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Immunohistochemical analysis of gastrin-releasing peptide receptor (GRPR) and possible regulation by estrogen receptor βcx in human prostate carcinoma

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Gastrin-releasing peptide (GRP) belongs to the family of bombesin-like peptides. GRP was demonstrated to stimulate the proliferation and invasiveness of androgen-independent prostate carcinoma. GRP mediates its action through the membrane-bound receptor, GRP receptor (GRPR), which is characterized by a high-affinity binding for both GRP and bombesin. In human prostate cancer tissue, GRPR mRNA was reported to be detectable in more than 90% but its immunolocalizaition has not been reported. Therefore, in this study we immunolocalized GRPR in 51 human prostate cancer cases and correlated the findings with several clinicopathological parameters in order to beter understand the function and regulation of GRPR in human prostate cancer. GRPR was immnolocalized in carcinoma cells and their values were significantly associated with Gleason score and immunoreactivity of estrogen receptor βcx (ERβcx) that is one of splicing variants of ligand dependent transcription factor, ERβ, and considered to be prognostic factor of prostate cancer patients. The amounts of GRPR and ERβcx mRNA in three prostate cancer cell lines PC-3, DU-145 and LNCaP evaluated by quantitative RT-PCR (qPCR) analysis were also significantly correlated. In addition, we established stable transformants of prostate carcinoma cell line PC-3 introduced with ERβcx, and confirmed that GRPR mRNA was induced in ERβcx over-expressing PC-3 cells by qPCR analysis. These results also suggest that ERβcx contributes to prostate cancer development possibly through mediating GRPR expression in carcinoma cells.

Key words: ERβcx, GRPR, prostate cancer

Gastrin-releasing peptide (GRP) belongs to the family of bombesin-like peptides that includes the amphibian peptide bombesin as well as the mammalian counter-parts GRP and neuromedin B [1]. Several previous studies demonstrated that growth stimulation of bombesin-like peptides played some roles in the process of human carcinogenesis [2-7]. In human prostate cancer, GRP was also reported to increase the proliferation and invasiveness of androgen-independent prostate cancer [8-11]. In addition, the secretion of GRP by neuroendocrine cells has been also proposed to cause prostate cancer progression, development of androgen independence, and poor prognosis [12]. GRP mediates its action through membrane-bound receptors. These receptors correspond to one of the subtypes of the bombesin-like peptide receptors, namely the GRP receptor (GRPR), which is characterized by a high-affinity binding for GRP and bombesin. GRPR also belongs to the members of the large superfamily of G-protein-coupled receptors with seven transmembrane domains. GRPR have been also detected in various types of tumor cell lines including prostate cancer cells [13-16]. In human prostate cancer tissue, GRPR mRNA was also reported to be detected in more than 90% of the cases examined, and to be involved in prostate cancer development by two different investigators [17, 18]. However, GRPR immunolocalizatoin has not been studied in normal human prostate.

In order to further understand the mechanism of functions and regulation of GRPR in human prostate cancer, we immunolocalized GRPR and correlated the findings with clinicopathological parameters including ages, proste specific antigen (PSA) level, Gleason score, stage, lymph node status,

Luciferase assay. The luciferase assay was performed according to a previous report with some modification [24]. Briefly, pERE-Luc, pRL-TK and pRc/CMV-ERβ plasmids were used to analyze dominant negative effects of ERβcx expressed in stable transformant. Cells were seeded at 1×10^5 cells/well in 24-well culture plates in RPMI 1640 supplemented with 10% FBS and allowed to adhere for 24hr. Cultures were subsequentry washed twice with PBS. Cells were then cultured for 24hr in phenol-red-free RPMI1640 without FBS for serum deprivation. Transient transfection was carried out using *Trans*IT-LT1 Transfection Reagents (Mirus Bio Corporation), and the luciferase activity of lysates were measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN(AB-2200) (Atto Co., Tokyo, Japan) after incubation with 100nM 17β-estradiol(Sigma-Aldrich) for 24hr. The cells were also treated with the same volume of ethanol (final dilution-0.05%) for 24hr as controls. The transfection efficiency was normalized against Renilla luciferase activity using pRL-TK control plasmids, and the luciferase activity for each sample was evaluated as a ratio (%) compared with that of controls.

qPCR analysis. Total RNA was extracted from the cells using TRIzol regents(Invitrogen Corporation), and cDNA was synthesized using QuantiTect reverse transcription kit (QIAGEN GmbH, Hilden, Germany). Real-time PCR was carried out using the LightCycler System and FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). The PCR primer sequences of ERβcx, GRPR, and ribosomal protein L13A (RPL13A) used in this study are as follows: ERβcx [AB006589; forward 5'-GATCTTGTTCTGGACAGGGAT-3' and reverse 5'-AGGCCTTTTCTGCCCTC-3'], GRPR [NM_005314; forward 5'-CTGATCCAGAGTGCTTACAA-3' and reverse 5'-CGGTACAGGTAGATGACATGA-3'], RLP13A [NM_012423; forward 5'-CCTGGAGGAGAAGAGGAAAGAGA-3' and reverse 5'-TTGAGGACCTCTGTGTATTTGTCAA-3']. An initial denaturing step of 95°C for 10 minutes was followed by 40 cycles of 95°C for 10 seconds; 10 seconds annealing at 68°C ; and elongation at 72°C for 10 seconds (GRPR and RPL13A) and 15 seconds (ERβcx). The fluorescence intensity of the double-strand specific SYBR GreenI was read at 72°C (GRPR and RLP13A) and 83°C (ERβcx) after the end of each extension step. In initial experiments, PCR products were purified and subjected to direct sequencing to verify amplification of the corresponding sequence. To determine the quantity of target cDNA transcript, cDNAs of known concentrations for target genes, and the housekeeping gene, RPL13A, were used to generate standard curves for qPCR [25-27]. The mRNA level in each case was represented as a ratio of RLP13A and was evaluated as a ratio (%) compared with that of each control [25-27]. Negative control experiments were done without cDNA substrate to examine the presence of exogenous contaminant DNA.

Immunoblotting. The cell protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) with Halt Protease Inhibitor Cocktail(Pierce Biotechnology), according to instruction manual. The concentration of the protein included in cell lysate was measured using Protein Assay Kit Wako (Wako Pure Chemical Industries, Osaka, Japan)). 20 μg of the protein (whole cell extracts) were subjected to SDS-PAGE(10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, England) using Mini Trans-Blot Cell and Power/Pac200 (Bio-Rad Laboratories, Hercules, CA). The blots were blocked in 5% non-fat dry skim milk for 1hr at room temperature, and were then incubated with a 1/1000 dilution of human ERβ antibody (GeneTex Inc., TX, USA), which detects both wtERβ and ERβcx, for overnight at 4 °C. After incubation with anti-mouse IgG horseradish peroxidase (GE Healthcare) for 1hr at room temperature, antibody-protein complexes on the blots were detected using ECL-plus western blotting detection reagents (GE Healthcare). The protein bands were visualized with LAS-1000 cooled CCD-camera chemiluminescent image analyzer (Fuji Photo Film Co, Tokyo, Japan).

Statistical Analysis. Statistical analysis was done using the StatView 5.0J software (SAS Institute, Cary, NC). In an analysis of the possible correlation between immunoreactivity and clinicopathologic variables, values for the patient age, serum PSA level, and LI for Ki-67, AR, ERβ and ERβcx were presented as the mean±95% confidence interval (95% CI). An association between GRPR immunoreactivity and each clinicopathological parameters were evaluated in a Spearman's rank correlation test. In the other studies, results were expressed as mean±SD, and analyzed by a Bonferroni test. In all statistical analysis, a *p-value*<0.05 was considered to indicate statistical significance.

Results

Immunnohistochemistry and correlation between GRPR immunoreactivity and clinicopathologic variables in 51 prostate carcinoma patients. GRPR immunoreactivity was detected in the cytoplasm of prostate carcinoma cells, while immunoreactivity of ERβcx was detectable in the nuclei of prostate carcinoma cells (Fig.1). Results of associations between GRPR immunoreactivity and clinicopathologic variables in 51 prostate carcinoma patients were summarized in Table1. The number of cases with immunoreactive GRPR in each group was summarized as follows: -, 28 cases (54.9%); +, 18 cases (35.3%); and ++, 5 cases (9.8%). The status of GRPR immunoreactivity was also significantly associated with Gleason score and ERβcx immunoreactivity of the cases, whereas there were no significant associations between GRPR and other clinicopathologic variables, including patients' age, serum PSA level, pathological stage, lymph node status, Ki-67 LI, AR LI, ERβ LI and NED status.

Table1. Association between GRPR immunoreactivity and clinicopathological parameters in 51 Prostate Carcinoma

	GRPR immunoreactivity						*P* value
	-		+		++		
	(*n*=28)		(*n*=18)		(*n*=5)		
Ages(years)	66.3 ± 2.2		64.1 ± 2.8		64.0 ± 8.3		0.1843
PSA(ng/mL)	11.9 ± 3.7		14.3 ± 7.0		16.8 ± 23.6		0.4075
Gleason score							
6	14	(27.5%)	0	(0.0%)	0	(0.0%)	
7	8	(15.7%)	11	(21.6%)	0	(0.0%)	
8	4	(7.8%)	4	(7.8%)	5	(9.8%)	
9	2	(3.9%)	3	(5.9%)	0	(0.0%)	**0.0003**
Stage							
pT2	14	(27.5%)	5	(9.8%)	2	(3.9%)	
pT3	14	(27.5%)	13	(25.5%)	3	(5.9%)	0.2156
Lymph node status							
Positive	1	(2.0%)	2	(3.9%)	0	(0.0%)	
Negative	27	(52.9%)	16	(31.4%)	5	(9.8%)	0.6045
Ki-67 LI (%)	7.3 ± 2.4		9.1 ± 2.5		6.0 ± 5.3		0.3431
AR LI (%)	71.6 ± 8.2		73.7 ± 13.1		81.2 ± 9.6		0.2946
ERβ LI (%)	39.1 ± 9.8		46.8 ± 16.5		40.0 ± 37.5		0.7310
ERβcx LI (%)	3.7 ± 2.2		11.9 ± 5.0		19.2 ± 16.9		**<0.0001**
NED status							
Positive	2	(3.9%)	4	(7.8%)	1	(2.0%)	
Negative	26	(51.0%)	14	(27.5%)	4	(7.8%)	0.3185

Data are presented as means±95% confidence interval. All other values represent the number of cases and percentage. Difference between each groups were determined by Spearman's rank correlation test. *P* values less than 0.05 were considered significant, and were represented in boldface.

Stable over-expression of ERβcx in PC-3 cells. ERβcx is expressed in PC-3 cell line at the basal level but its level of corresponding mRNA and protein were near or below the detection limit. PC-3 cells were therefore stably transfected with human ERβcx in order to clarify the function of ERβcx. Following the process of G-418 selection, the levels of ERβcx mRNA expression were evaluated using qPCR, and the clones that strongly expressed ERβcx mRNA were selected as positive clones in this experiment (Fig.2A). Expression of ERβcx protein was also evaluated using immunoblotting analysis (Fig.2B). We subsequently obtained two over expressing clones termed ERβcx-#7 and ERβcx-#8. Luciferase reporter gene assay was performed in order to further confirm the functionality of the ERβcx that was reported to block binding of ERs to estrogen responsive element and to function as dominant negative factors against ERs. ERβ expression vector and ERE-reporter vector were transiently transfected to Mock-#1 and ERβcx-#7 described above. ERE-dependent transactivation via ERβ by estradiol was significantly inhibited in ERβcx-#7 compared to Mock-#1 (Fig.2C).

GRPR mRNA expression in ERβcx over-expressing PC-3 cells using qPCR. We performed qPCR analysis for GRPR mRNA in the control strains transfected with empty vector, Mock-#1 and Mock-#2, and test strains over-expressing ERβcx, ERβcx-#7 and ERβcx-#8. Results of qPCR analysis were summarized in Fig.3A. The levels of GRPR mRNA expression in ERβcx-#7 and ERβcx-#8 were significantly higher (2 to 3fold) than that of Mock-#1 and Mock-#2.

ERβcx and GRPR mRNA expression in PC-3, DU-145 and LNCaP using qPCR. Results of qPCR analysis were summarized in Fig.3B. The amounts of ERβcx and GRPR mRNA expression were relatively higher in PC-3 than in DU-145 and LNCaP. The amounts of mRNA of these two genes were extremely low in androgen-dependent prostate cancer cell line LNCaP.

Discussion

GRPR expression was reported in human prostate cancer by qPCR analysis or receptor autoradiography using radiolabeled ligand for GRPR but GRPR immunolocalozation has not been reported in the literature to the best of our knowledge [17, 18]. Therefore, this is the first immunohistochemical study of GRPR in human prostate carcinoma. Results of our study demonstrated that 45% (+;35%, ++;10%) of the cases examined demonstrated positive immunoreactivity in the cytoplasm of the carcinoma cells. In addition, results of this our present study demonstrated statistically significant positive correlation between GRPR immunoreactivity and Gleason score, the most well-established prognostic factor

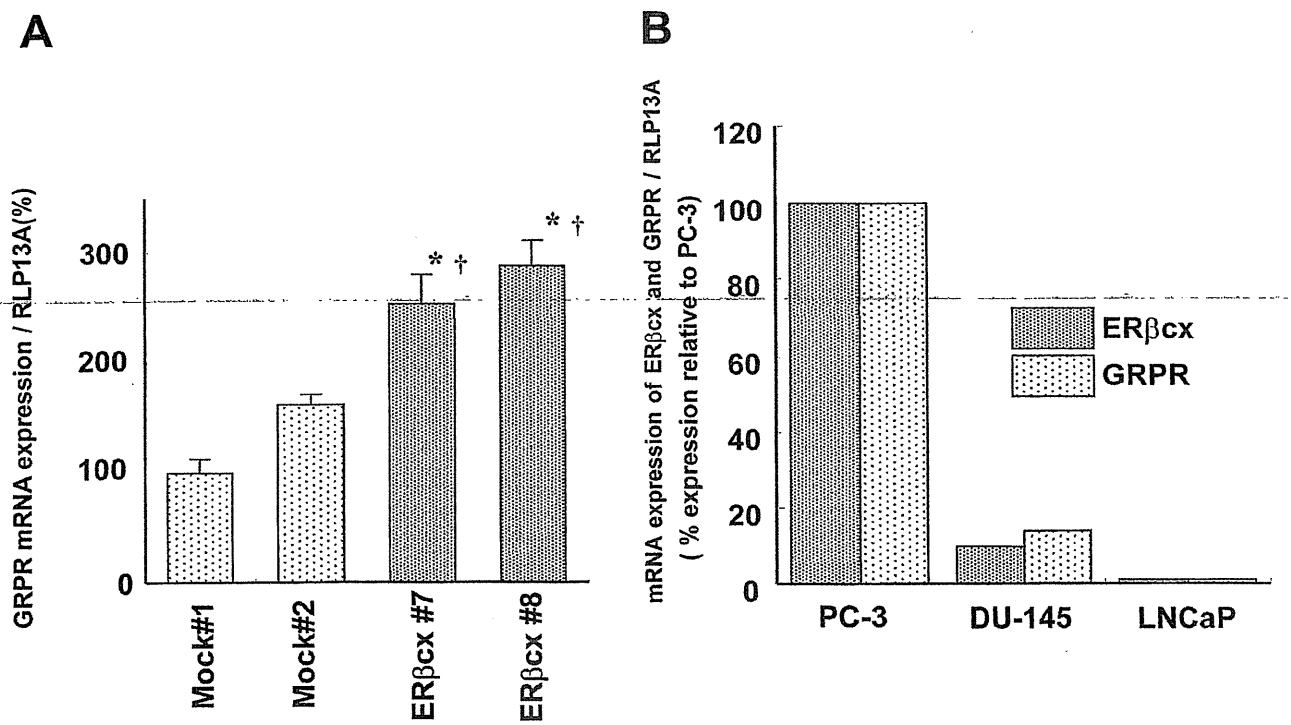

Figure 3. qPCR analysis of GRPR mRNA in ERβcx stably expressing PC-3 cells and human prostate cancer cell lines.
A: Relative expression of GRPR mRNA in stably trasnfected with pRc/CMV or pRc/CMV-ERβcx vector in prostate cancer cell lines PC-3 was analyzed by qPCR. 2 to 3 fold induction of GRPR mRNA was detected in ERβcx-#7 ERβcx-#8 compared to Mock-#1 and Mock-#2. The expression level of GRPR mRNA was evaluated as a ratio(%) compared to that of Mock-#1. Data are presented as mean±S.D.(n=3). $p<0.05$ vs Mock-#1(*) or Mock-#2(†).
B: Comparison of mRNA expression of ERβcx and GRPR in three prostate cancer cell lines, PC-3, DU-145 and LNCaP. Relative expression of ERβcx and GRPR mRNA in each cell lines were analyzed by qPCR. Expression pattern of ERβcx and GRPR were extremely resembled between three cell lines.

reported to significantly suppress the growth or proliferation of PC-3 tumors implanted into the nude mice [30]. Therefore, all of these data above and our present results suggested that the up-regulation of GRPR in prostate carcinoma cells may partially explain these clinical behaviors of prostate adenocarcinomas with neuroendocrine differentiation. However, GRPR immunoreactivity was not correlated with NED status in our study evaluated by chromogranine A and it awaits further investigations for clarification.

The significant correlations were also detected between the status of GRPR and immunoreactivity of estrogen receptor β (ERβ) isoform 2, called ERβcx in prostate carcinoma cases. ERβcx is the one of well-studied isoform of ERβ and expressed in several types of human malignancies including human prostate carcinoma [31-37]. ERβcx was also more abundant in high-grade prostate carcinomas than low grade ones [20]. In addition, clinical outcome of patients with higher ERβcx was worse than those with lower ERβcx in human prostate cancer [20]. Therefore, ERβcx is currently considered as one of the important prognostic factors in the patients with prostate carcinoma. These reports above and our finding suggest that the expression of GRPR could be, at least partially, mediated via ERβcx in direct or indirect manner in human prostate carcinoma cells and sequentially promote the progression of prostate cancer. Results of qPCR analysis in this our present study in which the amounts of ERβcx and GRPR mRNA were evaluated in three different prostate cancer cell lines also strongly supports the hypothesis discussed above. In addition, an induction of GRPR mRNA expression was confirmed in ERβcx over-expressing PC-3 cells. However, it awaits further investigations including some in vitro investigations to demonstrate the regulation of GRPR gene expression through ERβcx in detail.

In summary, we immunolocalized GRPR in 51 human prostate carcinomas and the status of GRPR was correlated with Gleson score and ERβcx LI. These findings also indicates that ERβcx contribute to prostate cancer development possibly through mediating GRPR expression in carcinoma cells.

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Review

The advantages of co-culture over mono cell culture in simulating *in vivo* environment

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ABSTRACT

Breast cancer tissue consists of both carcinoma cells and stromal cells, and intratumoral stroma is composed of various cell types such as fibroblasts, adipocytes, inflammatory including lymphocytes and macrophage and lymphatic and blood capillaries including pericytes and endothelial cells. Recently, cell–cell communications or interactions among these cells have been considered to play an important role to cancer initiation, promotion, and progression. In particular, intratumoral fibroblasts are well known as cancer-associated fibroblast (CAF). CAF is considered to be different from normal fibroblasts in terms of promoting cancer progression through the cytokine signals. Carcinoma cell lines have contributed to the advancement of our understanding of cancer cell biology. Numerous researches have employed these carcinoma cell lines as a single- or mono-culture. However, it is also true that this mono-culture system cannot evaluate interactions between carcinoma and intratumoral stromal cells. Co-culture compositions of two different cell type of cancer tissues *i.e.*, carcinoma cell lines and fibroblasts, were established in order to evaluate cell–cell interactions in these cancer microenvironment. This co-culture condition has the advantage of evaluating cell–cell interactions of cancer microenvironment. Therefore, in this review, we focused upon co-culture system and its application to understanding of various biological phenomenon as an *ex vivo* evaluation method of cancer microenvironment in breast cancer.

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Contents

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1. Introduction

Invasive breast carcinoma is the most common carcinoma among women. It accounts for 22% of all female cancers, which is more than twice the occurrence of malignancies in women at any other sites of the body [1]. Approximately 60–70% of breast carcinomas express the estrogen receptor (ER) α protein in carcinoma cells [2]. In postmenopausal breast cancer patients, an intratumoral production of estrogens occurs as a result of aromatization of androgens into estrogens and is catalyzed by the cytochrome P450 aromatase enzyme [3]. Aromatase inhibition has been the gold standard for treatment of early and advanced estrogen receptor positive breast cancer in postmenopausal women. Aromatase has been reported to be predominantly expressed in intratumoral stromal cells and adipocytes of human breast cancer tissues [4,5]. Intratumoral stromal cells have been considered to play an important role on estrogen paracrine supplying system in breast cancer tissues [4]. In addition, a new concept of stroma-derived prognostic predictor (SDPP) was reported by Finak and co-investigators [6], who proposed subtypes of tumor stroma corresponding to good and poor-outcome breast cancers using laser capture microdissection separating intratumoral stromal cells and subsequent microarray analysis.

Numerous different cell types are involved in the maintenance of cancer tissues and this phenomenon is generally called a cancer microenvironment, which has been studied by increasing number of investigators. This concept itself is by no means a new one and Paget [7] already reported in 1889 that cancer metastasis depends on the presence of cross-talk between carcinoma cells in primary site and metastatic target organ microenvironments based only upon light microscopic evaluation of the cancer tissues, which has been well-known as "*seed* and *soil* theory" in the fields of general pathology.

Cell or tissue culture techniques have provided numerous pivotal information as to understanding the basic biology of cancer. The cell culture allows cancer cells to grow and be maintained outside the body was originally developed in the middle of 20th century [8]. One of the most famous and pivotal cell lines of human malignancy, "HeLa" was first established cell line of human cancer, and originated from surgical pathology specimen of uterine cervical carcinoma from African American patient Henrietta Lacks in 1951 [9]. HeLa cells have been widely available in many laboratories of the world and provided numerous significant scientific findings toward our understanding of cancer. One of the most well-studied breast cancer cell line, MCF-7 was derived in the Michigan Cancer Foundation of the U.S.A. in 1973 from a cytology specimen of pleural effusion or pleuritis carcinomatosa from a 69-year-old Caucasian American woman [10]. This cell line is one of the most studied one among all cancer cell lines and PubMed (National Center for Biotechnology Information) search of "MCF-7" from 1973 to present (July 2011) revealed over 13,000 hits (including the category of review article). However, it is also true that MCF-7 cell line demonstrated some karyotype differences, which is generally considered due to the differences in cell passage number and maintenance or culture conditions among different laboratories [11]. These finding all suggest that the characteristics of cell lines might depend on the difference of the maintenance in each laboratory, which provided the cells, which may explain some of the differences reported among the results employing the same cell line. Despite these drawbacks above, it is very true that carcinoma cell lines have been established very important tools to evaluate in depth the biochemistry and molecular biology associated with individual cancer types and have contributed enormously to our understanding of normal as well as malignant cell physiology [8]. However, as mentioned above, it is practically impossible to reproduce the cancer microenvironment using the cancer cell line alone in *in vitro* analysis. Primary culture of the whole cancer tissue derived from surgery or biopsy can indeed provide very important information as to our understanding of cancer tissue microenvironment but technical difficulties have made it difficult to establish this technique as the standard of *in vitro* evaluation. Therefore, co-culture compositions of at least two different type of cell (*i.e.*, cancer/fibroblast, epithelial cell/lymphocyte, *etc.*) have been established in order to evaluate cell–cell interactions in cancer microenvironment through maximum simulation to *in vivo* microenvironment of human cancer.

2. Tumor microenvironment in breast cancer

Breast cancer is composed of both parenchymal or carcinoma cells and stromal cells. Tumor stroma consists of fibroblasts, adipocytes, inflammatory cells such as lymphocytes and macrophage and lymphatic and blood capillaries including pericytes and endothelial cells. Representative histological feature of invasive ductal carcinoma tissue obtained from postmenopausal breast cancer patient is illustrated in Fig. 1. Despite these complicated architecture of breast cancer tissue environment, the most of the research has been directed toward that in carcinoma or parenchymal cells. Or instance, proliferative activity of breast carcinoma cells has been focused in the great majority of breast cancer research published and is currently considered the most reliable prognostic parameter in early breast cancer or the cancer without metastasis. Toward the evaluation of this proliferative activity of breast carcinoma cells, mitotic count of carcinoma cells under light microscopic evaluation identifying the carcinoma cells undergoing cell division has been incorporated into histological grading system, which has been well-known to be very significantly correlated with the patient outcome [12,13]. However, the evaluation of mitotic activity may be subjective and immunohistochemistry of Ki-67, a non-histone nuclear protein detected in the G1 through M phase of cell cycle but not in resting cells, has been employed and Ki67 labeling index or the ratio of Ki67 positive cells is generally considered to represent the direct indicator of the growth fraction of carcinoma cells examined [14,15].

However, tumor/cancer microenvironment has been recently identified as a major factor influencing treatment resistance of cancer to radiotherapy and chemotherapy [16–18]. In addition, it is also well known that tumor microenvironment play a pivotal role in neoplastic cell initiation or tumorigenesis, progression/development, and metastatic spread of tumor cells [18–20]. For instance, the foci of marked fibrosis or desmoplasia present at invasive front of some invasive ductal carcinoma patients are characterized by the abundance of myofibroblasts [21]. These myofibroblasts contain abundant smooth muscle actin, calponin, desmin, and myosin heavy chain positive [21]. In addition, invasive ductal carcinoma cases associated with these foci of fibrosis or desmoplasia have been demonstrated to be associated with significantly poorer survival than invasive ductal carcinomas without fibrotic focus [22]. The presence of fibrotic focus was also reported to be positively correlated with angiogenesis and distant metastasis in invasive ductal carcinoma patients [23–25].

Fibroblasts or fibroblastic stromal cells are well known as cancer-associated fibroblast (CAF). Both normal epithelial-associated fibroblast (NAF) and CAF can inhibit the growth or cell proliferation of pre-cancerous breast epithelial cells. Normal breast-associated fibroblast demonstrated greater inhibitory capacity than CAF, which suggest that the ability of fibroblasts to inhibit epithelial cell proliferation detected in normal human breast tissue is lost during the process of breast carcinogenesis [26,27]. CAF also has key roles in transformation, proliferation, and invasion of several type of carcinoma including breast carcinoma

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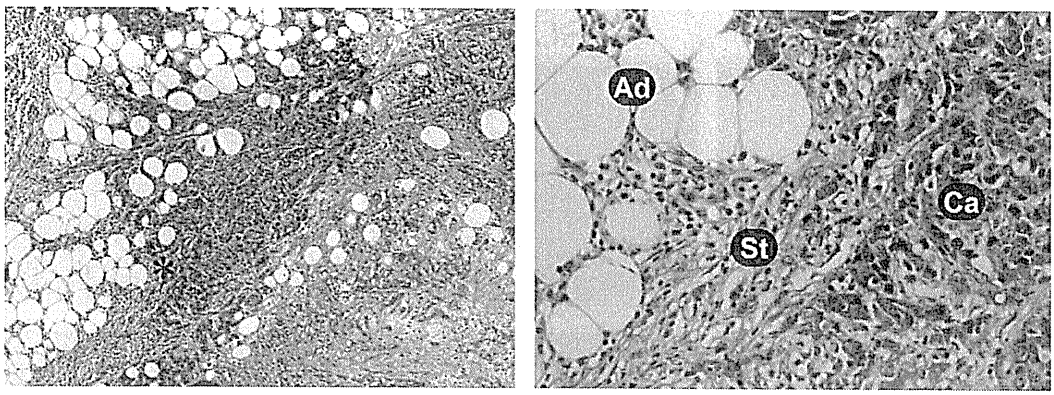

Fig. 1. Invasive ductal carcinoma tissue obtained from postmenopausal breast cancer patient. *Left*: low magnification; *Right*: high magnification of asterisk aria of *left*; Ad, adipocytes in intratumoral stroma; Ca, parenchymal carcinoma cells; St, stromal cells including inflammatory cells and fibroblasts.

[18,28,29]. CAF has been demonstrated to secrete growth factors and chemokines, which result in critical changes in the extracellular matrix and exert oncogenic signals resulting in increased cell proliferation and invasive properties of cancer [18]. In addition, one of the major components in stroma of human breast carcinoma tissues is inflammatory cell such as lymphocyte and macrophage. Cytokines derived from these inflammatory cells are considered to serve as a regulatory factor of invasion, angiogenesis, and growth in breast carcinoma cells [29–31]. Intratumoral macrophage or tumor-associated macrophage (TAM) promotes cancer metastasis through several mechanisms including promotion of angiogenesis, induction of tumor growth, and enhancement of tumor cell migration and invasion [32]. TAM has also been demonstrated to produce a lot of both growth and angiogenic factors such as interleukin-8, tumor necrosis factor-α (TNF-α), macrophage migration inhibition factor (MIF), and vascular endothelial growth factor (VEGF) [30–32].

Therefore, tumor/cancer stromal cells have been considered not only as a mere physical supporting cell of the parenchymal or carcinoma cells but also functional or regulatory cells in tumor/cancer microenvironment. Therefore, endocrine, autocrine and paracrine interactions between parenchymal and stromal cells are considered pivotal for breast carcinogenesis and malignancy such as metastasis, proliferation, and angiogenesis in breast cancer microenvironment. These findings above all indicated that it is required to establish the *in vitro* system that can examine this important carcinoma/stromal cell interaction in human malignancies.

3. Establishment of co-culture system

3.1. Primary culture or cell line: advantages and limitations

Primary tissue or cell culture of cancer derived from an individual patient certainly represents the microenvironment related to the original carcinoma tissue as mentioned above. Because of the difficulties involved in the tissue culture derived from clinical materials, numerous efforts have been done toward the establishment of primary carcinoma cell culture. However, it is also true that the primary culture of carcinoma cells dose have limitations. First, the growth or proliferation of primary cultured carcinoma cells is usually very slow and doubling time is markedly long [5]. In many cases of human malignancy, the primary cultured cells can survive only two to three passages [33]. McCallum and Lowther [34] reported that 135 unselected primary breast cancer tissues obtained from 133 patients were cultured and only 10 resulted in the establishment of the stable cell line in a very experienced laboratory. These findings suggest that the established cell lines, despite several drawbacks or limitations described above, can still provide inert information at least as to the biology of breast carcinoma cells.

In our laboratory of pathology, we have set up the system in isolating fibroblastic stromal cells of selected breast cancer tissues obtained from breast cancer patients with informed consent at operation theater [5,35]. Not all breast cancer cases are suitable for this isolation of stromal cells because of the requirement of certain volume of the cancer tissues for isolation. Therefore, the cases with extremely small foci of cancer are not suitable for this analysis in order not to interfere with histopathological diagnosis. In addition, every efforts should be done to avoid the areas of core needle biopsy, which may result in exaggerated inflammatory response by the procedure and altered characteristics of stromal cells. For mechanical disaggregation, tissues are cut into small pieces using surgical knife in cold culture medium under sterilized conditions. Following further tissue disaggregation with collagenase L derived from *Streptomyces parvulus* subsp. *citrinus* (Nitta Gelatin Inc., Osaka, Japan) for 1–1.5 h, these stromal cells grown to confluence were subsequently cultured in RPMI 1640 with 10% fetal bovine serum. An outgrowth of these cells was detected at 3–5 days of cell culture. These breast intratumoral stromal cells demonstrated typical morphological features of fibroblasts under light microscopy (Fig. 2). The cells corresponding to the passages of five to eight have been employed in the subsequent experiments. There were no morphological changes and cell viabilities between the cells of passage five and eight [5]. It is well known that the proliferation of the fibroblasts under primary culture conditions is faster than that of carcinoma primary cells [18]. Therefore, in generally, the number of passage in the fibroblast primary cultures is much more than that in the carcinoma primary cells [18].

3.2. Co-culture system between carcinoma and stromal cells

Co-culture is one of culture methodologies, which constitute more than two different types of the cells in one culture dish or well. The techniques of co-culture involved varied but this technique has been performed for the purpose of analyzing "cultivation of embryonic stem cells on feeder cells", "examination of immune cell interactions", and "evaluation of mesenchymal–stromal interactions" [5,35–39]. Direct co-culture can be performed in nearly all cell culture dishes, for instance by layering two cell types one on top of the other. Cell–cell direct physical contact was certainly reported to play important roles in the mechanisms of cancer invasion through actions of adhesion molecules such as N-cadherin [40] and EMMPRIN [41]. However, this direct physical contact

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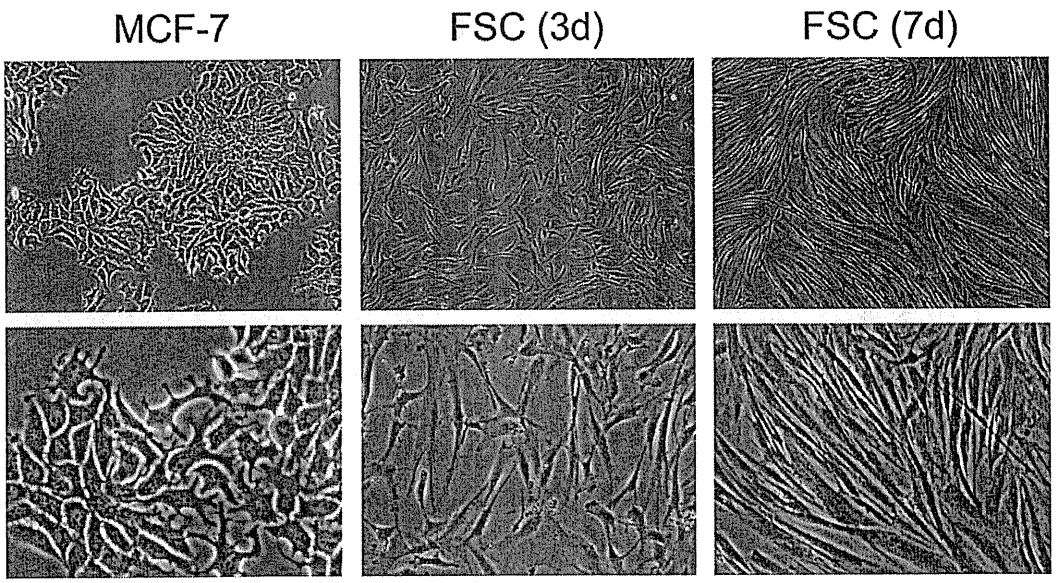

Fig. 2. Primary culture of fibroblastic stromal cells (FSC) derived from breast carcinoma patients and MCF-7. MCF-7 was provided from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). After 3 and 7 days of culture, morphologies of FSC were dendroidal-shaped and spindle-shaped, respectively. *Top*: low magnification; *bottom*: high magnification.

methodology is associated with numerous technical difficulties and indirect co-culture which takes advantage of cell culture inserted with porous membranes to keep the co-cultivated cell populations separated (Fig. 3), has provided more reproducible and reliable *in vitro* results. In our laboratory, we have employed 6 or 12 well ThinCert™ (Greiner Bio-One, Kremsmünster, Austria) cell culture inserts with translucent membranes (Fig. 4) [5,39]. The membrane material is polyester, which is clear film and can directly examine the cells under the light microscopy. Pore sizes and their density of membrane are 0.4 μm and 2×10^6 pores/cm^2, respectively. Study protocol of co-culture system employed in our laboratory is summarized as follows. First, breast carcinoma cell line (*i.e.* MCF-7, Fig. 2) and primary fibroblastic stromal cells are cultured in 6 well plate (bottom) and its insert (upper) the well, respectively [5]. The cells were allowed to adhere overnight at 37 °C and 5% CO_2. After reaching approximately 60–70% confluency of each cells, inserts (primary fibroblastic stromal cells) are placed on 6 well plate (MCF-7). Co-cultures are maintained for 24–72 h at 37 °C and 5% CO_2. After cultivation using this co-culture system, carcinoma and stromal cells were separated and each component was examined by real time RT-PCR, cell count, analysis of enzymatic activity, and cytokine analysis [5,39].

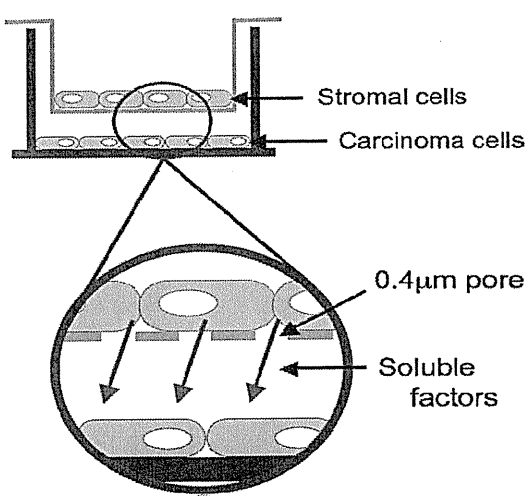

Fig. 3. Schema of co-culture system. The cells could not through the membrane separate each cells. This co-culture system can monitor the cell–cell interactions through the soluble factors derived from each cells.

4. Co-culture *vs.* mono-culture in aromatase expression and activity

In this chapter, we will summarize the advantages of co-culture system of breast cancer by citing the example of the analysis of intratumoral estrogen production in human breast cancer. In human breast cancer tissues, aromatase has been reported to be predominantly detected in intratumoral stromal cells including adipocytes [4,5,42]. The human aromatase expression is regulated through the alternative use of multiple exons 1 [43]. Harada and Honda demonstrated [43] that co-culturing the adipose stromal cells obtained from breast tissues with MCF-7 significantly increased aromatase mRNA and the frequency of alternative utilization of exon 1 (1b–1c). In our previously reported study, the results of combined laser capture microdissection and RT-PCR analysis of aromatase demonstrated that aromatase mRNA were indeed detected in breast parenchymal or carcinoma cells as well as stromal cells [5]. In breast carcinoma cell lines, however, it is also true that aromatase mRNA expression or enzymatic activity is in general below the detection level [43–45]. Therefore, aromatase transfected cell lines such as MCF-7aro and T-47Daro have been established by some investigators and used in many laboratories [46–48]. This discrepancy in terms of aromatase expression between intratumoral carcinoma cells derived from patients and breast carcinoma cell lines is considered to be at least partly due to the lack of interaction of parenchymal or carcinoma cells and stromal cells [5]. We therefore examined the effects of stromal cells on aromatase expression level in breast carcinoma cell lines using co-culture system described above in order to clarify this "discrepancy" between *in vivo* and *in vitro* breast cancer microenvironment [5]. Human breast carcinoma cell lines, MCF-7 and SK-BR-3 cells were cultured in co-culture chambers in the absence or presence of 32N or 74T cells and were cultivated on the bottom of the plates or dishes. Both 32N and 74T cells were intratumoral

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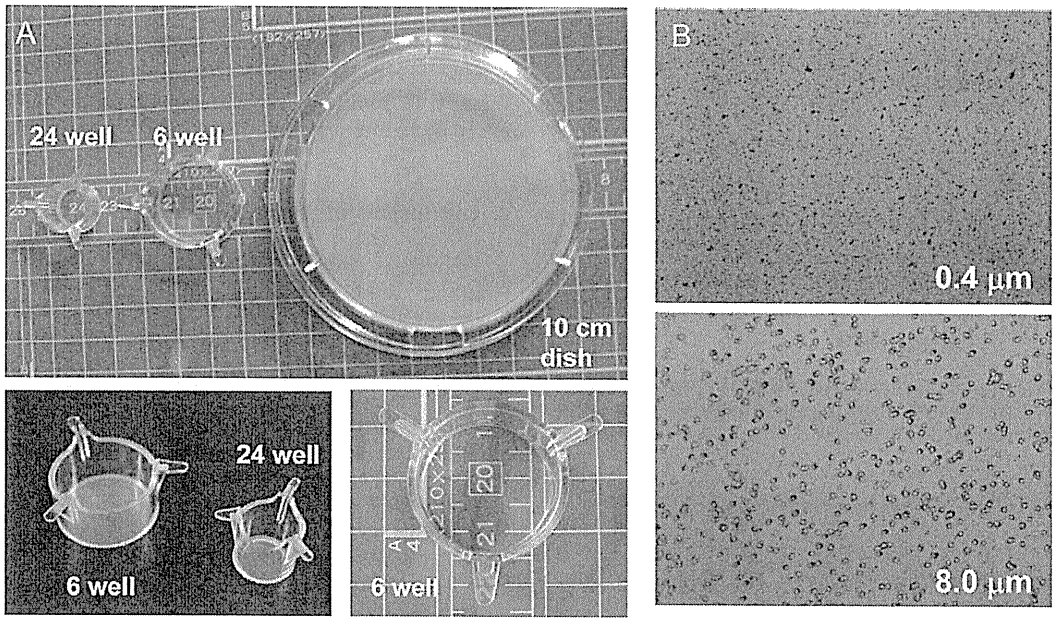

Fig. 4. Co-culture insert employed in our laboratory. (A) *Top*: Co-culture insert for 24 well plate (polyester membrane), 6 well plate (polyester membrane), and 10 cm dish (polycarbonate membrane). *Bottom, left*: Co-culture insert for 24 well and 6 well plate; *right*: translucent membranes of 6 well plate type. (B) High magnification of the surface of a 0.4 (*top*) and 8.0 (*bottom*) μm pore membrane.

fibroblastic stromal cells obtained from breast cancer patients by primary culture [39]. After 24 h cultivation using this co-culture system, both carcinoma and stromal cells were physically separated and each component was examined in the aromatization assay, estrogen production assays or by real time RT-PCR of aromatase. We demonstrated that co-culture of breast carcinoma cell lines with intratumoral fibroblastic stromal cells markedly induced aromatase enzymatic activity/mRNA expression (Fig. 5A and B) and ability of estrogen production [5] in breast carcinoma cell lines. The ability to induce the aromatase enzymatic activity/mRNA expression was by no means detected in the 3T3-L1, which is mouse embryonic fibroblast/adipose like cell line. Furthermore, aromatase activity in both 32N and 74T but not 3T3-L1 were significantly increased by co-culture with MCF-7 (Fig. 5C) [5].

Hayashi et al. [35,49] also established the *in vitro* monitoring system for analyze the estrogen-related cancer microenvironment of individual breast cancer tissues, which was stably transfected GFP reporter DNA inserted estrogen response element in MCF-7 cells (MCF-7/E10). In the presence of substrate for aromatase, testosterone, GFP was significantly induced in MCF-7/E10 cells co-cultured with intratumoral breast stromal cells while no GFP was observed without testosterone [35]. This finding suggests that stromal cells in breast carcinoma convert testosterone to estradiol and activate estrogen signaling in ERα positive MCF-7/E10 cells. They also reported that the ability of stromal cells to activate the ER varied among patients [35]. In addition, aromatase inhibitors suppressed an induction of GFP expression in the coculture, but sensitivities to these drugs also varied among individual cases [35].

We could also clearly demonstrate that using this co-culture technique, an interaction with fibroblastic stromal cells is important in the expression of aromatase in parenchymal or carcinoma cells of lung [39] and endometrial [50] as well as breast carcinoma. These findings in co-culture system confirmed that carcinoma–stromal interactions do play pivotal and critical roles in induction of estrogen dependency through the aromatase in several types of human estrogen dependent malignancies.

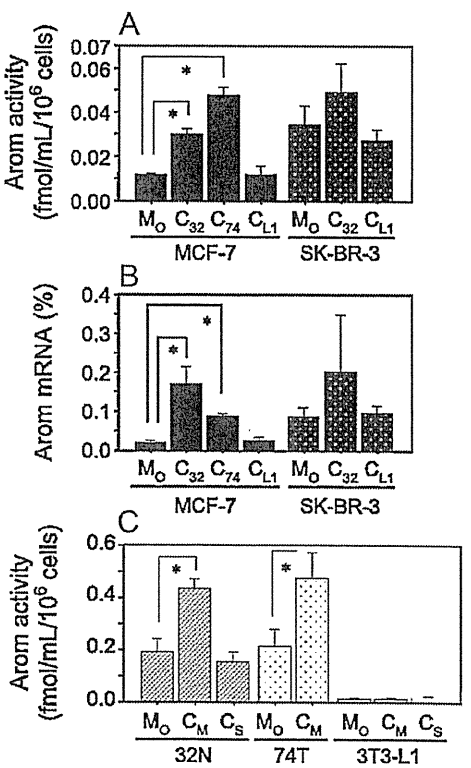

Fig. 5. The effects of co-culture on aromatase mRNA level and enzyme activity in breast carcinoma and stromal cells. (A) Aromatase mRNA level in MCF-7 or SK-BR-3 cells; M_O, monoculture. (B) After co-culture with 32N (C_{32}), 74T (C_{74}), 3T3-L1 (C_{L1}), MCF-7 (C_M), or SK-BR-3 (C_S) cells. (B) Aromatase enzyme activity in MCF-7 and SK-BR-3 cells. (C) Aromatase mRNA level in 32N, 74T, and 3T3-L1 cells.
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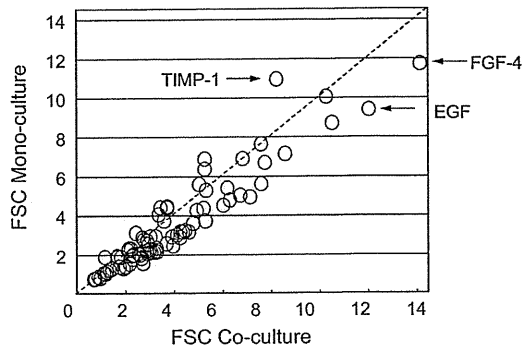

Fig. 6. Scattered plot of cytokine expression level in fibroblastic stromal cells (FSC). *X* axis, cytokine expression level in FSC after co-culture with MCF-7; *Y* axis, cytokine expression level in FSC mono-culture; dashed line, *Y*=*X*.

5. Co-culture *vs.* mono-culture in cytokine expression profile

Primary fibroblastic stromal cells (FSC, Fig. 2) derived from human breast cancer tissues were cultured in transwell chambers in the absence or presence of MCF-7 cells and were placed at the bottom of the co-culture 6 well plate. After 72 h of cultivation using this co-culture system, FSC cells were physically separated and the culture medium was replaced by FBS and phenol red-free medium. After 24 h, the conditioned medium (total 18 mL) was collected and concentrated to a total volume of 2 mL using Macrosep Centrifugal Devices (Pall Corporation) [39]. In this particular analysis, Human Cytokine Antibody Array 5 (RayBiotech, Inc.) [5,51], which detects 79 cytokines on one membrane, was used to determine the profiles of cytokines released from breast FSC. Cytokine antibody membranes were incubated for 2 h with 2 mL of condensed conditioned medium described above. The reacted membranes were subsequently incubated for 1 h with biotinconjugated anti-cytokines and then developed with horseradish peroxidase–streptavidin and chemiluminescence. Protein dots were visualized with Las-1000 cooled CCD-camera chemiluminescent image analyzer (Fuji Photo Film Co., Ltd.). The relative abundance of reacted signals was subsequently quantified as OD value with Science Lab 99 Image Gauge 3.2 software (Fuji Photo Film Co., Ltd.).

The results of the expression pattern of cytokines derived from breast intratumoral FSC are summarized in Fig. 6. The significant changes were detected in Fibroblast Growth Factor-4 (FGF-4), epidermal growth factor (EGF), and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression levels. The levels of FGF-4 and EGF in FSC were increased by co-culture with MCF-7 cells. The level of TIMP-1 in FSC was decreased following co-culture with MCF-7. EGF receptor (EGFR) was reported to be detected in breast carcinoma cells and its gene amplification was also detected in breast carcinoma expressed high levels of EGFR protein [52,53]. An overexpression of growth factor receptors including EGFR has been also reported as one of the mechanisms of endocrine therapy resistance in ER positive breast cancer patients [54,55]. FGF-4, initially isolated as a transforming gene from human tumors, is a member of the Fibroblast Growth Factor family. FGF-4 was detected in breast carcinoma tissues [56], and was reported to be the tamoxifen resistance factor in MCF-7 mice xenograft model [57]. High levels of TIMP-1 mRNA in breast carcinoma showed significant correlation with the presence of lymph node metastases, distant metastases, and early death of the disease [58]. Otherwise, TIMP-1 decreases cell proliferation of nonmalignant human breast epithelial MCF10A cells through cell cycle arrest at the G_1 phase [59]. TIMP-1-mediated G_1 arrest is associated with upregulation of $p27^{KIP1}$ and downregulation of cyclin D_1 expression *via* differential regulation of protein stabilization [59]. These finding all suggested that cytokine released from stromal cells exerted enormous effects upon progression of carcinoma cells including resistance of chemotherapy or endocrine therapy. In addition, cytokine expression profile was also influenced by an interaction with carcinoma cells through the cytokine.

6. Conclusions and future perspectives

Fibroblasts were first described in the late 19th century [60]. Fibroblasts are highly heterogeneous, and those isolated from different normal tissues reflect a substantial topographic diversity [60]. Co-culture system revealed that intratumoral fibroblastic stromal cells are also heterogeneous in individual patients. Therefore, this co-culture system makes it possible to study the cancer microenvironment in each individual breast cancer patients. In addition, this *in vitro* system can be also useful for predicting the prognosis or recurrence and the response to drug such as chemotherapy and endocrine therapy. In recent years, paradigm shift from two-dimensional (2D) to 3D cell culture techniques has been developed rapidly. 3D culture affects cell functions and behaviors including morphology and gene expression in a similar fashion to the *in vivo* response [61]. Human liver cells such as primary hepatocytes and HepG2 have been reported to be associated with significant changes of albumin or P450s expression under the different culture conditions such as 2D or 3D cultures [62–64]. Cell–cell interaction between bone cells has been also well examined in osteogenesis [65]. Osteoclasts including their precursor cells express receptor activator of nuclear factor kappa B (RANK) that recognizes RANK ligands (RANKL) through direct cell–cell interaction with osteoblasts or their immature putative precursor stromal cells [65–67]. In addition, this interaction between osteoblasts and osteoclasts was demonstrated to result in more pronounced cell differentiation when cultured in 3D than in 2D conditions [68]. The great advantage of the 3D co-culture system without using any scaffold compared to 2D co-culture is that it is possible to evaluate cell–cell interaction by both paracrine and direct cell–cell contacts. It is true that further studies are required for refinement of co-culture system but this technology can contribute greatly to the advancement of high-throughput screening, prediction of response to antineoplastic drugs and prediction of clinical patient outcome in individual breast cancer patients.

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EXPERT OPINION

Effects of estrogen depletion on angiogenesis in estrogen-receptor-positive breast carcinoma – an immunohistochemical study of vasohibin-1 and CD31 with correlation to pathobiological response of the patients in neoadjuvant aromatase inhibitor therapy

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Objectives: Tumor–stroma interactions, including angiogenesis, are pivotal in breast cancer. Changes of angiogenesis during endocrine therapy have not been reported in breast cancer patients. Vasohibin-1 (VASH-1) is a recently identified endothelium-derived negative feedback regulator of angiogenesis. Vasohibin-1 positive ratio (VPR) is proposed as an indicator of neovascularization of the tissues.

Methods: The status of neovascularization, based on VPR before and after steroidal aromatase inhibitor (AI) exemestane (EXE) treatment, was evaluated in 54 post-menopausal Asian patients. VPR changes were correlated with the pathobiological response of the patients using Ki67 labeling index (LI) changes.

Results: When using a decrement of more than 40% in post-treatment Ki67 LI as the definition of response, significant inverse correlation was detected between Ki67 LI and VPR changes in responders. Significant increment in neovascularization, as demonstrated by elevated VPR, was only detected in responders ($p = 0.039$). Increased angiogenesis detected in responders to neoadjuvant therapy may represent a stromal response to dying/dead cells, as part of tumor–stroma interaction following estrogen depletion.

Conclusions: VPR could be a potential surrogate marker for predicting neoadjuvant endocrine therapy response, incorporating features of both carcinoma and stromal cells, in the early stage of neoadjuvant endocrine therapy before any discernible clinical and/or histopathological changes became apparent.

Keywords: angiogenesis, aromatase inhibitors, breast cancer, immunohistochemistry, vasohibin

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1. Introduction

In human breast cancer, the overall tumorigenesis and disease progression/ metastasis, as well as the response of tumor to therapeutic agents were all closely correlated with complicated interaction and balance of cellular pathways within the tumor cells and their interaction with the stromal microenvironment [1-11]. One of the most important carcinoma-stromal cells interactions is angiogenesis [1-12], in which cross-talk between tumor and endothelial cells occur as a result of *in situ* production and secretion of numerous cytokines and growth factors including angiogenic factors such as vascular endothelial growth factor (VEGF) elevated in their microenvironment [3,6,8,12-14]. These cytokines and growth factors act upon their corresponding receptors present in the endothelial cells and subsequently induce angiogenesis.

Breast cancer is one of the well-known angiogenic dependent diseases and neovascularization or angiogenesis plays an important role in its development, subsequent invasion and metastasis [6-8,11,15,16]. Therefore, the tilt of balance of angiogenesis can determine the progression or halt of these processes above [5-10,13-15,17]. The status of *in situ* angiogenesis in breast cancer tissues has been reported to be correlated with the overall and relapse-free interval of the patients [18,19]. Therefore, the analysis of angiogenesis could provide important information as to understanding the biology of breast cancer. Most of the previous studies on tumor angiogenesis in breast cancer, however, employed the measurement of microvascular density (MVD) using immunohistochemistry endothelial markers like CD34 [18-20]. It is true that results of these studies have provided very important information relating to angiogenesis of human breast cancer but it is also true that they only evaluated static vascularity but cannot necessarily demonstrate the actual degrees of angiogenesis. Therefore, numerous investigators have looked for specific markers of angiogenesis in endothelial cells especially applicable to an evaluation in surgical pathology or archival specimens.

Vasohibin-1 (VASH-1) is a recently identified endothelium-derived negative feedback regulator of angiogenesis [21-23] and has been demonstrated to be the first physiologically specific suppressor of angiogenesis in human [21-23] by inhibiting several endothelial functions relevant to neovascularization *in vitro* [21-23]. VASH-1 is induced by angiogenic growth factors such as fibroblast growth factor 2 (FGF-2), basic FGF (bFGF) and VEGF (via the type 2 VEGF receptor activation and its downstream receptor, PKC-δ) [21-23]. CD31 is the most widely used and most sensitive immunohistochemical panendothelial marker in archival of 10% formalin fixed and paraffin-embedded tissue specimens. CD31 immunoreactivity is in general considered more specific compared with CD34 immunoreactivity because CD34 was also expressed in lymphatics and other non-endothelial cells [24]. However, it is also true that CD31 is expressed in both actively proliferating and quiescent endothelial cells (Figure 1A) [23-25]. VASH-1, on the other hand, is selectively expressed in proliferating endothelial cells (Figure 1B) [23,25]. The status of VASH-1 in particular tissues was reported to be directly related to the degree of angiogenesis, possibly as a result of an interaction between tumor and stromal cells [23,25]. Tamaki *et al.* [25] demonstrated a significantly positive correlation between Ki67-positive proliferating vascular endothelial cells and VASH-1-positive endothelial cells in human breast cancer cases. Therefore, VASH-1 is considered a more appropriate immunohistochemical marker for intratumoral neovascularization compared with CD31 and the use of vasohibin-1 positive ratio (VPR) is considered an indicator of the status of neovascularization in human breast cancer [25]. To the best of our knowledge, the alterations of *in situ* angiogenesis during the course of neoadjuvant endocrine therapy with aromatase inhibitors (AI) have not been reported at all in human breast cancer. Therefore, in this study, the changes of *in situ* angiogenesis were evaluated first, assessed by VPR during neoadjuvant AI treatment of estrogen-receptor-positive breast cancer and then the results were compared with pathobiological responses of individual patients. This was in order to elucidate the possible involvement of angiogenesis in estrogen depletion in estrogen receptor (ER)-positive postmenopausal breast cancer patients.

2. Materials and methods

2.1 Breast cancer cases

A total of 54 ethnic East Asian postmenopausal patients (32 Japanese and 22 ethnic Chinese from Hong Kong, China) were included in this combined analysis. The combined analysis was based on two sets of study which were all performed in East Asian population of ER-positive breast cancer patients with similar pre-treatment parameters. All had operable breast cancers referred to the tertiary referral centres in Kyoto between March 2006 and January 2008 [26]; and those in Hong Kong between November 2001 to April 2004 [27]. An approval was obtained from the individual local ethics committee or institutional review board. In addition, informed consents were obtained from all the patients before enrolment into this trial.

The combination of these groups of patients was justified based on the statistical analysis (results as shown in Table 1) of their baseline parameters which showed no statistically significant differences in any of the pre-treatment biomarkers between these two subgroups in the pre-treatment cohort.

2.1.1 Hong Kong subgroup of patients

The 22 patients in the treatment arm treated with exemestane only among those enrolled in the celecoxib anti-aromatase neoadjuvant (CAAN) trial were evaluated in this analysis. The detailed study design was previously reported by Chow *et al.* [27]. exemestane (25 mg) was given to each of this group of patients for three months [27].

2.1.2 Japanese subgroup of patients

The 32 Japanese post menopausal patients with invasive ductal carcinoma among those enrolled into the study of

Figure 1. Representative illustrations of immunohistochemical findings of one of the breast carcinoma cases examined. A. Post-treatment specimen at one of the hot spots, showing staining of the vessels positively for CD31, note the moderate intense staining of both the small sized (arrowheads) and large sized vessels (arrows). **B.** At the same site of hot spot of CD31, showing the positive staining for vasohibin-1. Note that not all vessels stained positive for CD31 were positive for VASH-1. VASH-1 positively stained vessels were all small sized vessels (arrowheads). The large sized vessels were not stained by VASH-1 (but stained by CD31).

Table 1. Comparison of individual IHC scores of biomarkers between specimens from CAAN and Kyoto subgroups.

Biomarkers	Pre-treatment			Post-treatment			Changes of biomarkers		
	CAAN (n = 22)	Kyoto (n = 32)	p*	CAAN (n = 22)	Kyoto (n = 32)	p*	CAAN (n = 22)	Kyoto (n = 32)	p*
VPR	0.523 ± 0.059	0.565 ± 0.048	0.711	0.619 ± 0.059	0.566 ± 0.054	0.497	0.490 ± 0.211	0.255 ± 0.191	0.083
Ki67	15.471 ± 2.645	11.750 ± 1.878	0.149	10.548 ± 2.125	6.063 ± 1.125	0.094	0.509 ± 0.634	-0.342 ± 0.109	0.692
CD31	71.705 ± 17.886	65.031 ± 7.027	0.860	62.750 ± 11.129	70.906 ± 7.856	0.184	0.139 ± 0.188	0.574 ± 0.342	0.283
Vasohibin-1	26.182 ± 1.981	34.875± 4.868	0.653	33.045 ± 3.435	39.078 ± 7.546	0.881	0.484 ± 0.207	0.345 ± 0.198	0.384
HER2	1.667 ± 0.174	1.406 ± 0.155	0.311	1.524 ± 0.214	1.281 ± 0.169	0.375	-0.143 ± 0.186	-0.125 ± 0.117	0.895
ER	7.227 ± 0.286	7.250 ± 0.311	0.658	7.023 ± 0.308	7.094 ± 0.309	0.481	-0.205 ± 0.458	-0.156 ± 0.120	0.734
PgR	6.455 ± 0.388	5.313 ± 0.468	0.100	5.227 ± 0.431	2.531 ± 0.447	0.000	-1.227 ± 0.530	-2.781 ± 0.555	0.015

Data is shown as mean ± SEM.

*Mann–Whitney U test was used for the analysis of the differences between pre-treatment specimens from CAAN and Kyoto subgroups. The results indicated the pre-treatment specimens from the two subgroups are homogeneous and the specimens could be treated as a whole cohort in subsequent analysis. See text for the details.

ER: Estrogen receptor; HER2: Human EGFR2; PgR: Progesterone receptor; VPR: Vasohibin-1 positive ratio.

JFMC34-0601 [26] of the Japanese Foundation of Multidisciplinary Treatment of Cancer between March 2006 and January 2008 were examined in our present study. The study design was previously reported [26,28]. Subjects were treated with exemestane at daily 25 mg dosage for 16 to 24 weeks [26].

2.2 Breast tissue specimens

The specimens available for this study were core needle biopsies of the treatment-naïve tumor and the surgical specimens obtained after the treatment at the time of surgery. All the biopsy and surgical specimens had been fixed in 10% formalin for 12 – 24 h and 24 – 48 h, respectively. Pre- and post-treatment specimens of these 54 patients were available for the study of pathobiological responses and immunohistochemical evaluation.

2.3 Immunohistochemistry

2.3.1 Immunostaining

Immunoreactivity of Ki67, CD31 and VASH-1 was evaluated. The proliferation of tumor cells was assessed by the percentage of Ki67 labelling index (LI) obtained by evaluating 1000 or more carcinoma cells in individual cases. The degree of angiogenesis was assessed by the pre- and post-treatment VPR which was defined as the ratio of vasohibin-1 to CD31 positive counts obtained at the same hot spots in same fields of the slides [25]. The details of immunohistochemistry (IHC) were previously described by Tamaki *et al.* [25] and Chanplakorn *et al.* [28]. In brief, serial tissue sections (4 μm) were prepared from selected pre- and post-treatment blocks from the endocrine therapy cohort for hemotoxylin–eosin staining and immunohistochemistry of Ki67, CD31 and VASH-1. The specimens were deparaffinized in xylene and hydrated with graded

alcohols and distilled water sequentially. Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 10 min at room temperature. Immunohistochemical staining was performed by a streptavidin–biotin amplification method using a Histofine Kit (Nichirei Bioscience, Tokyo, Japan). The methodology and the different antibodies used for immunohistochemical staining of different biomarkers were previously reported in detail by Chanplakorn *et al.* [28], Watanabe *et al.* [21] and Tamaki *et al.* [25].

2.3.2 *Evaluation of immunoreactivity*

Immunoreactivity of Ki67 was evaluated by three of the authors (M Chan, N Chanplakorn and T Suzuki) independently, with all being blinded to the treatment regimens and clinical course of the patients. The Ki67 LI was sub-classified, using the same criteria as of Chanplakorn *et al.* [28], Miller *et al.* [29] and Yu *et al.* [30], into two different groups according to the changes in percentage of Ki67 LI before and after treatment. They are summarized as follows:

Group 1 or non-responders: Ki67 LI in this group was associated with increased, unchanged or reduction of less than 40% of the post-treatment Ki67 LI compared with the pre-treatment Ki67 LI.

Group 2 or responders: the Ki67 LI demonstrated a reduction of more than 40% compared with the pre-treatment level.

The immunoreactivity of CD31 and VASH-1 of the endocrine therapy cohort were evaluated by two of the authors (M Chan and N Chanplakorn) independently, both of whom were blinded to the treatment regimens and clinical course of the patients, by the Chalkley counting procedure. In brief, three hot spots in each of the specimens were selected for the assessment in the pre-treatment and post-treatment specimens (Figure 1A & Figure 1B). The VASH-1 positivity at the corresponding sites as of the CD 31 hot spots was then evaluated. The VPR was then derived based on the CD31 and VASH-1 positivity for each of the specimens (as determined by the site with highest MVD on CD31 immunohistochemistry). The VPR was defined as the ratio of vasohibin-1 positive counts to CD31 positive counts obtained in the same field of the slide [25]:

$$\text{VPR} = \text{vasohibin-1 positive counts} / \text{CD31 positive counts}$$

The scoring of the ER and progesterone receptor (PgR) immunoreactivity was evaluated by two of the authors (Hong Kong subgroup by M Chan and N Chanplakorn, Kyoto subgroup by T Suzuki and H Sasano), independently. The scoring was by assigning proportion and intensity scores, with reference to the Allred's procedure [31]. Human EGFR 2 (HER2) was independently evaluated in both of the series by two of the authors (T Suzuki and H Sasano). The membrane staining pattern was estimated in HER2 immunohistochemistry and scored on a scale of 0 – 3 described by Wolff *et al.* [32].

Olympus BX50 (Olympus, Tokyo, Japan), 20× and 40× objectives were used for the analysis.

The average numbers counted by investigators were used for subsequent data analysis.

2.4 Statistical analysis

Statistic analyses were performed using SPSS 13.0 statistic analysis software package (IBM, Corporation, Route 100, Somers, NY 10589, USA). The non-parametric Mann–Whitney U test was used to determine the homogeneity of the Hong Kong CAAN and Kyoto subgroups. Wilcoxon matched-pairs signed ranks test was used to determine the statistical significance of the mean changes of IHC scores of individual biological markers including the Ki67 LI, CD31, VASH-1 and positivity as well as that of VPR between pre- and post-treatment specimens. Statistical significance was considered to correspond to a p value < 0.05.

3. Results

3.1 Pre-treatment status of biological markers between Hong Kong and Kyoto subgroups

There were no statistically significant differences in any of the pre-treatment biomarkers between the two subgroups in the pre-treatment cohort. In post-treatment groups, the overall decrement in Ki67 is more pronounced in the Kyoto group than in the CAAN group but this difference did not reach statistical significance. Neither were there statistically significant differences in other post biomarkers between the two subgroups in our cohort. Therefore, the CAAN and Kyoto subgroups were analyzed as a single cohort. The details were summarized in Table 1.

3.2 Analysis of alterations of different biomarkers with respect to treatment response

Following AI treatment, IHC scores of CD31, VASH-1, ER and HER2 as well as VPR did not demonstrate statistically significant differences or alterations. Only Ki67 LI and PgR demonstrated significant decrements following AI treatment. The details were summarized in Table 2.

Responders to endocrine therapy were defined as those associated with decrement of the Ki67 LI of more than 40% following the treatment, those with a decrement of less than 40% and/ or increment of Ki67 LI after treatment were classified as non-responders according to the criteria of Chanplakorn *et al.* [28], Miller *et al.* [29] and Yu *et al.* [30].

In the responder group of patients, VPR demonstrated significant increment following AI treatment ($p = 0.039$); while ER and PgR status decreased significantly following the therapy ($p = 0.008$ and $p < 0.001$ respectively).

In non-responder group of the patients, no significant alterations of the VPR were detected. Only significant decrement of PgR was noted ($p = 0.016$).

In order to further evaluate the correlation between Ki67 LI of the tumor cells and VPR, sub-classifications were created, separating the entire cohort of the patients into VPR-increased and decreased groups using the 'zero' value of VPR changes following AI treatment as the cut off value: the VPR-increased

Table 2. Comparisons of pre- and post-treatment scores of biomarkers in tumors of whole aromatase inhibitor (AI) cohort (n = 54). As a whole cohort, after AI treatment, only Ki67 and PgR show significant decrement.

Biomarkers	Pre-treatment	Post-treatment	Mean change [95% CI]	p value‡
VPR	0.548 ± 0.037	0.587 ± 0.040	-0.039 [-0.134, 0.055]	0.270
Ki67	13.266 ± 1.554	7.890 ± 1.122	5.376 [2.670, 8.083]	< 0.001*
CD-31	67.750 ± 8.304	67.583 ± 6.457	0.167 [-14.123, 14.457]	0.846
Vasohibin	31.333 ± 3.032	36.620 ± 4.670	-5.287 [-12.190, 1.616]	0.394
HER2	1.509 ± 0.116	1.377 ± 0.133	0.132 [-0.070, 0.335]	0.196
ER	7.241 ± 0.216	7.065 ± 0.220	0.176 [-0.219, 0.571]	0.289
PgR	5.778 ± 0.326	3.630 ± 0.364	2.148 [1.338, 2.958]	< 0.001*

For scores of biomarkers in pre- or post-treatment specimen, data is shown as mean ± SEM.
For the change of scores of biomarkers after treatment, data is shown as means with 95% CI [lower, upper values].
*A p value less than 0.05 is considered significant.
‡Wilcoxon matched-pairs signed-ranks test for the difference between groups.
ER: Estrogen receptor; HER2: Human EGFR2; PgR: Progesterone receptor; VPR: Vasohibin-1 positive ratio.

group was defined as post-treatment > pre-treatment VPRs for each individual paired specimens; those paired specimens associated with post-treatment ≤ pre-treatment VPR were tentatively assigned to the VPR-decreased group. The Ki67 LI of tumor cells was associated with significant decrement only in the VPR-increased group but not in the decreased one ($p < 0.001$) (Table 3).

3.3 Alterations of biomarkers with respect to different baseline Ki67 LI groups

The cohort in this study was tentatively divided into high- and low-baseline Ki67 LI groups according to the mean IHC scores of baseline Ki67 LI. The subsequent changes of IHC scores of individual biomarkers were analyzed in these groups. In high- and low-Ki67 LI groups, both Ki67 LI and PgR status demonstrated significant decrement following AI treatment (change in Ki67: $p = 0.001$ and $p = 0.033$; change in PgR: $p = 0.004$ and $p < 0.001$, for high- and low-Ki67 LI groups respectively) but the mean changes of Ki67 LI were not different between these two groups ($p = 0.690$). In addition, VPR increment was more frequent in the high-baseline Ki67 group, although statistically not significant ($p = 0.058$). This tendency was not detected in the low-Ki67 group ($p = 0.884$) (Table 4).

3.4 Alterations of biomarkers with respect to different baseline VPR groups

In order to determine whether baseline VPR is associated with alterations of different biomarkers following AI treatment, the entire cohorts of the patients were tentatively divided into high- and low-baseline VPR groups according to the mean of IHC scores of baseline VPR, as established normal values of VPR are not available. Within our cohort, the mean value of VPR was 0.549, those cases having a VPR value ≥ 0.549 were therefore tentatively classified as the high-VPR group, those that had a VPR value < 0.549 as the low-VPR group. Within these two groups of low- and high-baseline VPR, only Ki67 LI and PgR status demonstrated significant decrement (change in Ki67: $p < 0.001$ and $p = 0.035$; change in PgR: $p = 0.002$ and $p = < 0.001$, for the low- and high-baseline VPR groups respectively) following AI treatment (Table 5).

4. Discussion

Breast cancer is still the leading cause of cancer death in women all around the world, despite efforts in early detection and availability of new treatments. Different systemic treatment modalities have been used in the adjuvant, neoadjuvant and metastatic settings. Neoadjuvant systemic treatment not only can decrease the size of the tumors for less invasive surgery but also allows an assessment of the efficacy of that particular agent on the tumor which may help predict the long-term treatment response and in tailoring the selection of subsequent adjuvant therapy of the patients [33,34]. Neoadjuvant endocrine therapy with aromatase inhibitors is currently considered one of the standard endocrine therapies in neoadjuvant treatment of ER-positive breast cancers in postmenopausal patients.

Tumor–stroma interactions, particularly angiogenesis, play an indispensable role in tumorigenesis and tumor development [1,2,4-6,8,9,11,12,15,17,35]. Results of previous studies demonstrated that antiestrogens, including tamoxifen and pure antiestrogens, also inhibit angiogenesis [36]. Tamoxifen was reported to inhibit the VEGF-mediated angiogenesis by attenuating the effects of VEGF upon the endothelial cells, in addition to the reduction of serum VEGF levels [37]. Results of *in vitro* studies also demonstrated that pure antiestrogen ICI compounds such as fulvestrant suppressed tumor cell proliferation in an ER-dependent manner and further inhibited the process of angiogenesis by preventing branching and capillary-like tubule formation, and by activating apoptotic pathways in endothelial cells [38]. The impairment of angiogenesis caused by fulvestrant is also partially related to inhibition of progestin induction of VEGF at both the mRNA and protein levels, which inhibits angiogenesis secondary to

Table 3. Comparisons of pre- and post-treatment immunohistochemical scores of biomarkers in tumors according to different groups of vasohibin-1 positive ratio (VPR) change after aromatase inhibitor treatment.

Biomarkers	VPR-decreased (n = 22)				VPR-increased (n = 32)			
	Pre-treatment	Post-treatment	Mean change [95% CI]	p value‡	Pre-treatment	Post-treatment	Mean change [95% CI]	p value‡
Ki67	9.210 ± 2.183	6.488 ± 1.495	2.723 [-0.329, 5.774]	0.089	16.054 ± 2.033	8.854 ± 1.587	7.201 [3.128, 11.273]	< 0.001*
HER2	1.619 ± 0.161	1.238 ± 0.181	0.381 [0.076, 0.685]	0.021*	1.438 ± 0.162	1.469 ± 0.185	-0.031 [-0.298, 0.235]	0.813
ER	7.318 ± 0.266	7.523 ± 0.125	-0.205 [-0.746, 0.337]	0.610	7.188 ± 0.319	6.750 ± 0.353	0.438 [-0.121, 0.996]	0.106
PgR	6.409 ± 0.313	3.091 ± 0.538	3.318 [2.229, 4.408]	< 0.001*	5.344 ± 0.495	4.000 ± 0.486	1.344 [0.243, 2.445]	0.007*

For IHC scores of biomarkers in pre- or post-treatment specimen, data is shown as mean ± SEM; for the change of scores of biomarkers after treatment, data is shown as means with 95% CI (lower, upper values).
*A p value less than 0.05 is considered significant.
‡Wilcoxon matched-pairs signed-ranks test for the difference between groups.
ER: Estrogen receptor; HER2: Human EGFR2; PgR: Progesterone receptor.

blockade of VEGF induction [39]. It awaits further investigations to clarify the differences in angiogenesis in ER-positive breast cancer tissues between estrogen receptor blockade and estrogen depletion by aromatase inhibition. However, nothing has been reported on the changes of angiogenesis during the course of endocrine therapy with aromatase inhibitors in human breast cancer.

Among the cases with estrogen depletion, regardless of duration or possibly accumulating dosages of AI, some cases were shown to be associated with decreased tumor cell proliferation whilst the others were not. It therefore becomes important to evaluate the potential differences in neoangiogenesis between those associated with decreased tumor cell proliferation caused by estrogen withdrawal by AI and those not.

VASH-1, being only expressed in the proliferative endothelial cells, is considered a more appropriate biomarker for intratumoral neovascularization which reflects the degree of angiogenesis as compared with CD31 [25]. Tamaki *et al.* demonstrated the use of VPR as an indicator of neovascularization in human breast cancer [25]. Therefore, the changes in *in situ* neovascularization before and after AI treatment of tumor tissues were evaluated, in which estrogen depletion incurred by AI treatment using VASH-1 and CD31 immunohistochemistry in the patients with breast cancer.

In our present study, when using the same criteria used by Chanplakorn *et al.* [28], Miller *et al.* [29] and Yu *et al.* [30] with 40% decrement of Ki67 LI as the cut-off point for treatment response, statistically significant increment of VPR was detected only in the responders but not in non-responders. These changes of VPR in the responders were not correlated with the baseline VPR. Similar changes were also detected according to the VPR alterations. In addition, significant decrement of Ki67 LI was also detected in the group of the patients demonstrating increased VPR. No such changes were detected in those associated with decreased VPR. The results of this study demonstrated that angiogenesis or neovascularization, as defined by an increased VPR, was indeed correlated with the therapeutic effectiveness of AI treatment in ER-positive postmenopausal breast cancer patients. This finding appeared to be paradoxical to the conceptual or putative involvement of angiogenesis in tumorigenesis and progression of breast cancer [4-10,12,16,17,35]. The roles of tumor–stroma interactions in estrogen signaling in breast cancers were first reported by Yamaguchi *et al.* [40] and previous studies all demonstrated that estrogen deprivation resulted in changes of biological features of not only tumor cells but also of stromal cells, leading to apoptosis, necrosis, desmoplasia, hyalinosis and finally fibrosis in tumor tissues [41,42]. AI's antiproliferative effects upon ER-positive tumor cell proliferation as a result of estrogen depletion have been reported [42,43], possibly due to disruption of cell cycle progression resulting in growth arrest at the G1 phase of the cell cycle [44]. AI was also shown to induce apoptosis of carcinoma cells [29,33,42,43,45,46], despite the fact that controversies exist