
2+	40
3+	24
Microvessel density median (range)	30.2 (8.4–68.1)

Table 3
Correlation between Cap43 expression and VEGF expression

Factor	Cap43 expression		
	Low	High	*P* value
VEGF expression			
Low	56	20	0.0439
High	12	12	

expression, as compared with 27.3 months in those with tumors showing high Cap43 expression ($P = 0.0017$). The median overall survival time was 54.1 months in patients with tumors showing low Cap43 expression, as compared with 36.4 months in those with tumors showing high Cap43 expression ($P = 0.0018$). Kaplan–Meier analysis showed that the intensity of Cap43 expression was significantly associated with survival; high Cap43 expression was associated with unfavorable outcomes (Fig. 3).

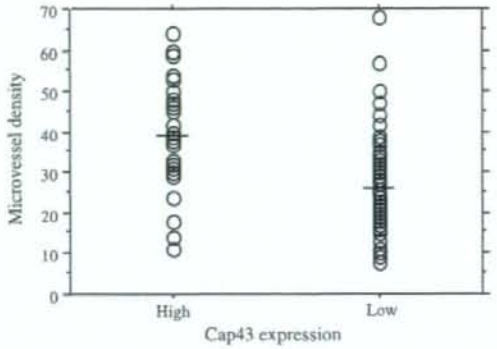

Fig. 2. Correlation between Cap43 expression and microvessel density (MVD). Median MVD was 39.4 in tumors with high Cap43 expression and 26.1 in tumors with low Cap43 expression ($P < 0.0001$).

Table 4
Clinicopathological significance of Cap43 expression

Factor	Cap43 expression		*P* value
	Low	High	
Stage			
I	57	25	0.5788
II	11	7	
Tumor diameter			
<40 mm	51	14	0.0034
⩾40	17	18	
Stromal invasion			
<2/3	42	8	0.0011
⩾2/3	26	24	
Lympho vascular space invasion			
Negative	40	9	0.0053
Positive	28	23	
Lymph node metastasis			
Negative	55	16	0.0022
Positive	13	16	
Differentiation			
Well	60	20	0.0060
Moderate, poorly	8	12	

4. Discussion

Our study showed that the intensity of Cap43 expression was significantly associated with tumor angiogenesis and other poor prognostic factors in cervical adenocarcinoma. Survival analysis showed that high tumor expression of Cap43 was associated with poor progression-free survival and overall survival.

The controversy surrounding the relevance of Cap43 expression in cancer may be attributed in

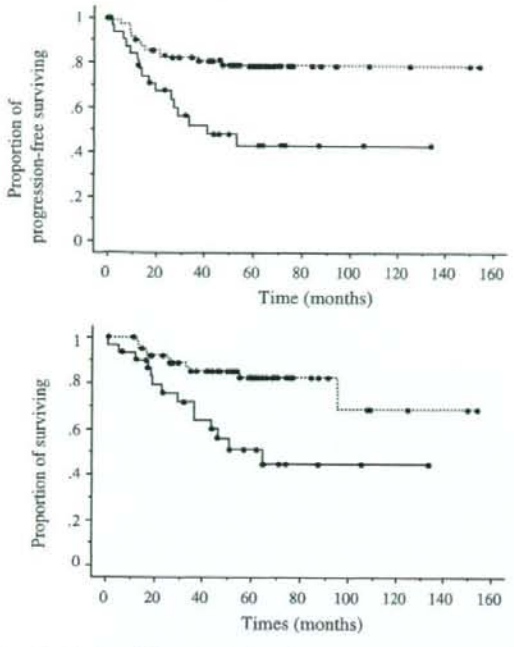

Fig. 3. Kaplan–Meier analysis of progression-free survival (above) and overall survival (bottom) according to Cap43 expression levels in cervical adenocarcinoma. High Cap43 expression (solid line) was associated with significantly poorer outcomes ($P = 0.0017$ and $P = 0.0018$, respectively) than was low Cap43 expression (dotted line). Bold dots indicate censored cases.

part to the fact that Cap43 expression is highly influenced by pleiotropic factors and stimuli, including various metal ions, hypoxia, oncogenes, tumor-suppressor genes, hormones, and vitamins. The expression of Cap43 in patients with cancer thus depends on which factor predominates in a particular case [16]. For example, Cap43 expression in prostate cancer cells is influenced by androgens [34], whereas that in breast cancer cells depends primarily on estradiols [14]. Cap43 expression may vary greatly according to the presence or absence of hormone dependence in hormone-susceptible cancers such as prostate cancer and breast cancer. However, confirmation of possible roles of Cap43 in human malignancies must await the results of future studies that comprehensively evaluate various biologic factors intrinsically related to different types of cancer.

We immunohistochemically studied the intensity of Cap43 expression in surgical specimens. Three patterns of Cap43 expression were observed, consistent with the results of Caruso and colleagues [35]: intense, predominantly membranous staining; intense nucleocytoplasmic localization; and low or undetectable expression. These different patterns of Cap43 expression might be attributed to differences among tumors in factors that either stimulate or inhibit its expression.

Recent advances in cancer research have revealed the importance of angiogenesis to cancer progression. Among the various angiogenic factors identified to date, VEGF and MVD are know to have a pivotal role in tumor angiogenesis and to participate in neovascularization by promoting the differentiation of vascular endothelial cells and increasing capillary permeability. Correlations between neovascularization and metastasis or poor outcomes have been reported in various cancers [36–39]. Previous retrospective studies have reported finding that VEGF and MVD are independent prognostic factors in cervical adenocarcinoma [32]. Our results showed that the significant association of VEGF and MVD with the intensity of Cap43 expression was closely related to high angiogenic activity in cervical adenocarcinoma.

We evaluated Cap43 expression on the basis of the intensity of cellular membrane staining. Cap43 is most often localized in the nucleus, cytoplasm, cell membrane, and intracellular organelles [11]. During the differentiation of various organs, localization of Cap43 may vary between the nuclear membrane and cytoplasm [12,40]. However, Cap43 proteins appear to have no transmembrane domain, signal sequence, or endoplasmic reticulum retention sequence [9]. Cap43 has more than seven phosphorylation sites, two of which are susceptible to protein kinase A and calmodulin kinase II [8]. Recent studies have clearly demonstrated which sites bind to each kinase [16]. These findings suggest that Cap43 has a regulatory role in cells. This regulatory role as well as the cellular localization of Cap43 may be controlled at least in part by phosphorylation. The Cap43 gene is localized in the nucleus of some cell types, but its protein structure has no apparent nuclear localization signals, suggesting that interactions with other protein(s) are required for its nuclear localization [11]. Cap43 protein interacts with a nucleocytoplasmic transport protein, heat-shock cognate protein 70, in mast cells [41,42]. Whether this interaction is required for the nuclear localization of Cap43 remains unclear. In cervical adenocarcinoma, Cap43 was localized primarily in tumor cell membranes. The reasons for this localization pattern are unknown. Further studies are

required to determine the function of this membrane-associated Cap43.

Our study had several limitations. We studied only patients with early-stage cervical adenocarcinoma treated by surgery. Because of the selection bias, the results cannot be directly extrapolated to larger populations of women with cervical adenocarcinoma (e.g., women with stage III or IV cervical adenocarcinoma). Cervical adenocarcinoma continues to have unfavorable outcomes. Assessment of Cap43 expression in cervical adenocarcinomas may provide a useful biomarker for the prediction of outcomes, independently of conventional clinical variables.

In conclusion, our study demonstrated, for the first time to our knowledge, that high expression of Cap43 in patients with cervical adenocarcinoma is associated with tumor angiogenesis and poor outcomes. Our findings remain preliminary and should be confirmed in prospective clinical trials. Moreover, further basic research is required to identify the pathways by which Cap43 protein modulates the malignant characteristics of tumors and to delineate the mechanisms underlying tumor progression in cervical adenocarcinoma.

Disclosure/conflict of interest

The authors declare no potential conflict of interest.

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