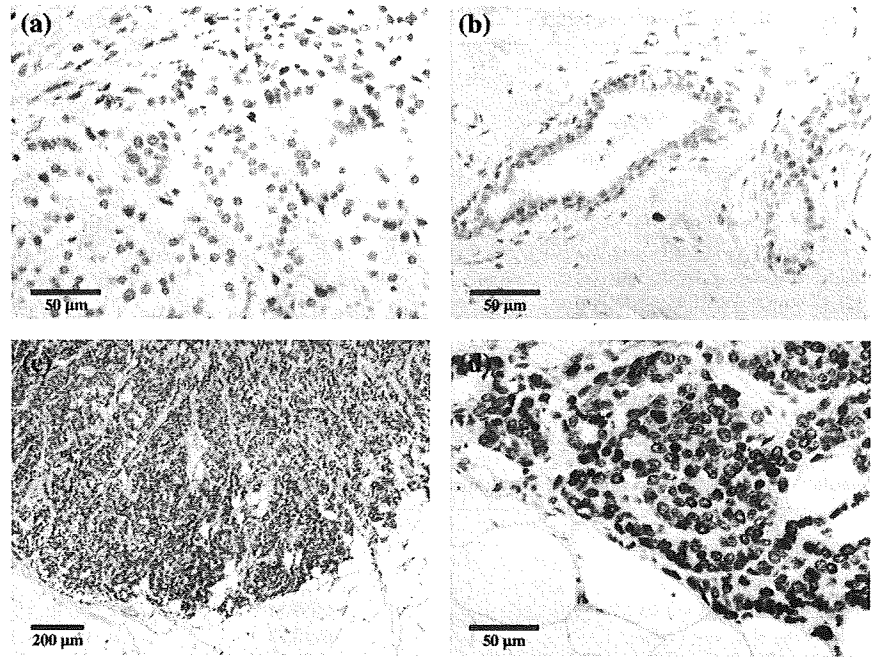


**Fig. 1.** Representative illustrations of chicken ovalbumin upstream promoter transcription factor (COUP-TF) II immunohistochemistry in invasive ductal carcinomas of the breast. (a) Adrenal gland was used as a positive control for immunohistochemistry. (b) Immunoreactivity for COUP-TFII was detected in a few normal epithelial cells but its relative immunointensity was low compared to that in carcinoma cells. (c,d) Marked COUP-TFII immunoreactivity was detected in carcinoma cells of invasive ductal carcinoma. Low (c) and High (d) magnification.

and adverse clinical outcomes (Supporting Information Fig. 1). However, the tendency of the two groups '–' and '+' were relatively similar. The data from these two groups were subsequently merged and the prognostic analysis above was carried out using dichotomous data on the basis of classification into two patient groups, '– or +' and '++'. The DFS and OS curves in these analyses are shown in Figure 2. The statistical analysis demonstrated that high COUP-TFII status (++) in breast carcinoma cases was significantly associated with poor survival or adverse clinical outcome (log-rank test: DFS, $P = 0.0224$; OS, $P = 0.0183$). The results of univariate analysis (Tables 1,2) demonstrated that lymph node status (DFS, $P < 0.0001$; OS, $P = 0.0001$), histological grade (DFS, $P = 0.0043$; OS, $P = 0.0019$), tumor size (DFS, $P = 0.0048$; OS, $P = 0.0143$), COUP-TFII status (DFS, $P = 0.0266$; OS, $P = 0.0234$), and HER2 status (DFS, $P = 0.0172$) were all significant prognostic factors for DFS and OS in the 119 patients examined. A subsequent multivariate analysis, however, revealed that lymph node status (DFS, $P < 0.0001$; OS, $P = 0.0044$), histological grade (OS, $P = 0.0161$), and HER2 status (DFS, $P = 0.0231$) were independent prognostic factors for DFS and OS in these patients, but COUP-TFII status was not (DFS, $P = 0.8974$; OS, $P = 0.4020$) (Tables 1,2).

**Correlation between COUP-TFII LI and clinicopathological variables in 119 breast carcinoma patients.** The associations between COUP-TFII LI and the clinicopathological variables in 119 breast carcinomas are summarized in Table 3. COUP-TFII LI was significantly associated with clinical stage (I vs IV, $P = 0.0222$), lymph node status ($P = 0.0369$), histological grade (1 vs 3, $P = 0.0222$), and ERα status ($P = 0.0050$). No significant correlations were detected between COUP-TFII LI and menopausal status ($P = 0.7616$), tumor size ($P = 0.582$), PR status ($P = 0.2964$), Ki-67 status ($P = 0.1157$), or HER2 status ($P = 0.8364$).

**Knockdown of COUP-TFII protein and downregulation of VEGF-C mRNA expression.** Decreased amounts of COUP-TFII protein were also detected by immunoblotting analysis 3 days following the transfection of specific siRNA (Fig. 3a). VEGF-C mRNA expression was decreased (Fig. 3b) in both COUP-TFII-knockdown cell lines compared to the cells transfected with non-specific siRNA.

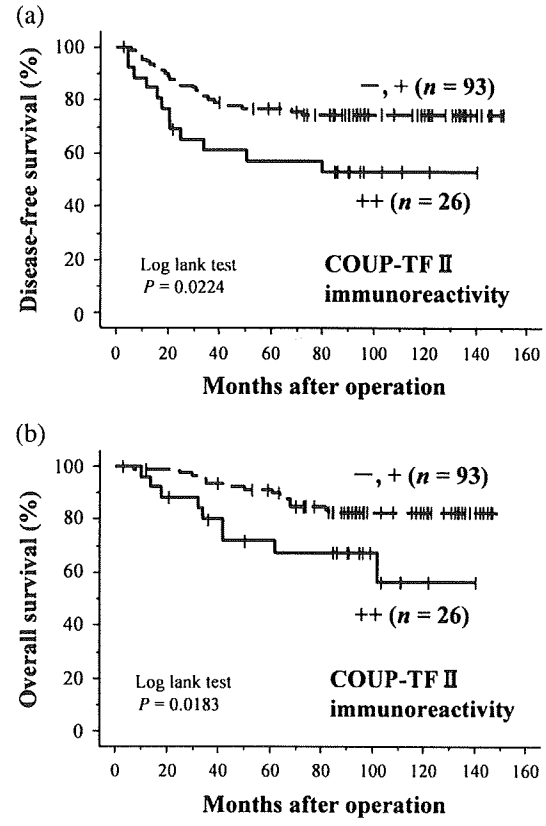


**Fig. 2.** (a) Disease-free and (b) overall survival of 119 patients with breast carcinoma according to the status of chicken ovalbumin upstream promoter transcription factor (COUP-TF) II immunoreactivity of carcinoma cells (Kaplan–Meier method). Cases were classified into two groups according to COUP-TFII labeling index (LI): ++, >50% LI; +, 10–49% LI; and –, 0–9% LI. COUP-TFII status was significantly associated with an increased risk of adverse clinical outcome.

**Table 1. Univariate and multivariate analysis of disease-free survival in 119 breast cancer patients examined**

| Variable | Univariate | | Multivariate | |
|---|---|---|---|---|
| | *P*-value | Relative risk (95% CI) | *P*-value | Relative risk (95% CI) |
| Lymph node status (positive/negative) | **<0.0001** | 9.561 (4.162–21.963) | **<0.0001** | 7.934 (3.272–19.241) |
| Histological grade (3/1,2) | **0.0043** | 2.640 (1.357–5.139) | 0.0708 | 1.919 (0.946–3.893) |
| Tumor size (≥2.5 cm/<2.5 cm) | **0.0048** | 3.117 (1.415–6.869) | 0.2603 | 1.621 (0.699–3.758) |
| COUP-TF status (++/–, +) | **0.0266** | 2.205 (1.096–4.436) | 0.8974 | 1.051(0.491–2.254) |
| Estrogen receptor α status (positive/negative) | 0.2571 | 0.673 (0.339–1.336) | | |
| Ki-67 status (positive/negative) | 0.7724 | 0.906 (0.464–1.770) | | |
| HER2 status (positive/negative) | **0.0172** | 2.306 (1.160–4.585) | **0.0231** | 2.278 (1.119–4.634) |

Data considered significant ($P < 0.05$) in the univariate analysis are shown in bold, and were examined in the multivariate analysis. CI, confidence interval; COUP-TF, chicken ovalbumin upstream promoter transcription factor; HER, human epidermal growth factor receptor.

**Table 2. Univariate and multivariate analysis of over-all survival in 119 breast cancer patients examined**

| Variable | Univariate | | Multivariate | |
|---|---|---|---|---|
| | *P*-value | Relative risk (95% CI) | *P*-value | Relative risk (95% CI) |
| Lymph node status (positive/negative) | **0.0001** | 6.204 (2.458–15.660) | **0.0044** | 4.338 (1.582–11.897) |
| Histological grade (3/1,2) | **0.0019** | 3.719 (1.623–8.521) | **0.0161** | 2.888 (1.217–6.850) |
| Tumor size (≥2.5 cm/<2.5 cm) | **0.0143** | 3.427 (1.278–9.187) | 0.2413 | 1.860 (0.659–5.254) |
| COUP-TF status (++/–, +) | **0.0234** | 2.605 (1.138–5.964) | 0.4020 | 1.467 (0.599–3.596) |
| Estrogen receptor α status (positive/negative) | 0.1679 | 0.565 (0.251–1.272) | | |
| Ki-67 status (positive/negative) | 0.2209 | 1.734 (0.718–4.184) | | |
| HER2 status (positive/negative) | 0.0644 | 2.184 (0.954–4.996) | | |

Data considered significant ($P < 0.05$) in the univariate analysis are shown in bold, and were examined in the multivariate analysis. CI, confidence interval; COUP-TF, chicken ovalbumin upstream promoter transcription factor; HER, human epidermal growth factor recep*or.

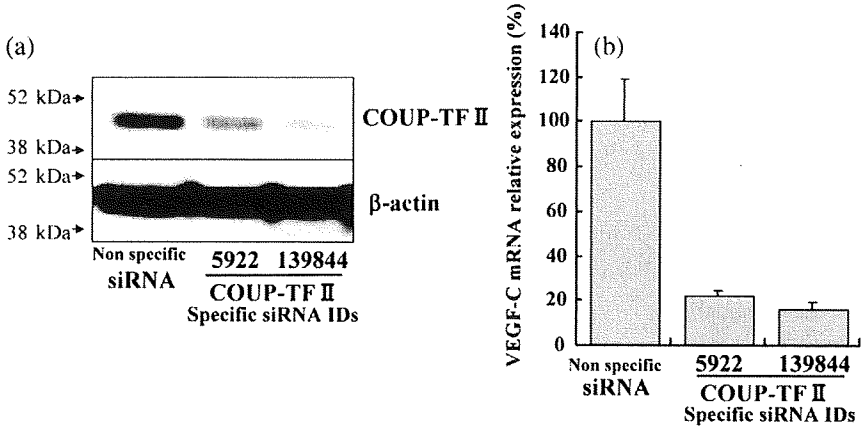


**Fig. 3.** Short interfering RNA (siRNA)-mediated knockdown of endogenous chicken ovalbumin upstream promoter transcription factor (COUP-TF) II protein in MCF-7 cells. Knockdown cells were subjected to immunoblotting and reverse transcription–polymerase chain reaction analysis 3 days after transfection of siRNA. (a) COUP-TFII immunoreactivity was detected in the protein extracts of cells transfected with non-specific siRNA (lane 1). COUP-TFII immunoreactivity was decreased in the cells treated with COUP-TFII-specific siRNA (lanes 2 and 3). Vascular endothelial growth factor (VEGF)-C mRNA expression was also repressed in the cells treated with COUP-TFII-specific siRNA compared to cells treated with non-specific siRNA in the reverse transcription–polymerase chain reaction analysis.

## Discussion

To the best of our knowledge, this is the first report to demonstrate immunolocalization of COUP-TFII in human breast carcinoma. Our results demonstrated that 59% of the cases examined had moderate (+, 37.0%) or high (++, 21.8%) positivity in the nuclei of carcinoma cells. The rate of COUP-TFII-positive cases was relatively high compared with the number of cases positive for ERα (69.7%) and PR (65.5%) in our present study. The prognostic analyses in our present study indicated that a relatively high abundance of COUP-TFII in breast carcinoma cells was significantly associated with poor or adverse clinical outcome. The results of multivariate analysis demonstrated that a relatively high abundance of COUP-TFII was not necessarily an independent prognostic factor, but in the multivariate analysis without including the 'lymph node' factor, COUP-TFII status reached statistical significance in terms of an independent prognostic factor (DFS, $P = 00359$; OS, $P = 0.0356$). Therefore, the COUP-TFII status of carcinoma cells is postulated, at least partly, to be involved in the process of lymph node metastasis.

Chicken ovalbumin upstream promoter transcription factor II LI and lymph node status were significantly correlated, as expected based on the prognostic analysis described above. Schafer *et al.* reported that *VEGF-D* gene expression is directly regulated by hepatocyte nuclear factor (HNF)-4α, COUP-TFI, and COUP-TFII through binding of these orphan nuclear receptors to the response element existing in the *VEGF-D* gene promoter.[17] VEGF-C and VEGF-D were originally discovered as members of the VEGF family, which is involved in lymphangiogenesis as well



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**Table 3. Association between chicken ovalbumin upstream promoter transcription factor (COUP-TF) II immunoreactivity and clinicopathological parameters in 119 breast carcinomas**

| Variable | Patient number | COUP-TFII labeling index (%) | *P*-value | *P*-value adjusted by Bonferroni |
|---|---|---|---|---|
| Menopausal status | | | | |
| pre- | 51 | 24.9 ± 3.4 | | |
| post- | 68 | 27.5 ± 3.3 | 0.7616 | |
| Stage | | | | |
| I | 32 | 21.9 ± 4.5 | | |
| II | 58 | 22.6 ± 3.0 | 0.9071 (I vs II) | |
| III | 13 | 26.0 ± 7.6 | 0.6247 (I vs III) | |
| IV | 16 | 44.8 ± 7.4 | 0.0037 (I vs IV) | 0.0222 |
| Tumor size (cm) | | | | |
| ≥2.5 | 65 | 26.6 ± 3.1 | | |
| <2.5 | 54 | 24.8 ± 3.6 | 0.582 | |
| Lymph node status | | | | |
| Positive | 46 | 34.1 ± 4.3 | | |
| Negative | 73 | 20.5 ± 2.6 | 0.0369 | |
| Histological grade | | | | |
| 1 (well) | 34 | 15.3 ± 3.3 | | |
| 2 (moderate) | 44 | 28.6 ± 4.0 | 0.0231 (1 vs 2) | 0.0693 |
| 3 (poor) | 41 | 31.3 ± 4.4 | 0.0074 (1 vs 3) | 0.0222 |
| Estrogen receptor α status | | | | |
| Positive | 83 | 29.8 ± 3.0 | | |
| Negative | 36 | 16.5 ± 3.6 | 0.0050 | |
| Progesterone receptor status | | | | |
| Positive | 78 | 26.7 ± 2.9 | | |
| Negative | 41 | 24.0 ± 4.2 | 0.2964 | |
| Ki-67 status | | | | |
| Positive | 72 | 28.8 ± 3.1 | | |
| Negative | 47 | 21.1 ± 3.6 | 0.1157 | |
| HER2 status | | | | |
| Positive | 27 | 28.2 ± 5.5 | | |
| Negative | 92 | 25.4 ± 2.6 | 0.8364 | |

Data are presented as means ± SEM. All other values represent the number of cases. *P*-values less than 0.05 were considered significant, and are shown in bold.

as angiogenesis[18] via binding to VEGFR-2 and VEGFR-3 expressed on the surface of vascular and lymphatic endothelial cells.[18] Skobe *et al.* reported that VEGF-C expression in breast tumor cells induces intratumoral lymphangiogenesis and enhances metastasis to the lymph nodes.[19] Sun *et al.* also reported that migration of human lymphatic endothelial cells cocultured with human breast carcinoma cell line MCF-7 transfected with siRNA against VEGF-C was significantly suppressed.[20] These findings above indicate the importance of evaluating VEGF-C and VEGF-D regulation by COUP-TFII in human breast carcinoma cells. In our present study, the expression of VEGF-C mRNA was markedly suppressed in the COUP-TFII-knockdown MCF-7 cells, as expected. The expression of VEGF-D in MCF-7 cell was extremely low, but decreased expression of VEGF-D was detected in these circumstances (data not shown). COUP-TFII LI was also significantly higher in the tissue of carcinoma cases diagnosed with stage IV compared with other clinical stages. These findings strongly suggest that COUP-TFII regulates the expression of VEGF-C or VEGF-D in breast carcinoma cells and contributes to lymph node or distant metastasis, which consequently results in poor patient prognosis.

Pereira *et al.* reported that targeted deletion of the *COUP-TFII* gene results in embryonic lethality associated with defects in angiogenesis and decreased expression of the angiogenesis factor ANG-1.[21] This result suggests that COUP-TFII regulates the organized process of angiogenesis during development and that COUP-TFII accelerates angiogenesis in human breast carcinoma by inducing ANG-1 expression. However, the results of the COUP-TFII knockdown assay in our study demonstrated that decreased ANG-1 mRNA expression was not necessarily detected in the RT-PCR analysis because of extremely low ANG-1 expression in MCF-7 cells. Therefore, the regulatory mechanism of ANG-1 by COUP-TFII awaits further investigation for clarification.

Pereira *et al.* also reported that COUP-TFII is markedly expressed in mesenchymal cells that undergo differentiation to epithelium, but not in terminally differentiated epithelium.[22] We have previously reported that COUP-TFII expression is not detected in the great majority of glandular epithelium of non-pathological human adult breast tissue.[23] These results are clearly consistent with our present results in which the expression of COUP-TFII tended to be more abundant in poorly differentiated high histological-grade carcinoma cases. COUP-TFII is, therefore, considered to be involved in dedifferentiation of cancer cells in advanced breast cancer.

Riggs *et al.* demonstrated that treating MCF-7 cells with estradiol increases COUP-TFII mRNA expression, and transient transfection of MCF-7 with siRNA against ERα decreases the expression of COUP-TFII mRNA.[24] These results are consistent with our finding in which the COUP-TFII LI was significantly higher in the ERα-positive group than in the ERα-negative group. Therefore, ERα is also considered to be one of the regulatory factors for COUP-TFII expression, which may explain why a correlation between COUP-TFII LI and ERα status was detected in our present study. In this study, the prognostic analysis indicated that COUP-TFII status was associated with a worse prognosis or clinical outcome for the patients, and a correlation between COUP-TFII LI and ERα status was detected. However,

it is also true that ERα is generally considered to be a modest but good prognostic factor[25] in breast carcinoma patients because it is a potential target of hormone therapy. In fact, the results of the univariate analysis in our present study demonstrated that ERα status in carcinoma cells tended to be a good prognostic factor (ERα-positive patients: DFS, relative risk 0.673; OS, relative risk 0.565) even though this association did not reach statistical significance (DFS, $P = 0.2571$; OS: $P = 0.1679$), possibly due to the relatively small number of cases examined. More *et al.* reported that COUP-TFII is present in several ERα-negative breast cancer cell lines, such as MDA-MB-231, HS-578, MT-SV1-7, and SK-BR-3.[26] In addition, they demonstrated that epidermal growth factor and transforming growth factor-α, both of which are well-known growth factors for breast carcinoma cells,[27] induce COUP-TFII mRNA expression in MCF-7 cells.[26] Therefore, the expression of COUP-TFII may be considered to be regulated not only by ERα but also other factors in breast carcinoma cells. COUP-TFII induced by factors other than ERα is reasonably postulated not to be affected by hormonal therapy and to induce aggressive events such as lymphangiogenesis, which bestows a poor prognosis regardless of the patients' ERα status. Indeed, the expression of COUP-TFII was associated with poor prognosis and adverse clinical outcome in each group of ERα-positive ($n = 83$) and ERα-negative ($n = 36$) patients in the same fashion in our study (Supporting information Fig. 2).

Riggs *et al.* reported that COUP-TFII expression is required for growth inhibition induced by tamoxifen treatment,[24] even though the status of COUP-TFII in breast carcinoma tissue was not associated with the therapeutic effects of tamoxifene in our study (data not shown). In addition, Nakshatri *et al.* demonstrated that COUP-TFII induces both p21 expression and growth inhibition in breast cancer cells.[28] Therefore, together with our data, the expression of COUP-TFII could be detrimental to cell growth *in vitro*, whereas its expression may affect the microenvironment of tumor tissue in which lymphangiogenesis or angiogenesis are induced. This effect is considered to overcome the detrimental effects described above and contributes to poor prognosis.

In summary, we demonstrated the immunolocalization of COUP-TFII in human breast carcinoma. COUP-TFII immunoreactivity was correlated with several adverse clinicopathological parameters associated with aggressive biological behavior and a worse prognosis for patients. Therefore, COUP-TFII is considered to be involved in the development of advanced human breast carcinoma, possibly via lymphangiogenesis and angiogenesis.

## Acknowledgments

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. 1.** (a) Disease-free and (b) overall survival of 119 patients with breast carcinoma according to the status of chicken ovalbumin upstream promoter transcription factor (COUP-TF) II immunoreactivity of carcinoma cells (Kaplan–Meier method). Cases were classified into three different groups according to COUP-TFII labeling index (LI): ++, >50% LI; +, 10–49% LI; and –, 0–9% LI.

**Fig. 2.** Overall survival of patients with estrogen receptor (ER) α (a) negative and (b) positive breast carcinoma according to the status of chicken ovalbumin upstream promoter transcription factor (COUP-TF) II immunoreactivity (Kaplan–Meier method). Cases were classified into two groups according to COUP-TFII LI: ++, >50% LI; +, 10–49% LI; and –, 0–9% LI.

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# Vasohibin-1 in human breast carcinoma: A potential negative feedback regulator of angiogenesis

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**Vasohibin-1 is a recently identified negative feedback inhibitor or suppressor of angiogenesis induced by vascular endothelial growth factor (VEGF)-A. The status of vasohibin-1 in human breast carcinoma has not been examined. We examined 151 breast specimens including 98 cases of invasive ductal carcinoma (IDC), 12 of ductal carcinoma *in situ* (DCIS), 16 of fibroadenoma (FA), six of inflammatory lesion, nine of fibrocystic change and seven of non-pathological breast tissue. We immunolocalized vasohibin-1 and compared its immunoreactivity to that of VEGF-A, basic fibroblastic growth factor (bFGF), VEGF receptor 2 (Flk-1), CD31, CD34 and Ki-67/MIB-1. The correlation of vasohibin-1 immunoreactivity with overall survival (OS), and disease-free survival (DFS) of the patients with breast carcinoma was also evaluated. In addition, we evaluated Ki-67 and CD31, and Ki-67 and vasohibin-1 double-immunostaining for further characterization of neovascularization. Vasohibin-1 was detected in endothelial cells of human breast and its immunodensity was significantly higher in IDC and inflammatory lesions than the other types ($P < 0.001$). In addition, a significant positive correlation was detected between vasohibin-1 and VEGF-A, bFGF or Flk-1 ($P < 0.001$). There was also positive associations between vasohibin-1 and OS ($P = 0.004$) and between vasohibin-1 and DFS ($P \leq 0.001$) in carcinoma cases. Results of double-immunostaining demonstrated the ratio of Ki-67-positive cells among vasohibin-1-positive endothelial cells (46.5%) was significantly higher than those among CD31-positive cells (23.5%). This is the first study demonstrating the status of vasohibin-1 in human breast lesions, which indicates that vasohibin-1 is associated with neovascularization and may especially play important roles in the regulation of intratumoral angiogenesis in human breast cancer. (*Cancer Sci* 2009; 100: 88–94)**

Angiogenesis or the formation of new blood vessel networks, not only plays a pivotal role in human normal development, but also in pathophysiological conditions such as inflammatory diseases and neoplasms. Angiogenesis is generally regulated by an *in situ* balance between stimulatory and inhibitory factors of angiogenesis.[1,2] However, this "angiogenic homeostasis" may be disrupted in pathological conditions such as cancer and dysregulated or excessive production and/or secretion of angiogenesis inducers result in excessive formation of abnormal blood vessels. In general, various biological phenomena in physiological conditions are under stringent control by numerous negative feedback systems as seen in endocrine mechanisms the including hypothalamic–pituitary–adrenal system to maintain their homeostasis. However, little has been known about such negative feedback mechanisms of angiogenesis in both physiological and pathological conditions.

Vasohibin-1 has been very recently identified as one of the first established negative feedback regulators of angiogenesis.[2–5] This interesting factor was identified as one of vascular endothelial growth factor (VEGF)-induced genes with anti-angiogenic properties in endothelial cells (EC) using cDNA microarray analysis.[3,4,6] Vasohibin-1 was subsequently demonstrated to be specifically expressed in EC in response to angiogenic stimulators such as VEGF and basic fibroblastic growth factor (bFGF).[3,6] Vasohibin-1 is also abundantly present in human placenta and fetus[2,3,5] in which angiogenic events markedly occur *in vivo*. VEGF-A is the most potent factor for angiogenesis among known VEGF family members, stimulating protease synthesis, migration and proliferation of EC.[7] In addition, the great majority of VEGF-A-mediated signals are transduced via VEGF receptor 2 (Flk-1)[8] and protein kinase Cδ (PKCδ), one of the signals located in important downstream intrasignaling pathway of Flk-1, and they also induced vasohibin-1 expression markedly.[4] Yoshinaga *et al.* demonstrated that the VEGF-A-mediated induction of vasohibin-1 was preferentially mediated via the Flk-1 signaling pathway in human endometrial carcinoma.[9] However, the status of vasohibin-1 in other human malignancies has not been examined in detail.

Therefore, in this study, we first immunolocalized vasohibin-1 in human breast disorders including breast cancer in order to examine whether this factor is expressed in endothelial cells or not in human breast tissues. We then correlated the findings with various clinicopathological factors of the cases including microvessel density (MVD)[10,11] in order to correlate the status of vasohibin-1 with vascularity of the lesions. We also correlated vasohibin-1 immunoreactivity with neovascularization or proliferating endothelial cells using double immunostaining of Ki-67 in order to further characterize vasohibin-1 expression and its clinical and/or biological significance in human breast disorders.

## Materials and Methods

**Breast tissue specimens.** We retrieved 151 Japanese female cases of breast tissues from surgical pathology files of Tohoku University Hospital (Sendai, Japan). These subjects were operated on between 1995 and 1998 at the Department of Surgery, Tohoku University Hospital. The median age of the patients was 48 years (range, 15–81). The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan). The relevant clinicopathological information including age, histological type, stage classification, histological grade for invasive ductal carcinoma (IDC), grading scheme for ductal carcinoma *in situ* (DCIS) (van Nuys classifications[12] for DCIS and T1mic) are summarized in Table 1. Histological findings were 98 cases of IDC including eight cases of T1mic, 12 of DCIS, 16 fibroadenoma (FA), six of

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**Table 1. Clinicopathological characters of examined cases**

| Histological type | |
|---|---|
| IDC | 98 |
| (T1mic) | 8 |
| DCIS | 12 |
| FA | 16 |
| Inflammatory lesion | 6 |
| Fibrocystic change | 9 |
| Non-pathological breast tissue | 7 |
| Age, years (range) | |
| All cases | 48 years (15–81) |
| IDC | 53 years (28–81) |
| DCIS | 47 years (40–81) |
| FA | 39 years (15–52) |
| Inflammatory lesion | 49 years (35–70) |
| Fibrocystic change | 47 years (39–48) |
| Non-pathological breast tissue | 40 years (34–48) |
| UICC stage grouping | |
| Stage 0 | 12 |
| Stage I | 38 |
| Stage II | 36 |
| II A | 23 |
| II B | 13 |
| Stage III | 18 |
| III A | 8 |
| III B | 6 |
| III C | 4 |
| Stage IV | 6 |
| Histological grade (for IDC) | |
| G 1 | 34 |
| G 2 | 42 |
| G 3 | 14 |
| Van Nuys scheme (for DCIS and T1mic) | |
| Group 1 | 8 |
| Group 2 | 12 |

DCIS, ductal carcinoma *in situ*; FA, fibroadenoma; IDC, invasive ductal carcinoma; UICC, International Union Against Cancer staging.

inflammatory lesion, nine of fibrocystic change and seven of non-pathological breast tissue taken from the lumpectomy specimen for breast cancer operation. In the nine cases of fibrocystic change, we evaluated vessels in the areas adjacent to adenosis or ductal hyperplasia. Stage grouping was based on *TNM Classification of Malignant Tumors Sixth Edition* by the International Union Against Cancer (UICC).[13] The tumor grade was determined according to the criteria of Elston and Ellis.[14]

**Immunohistochemistry.** We performed immunohistochemical staining for vasohibin-1, Flk-1, CD31, Ki-67, VEGF-A and FGF-2. The specimens had been fixed in 10% formalin, embedded in paraffin, cut into 4-μm thick sections and placed on glue-coated glass slides. Sections were deparaffinized in xylene, and hydrated with graded alcohols and distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 10 min at room temperature. Antigen retrieval was performed using Autoclave (TOMY SX-500 HIGH PRESSURE STEAM STERILIZER, TOMY SEIKO CO., LTD, Tokyo, Japan) in 10 nmol ethylene diamine tetra acetate (EDTA; pH 8) for vasohibin-1 and in citrate buffer for Flk-1, CD31, Ki-67 and FGF-2, heated at 121°C for 5 min, and for VEGF-A using microwave in citrate buffer for 15 min. Sections were subsequently incubated for 30 min at room temperature (RT) in a blocking solution of 10% rabbit serum (Nichirei Biosciences, Tokyo, Japan) for vasohibin-1, Flk-1, CD31, CD34 and Ki-67, and a blocking solution of 10% goat serum (Nichirei Bioscience) for VEGF-A and FGF-2, and then immunostained for 16 h at 4°C with primary antibodies. The primary antibodies of vasohibin-1, Flk-1, CD31, Ki-67, VEGF-1 were mouse monoclonal antibodies, whereas the primary antibody against FGF-2 was a rabbit polyclonal antibody, and were used as follows: antihuman vasohibin-1 monoclonal antibody[9,15] diluted at 1:3200; anti-VEGFR-2 (Flk-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:3200; anti-CD31 (Dako, Copenhagen, Denmark) diluted at 1:40; anti-CD34 (Nichirei Bioscience) diluted at 1:100, Ki-67 (Dako) diluted 1:300; anti-VEGF-A (Laboratory Vision, Fremont, CA, USA) diluted at 1:50; and anti-FGF-2 (Santa Cruz Biotechnology) diluted at 1:100. Antihuman vasohibin-1 monoclonal antibody (mAb) was raised against the synthetic fragment (Gly286-Arg299) of human vasohibin-1 as described by Watanabe *et al.*[3] The specificity and sensitivity of this mAb was confirmed by both western blotting and immunohistochemical analysis.[3] For vasohibin-1, Flk-1, CD31, CD34 and Ki-67 immunohistochemistry, secondary antibody reactions were performed using biotinylated rabbit antimouse antibody (Nichirei Bioscience) at a dilution of 1:100 for 30 min at RT and peroxidase-conjugated avidin (Nichirei Bioscience) was used according to the manufacturer's instructions. Envision (Dako) was used for immunostaining of VEGF-A and FGF-2. Reacted sections were visualized using 3,3′-diaminobenzidine-tetrachloride (DAB)/30% $H_2O_2$ in 0.05 mol/L Tris buffer (pH 7.6) and counterstained with hematoxylin–eosin (HE) for nuclear staining.

**Double staining procedure.** For the quantification of proliferating endothelial cells, Ki-67/CD31 and Ki-67/vasohibin-1 double-labeling immunohistochemical staining was performed. A mAb directed against Ki-67 (Dako) was diluted at 1:300 following antigen retrieval using Autoclave in a citrate buffer, and incubated for 30 min at RT in a blocking solution of 10% rabbit serum (Nichirei Bioscience). A secondary antibody reaction was performed using biotinylated rabbit antimouse antibody (Nichirei Bioscience) at a distribution of 1:100 for 30 min at RT. Peroxidase-conjugated avidin (Nichirei Bioscience) was subsequently used in this study. DAB was used to visualize the binding of the first antibody. Antigen retrieval was then performed using a microwave for 15 min in 10 nmol EDTA (pH 8) for vasohibin-1 and in a citrate buffer for CD31. The reacted sections were then incubated for 30 min with antibodies against vasohibin-1 diluted at 1:3200 and CD31 (Dako) diluted at 1:40. Following the reaction with biotinylated rabbit antimouse antibody (Nichirei Bioscience) diluted at 1:100 as a secondary antibody and alkaline phosphatase-conjugated avidin (Nichirei Bioscience), an alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA, USA) was employed.[16,17]

**Immunohistochemical analysis.** Two of the authors (K. T. and Y. M.) independently evaluated the immunohistochemical staining of the tissue sections. They were blinded to the clinical course of the patients and the average of numbers counted by the two investigators was used for subsequent analysis. We used Olympus (Tokyo, Japan) BX50 and 20X objectives for the analysis.

The number of microvessels was counted within the tumor of IDC and FA, whereas in DCIS, the number of vessels in the stroma among intraductal components was evaluated. In inflammatory lesions, fibrocystic change and non-pathological breast tissues, the greatest number of vessels in the tissue sections was determined as MVD.[10,11,18–20] Microvessels were identified based on the architecture, lumen lined by endothelial cells, complemented by positivity of the endothelial cells with anti-CD31 after scanning the immunostained section at low magnification (×40 and ×100).[10,11] The areas with the greatest number of distinctly highlighted microvessels were selected, and counted at one higher power (×200).[10,11] Any immunostained endothelial cells or clusters separated from adjacent vessels were counted as a single microvessel, even in the absence of vessel lumen. Each single count was defined as the highest number of microvessels identified at the "hot spot". Vasohibin-1- and Flk-1-positive signals

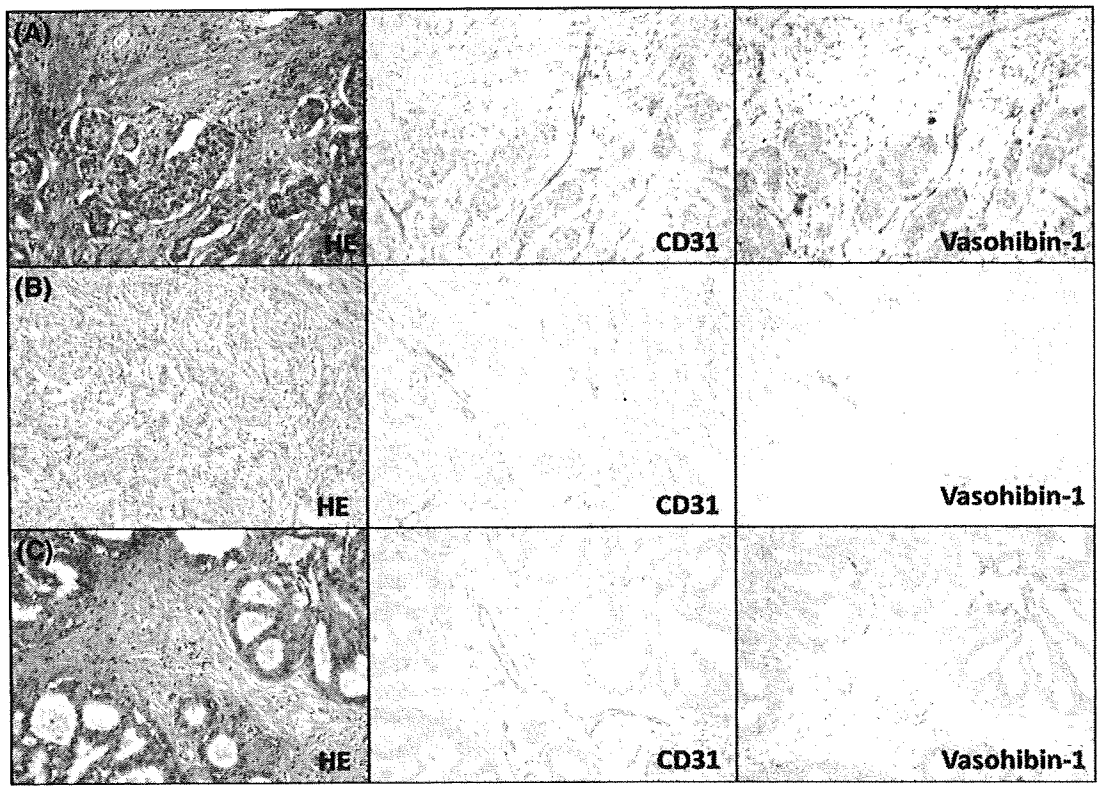


**Fig. 1.** Representative illustrations of histological and immunohistochemical findings of breast carcinoma cases examined. (A,B) Two invasive ductal carcinoma (IDC) cases stained positively for CD31 and vasohibin-1, whereas (C) a ductal carcinoma *in situ* (DCIS) case stained positive only for CD31 and not for vasohibin-1. (Original magnification, ×200.)

were counted in the hot spot in which the highest number of anti-CD31-positive vessels was identified. We also counted the average of vasohibin-1-positive vessels in 10 representative fields per case (×200). We defined vasohibin-1-positive ratio as the number divided by the number of vasohibin-1-positive vessels by that of CD31-positive vessels in the hot spot. An evaluation of Ki-67 immunoreactivity was performed at high power field (×400) and used as a marker of cell proliferation. More than 500 tumor cells from each of three different representative fields were evaluated and the labeling index was subsequently obtained. VEGF-A immunoreactivity was evaluated using grading, interpreting both relative immunointensity and the proportion of tumor cells associated with an unequivocal positive reaction.[21,22] Relative immunointensity was graded 0 (no staining) to 3 (strong staining), percentage of cells staining positive as 0 (no tumor cells positive), 1 (positive staining in <10% of the tumor cells), 2 (positive staining in 10–50% of the tumor cells) and 3 (positive staining in >50% of the tumor cells).[20,21] A semiquantitative method was used to evaluate the degrees of FGF-2 immunostaining ranging from 0 (no expression), 1 (weak), 2 (moderate) to 3 (highest level of expression).[23] The proportion of proliferating endothelial cells (CD31 and vasohibin-1-positive vessels) was defined as the number of endothelial cells with Ki-67-stained nuclei divided by the total number of endothelial cells.

Analyzes of OS and disease-free survival (DFS) curves were performed by employing the Kaplan–Meyer method. The segregation point of the parameter at 21 for vasohibin-1-positive vessels was determined by the Cox proportional hazards regression model. The values of survival rates represented estimated survival rates. Factors independently associated with OS and DFS – vasohibin-1, MVD, VEGF-A and Ki-67 – were identified by multivariate analyses using multiple regression analysis.

Statistical analysis, such as the one-factor ANOVA and simple regression analysis, were performed using StatMate III for Windows ver. 3.18 (ATMS, Tokyo, Japan). The results were considered significant at $P < 0.05$.

## Results

**MVD.** The representative findings of immunostaining for HE, CD31 and vasohibin-1 are illustrated in Fig. 1. The average number of microvessels detected by CD31 was 24.6 ± 8.3 in IDC, 21.7 ± 11.7 in DCIS, 26.3 ± 15.7 in FA, 34.2 ± 15.4 in inflammatory lesions, 20.6 ± 14.4 in fibrocystic change and 13.6 ± 10.3 in non-pathological breast tissue, respectively. Statistically significant differences of MVD among the lesions were detected only between IDC and non-pathological breast tissue ($P = 0.001$).

**Vasohibin-1 immunohistochemistry.** Vasohibin-1 immunoreactivity was detected only in endothelial cells (Fig. 1). Vasohibin-1-positive microvessels in the hot spot were 20.9 ± 7.7 in IDC, 5.3 ± 5.5 in DCIS, 4.6 ± 4.1 in FA, 23.7 ± 9.7 in inflammatory lesions, 4.6 ± 6.3 in fibrocystic change and 1.3 ± 1.8 in non-pathological breast tissue. There were statistically significant differences between IDC and four other histological types of breast tissues examined (DCIS, FA, fibrocystic change and non-pathological breast tissue; $P < 0.001$) (Fig. 2A). The ratio of vasohibin-1/CD31[4] was 0.857 ± 0.193 in IDC, 0.279 ± 0.308 in DCIS, 0.183 ± 0.146 in FA, 0.713 ± 0.200 in inflammatory lesions, 0.237 ± 0.332 in fibrocystic change and 0.112 ± 0.136 in non-pathological breast tissue. There were significant differences between IDC and all other histological types ($P < 0.001$) (Fig. 2B). The average number of vasohibin-1-positive vessels per 10 fields (×200) were 15.3 ± 6.1 in IDC, 4.4 ± 4.1 in



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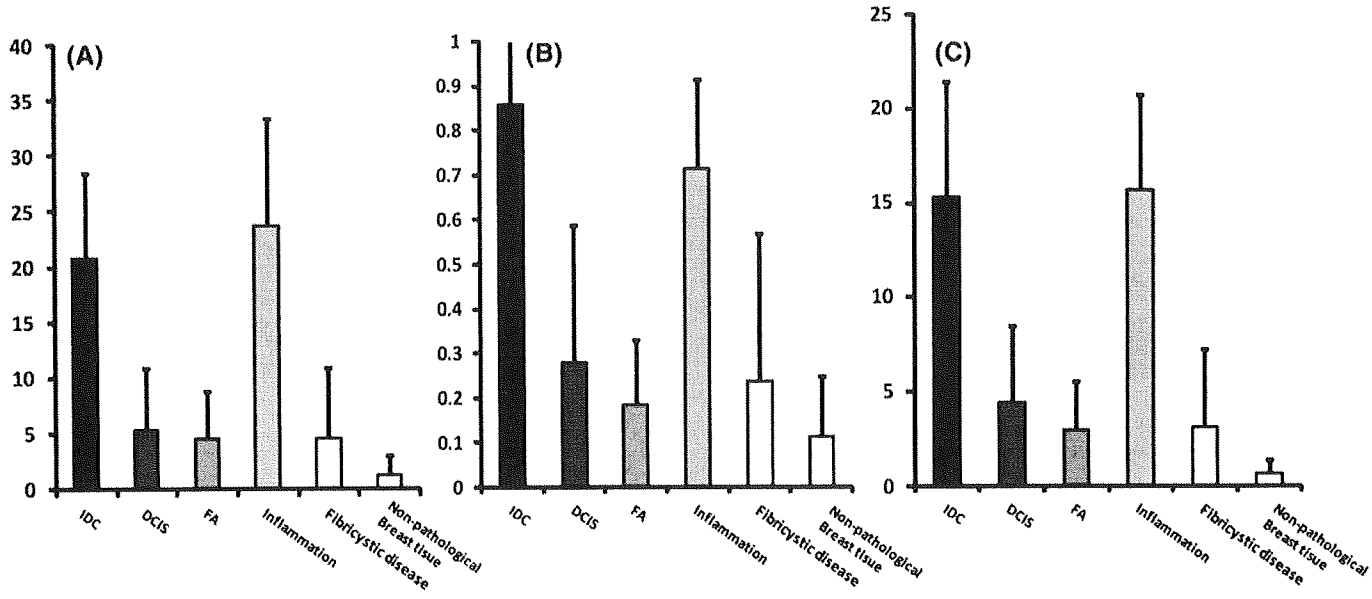



| IDC | 20.9 ± 7.7 |
|---|---|
| DCIS | 5.3 ± 5.5 |
| FA | 4.6 ± 4.1 |
| Inflammation | 23.7 ± 9.7 |
| Fibrocystic disease | 4.6 ± 4.1 |
| Non-pathological breast tissue | 1.3 ± 1.8 |

| IDC | 0.857 ± 0.193 |
|---|---|
| DCIS | 0.279 ± 0.308 |
| FA | 0.183 ± 0.146 |
| Inflammation | 0.713 ± 0.200 |
| Fibrocystic disease | 0.237 ± 0.332 |
| Non-pathological breast tissue | 0.112 ± 0.136 |

| IDC | 15.3 ± 6.1 |
|---|---|
| DCIS | 4.4 ± 4.1 |
| FA | 2.9 ± 2.6 |
| Inflammation | 15.7 ± 5.0 |
| Fibrocystic disease | 3.1 ± 4.1 |
| Non-pathological breast tissue | 0.7 ± 0.7 |

**Fig. 2.** Analysis of vasohibin-1 immunohistochemistry according to histological subtypes. (A) Number of vasohibin-1-positive vessels in the 'hotspot'. (B) Vasohibin-1-positive ratio defined as the vasohibin-1-positive vessels/CD31-positive vessels. (C) Average of vasohibin-1-positive vessels in 10 different fields. The lower boxes are the statistical analysis compared with invasive ductal carcinoma (IDC) cases.

DCIS, 2.9 ± 2.6 in FA, 15.7 ± 5.0 in inflammatory lesions, 3.1 ± 4.1 in fibrocystic change and 0.7 ± 0.7 in non-pathological breast tissue. There were also statistically significant differences between IDC and four histological types (DCIS, FA, fibrocystic change and non-pathological breast tissue, $P < 0.001$). No significant differences were detected between IDC and inflammatory lesions ($P = 0.781$) (Fig. 2C).

**Correlation between vasohibin-1-positive vessels and Ki-67 labeling index in carcinoma cells.** A significant positive correlation was detected between the number of vasohibin-1-positive vessels and Ki-67 labeling index in breast tumor cells ($P < 0.001$).

**Correlation between vasohibin-1-positive vessels and VEGF-A status in carcinoma cells.** The number of vasohibin-1-positive vessels was 5.8 ± 5.5 in VEGF-A of score 0, 11.0 ± 9.4 of score 2, 15.1 ± 10.0 of score 3, 17.5 ± 10.1 of score 4, 22.1 ± 8.9 of score 5 and 22.7 ± 5.7 of score 6. There was a statistically significant association between vasohibin-1 in the vessels and VEGF-A scores in carcinoma cells ($P < 0.001$) (Fig. 3A).

**Correlation between vasohibin-1-positive vessels and FGF-2 in carcinoma cells.** The number of vasohibin-1-positive vessels was 6.3 ± 6.1 in FGF-2 of score 0, 19.1 ± 6.5 of score 1, 21.9 ± 7.2 of score 2 and 26.8 ± 8.4 of score 3. A statistically significant association was detected between vasohibin-1 immunoreactivity in the vessels and FGF-2 scores in carcinoma cells ($P < 0.001$) (Fig. 3B).

**Correlation between vasohibin-1 and Flk-1 in microvessels in breast carcinoma.** A significantly positive correlation was detected between vasohibin-1 and Flk-1 positive ratios in microvessels ($P < 0.001$) (Fig. 3C).

**Correlation between vasohibin-1 and clinical stage of breast carcinoma cases.** The number of vasohibin-1-positive vessels was 5.3 ± 5.5 in TNM Stage 0, 19.6 ± 6.7 in Stage I, 18.7 ± 8.6 in Stage II A, 22.1 ± 8.3 in Stage II B, 23.8 ± 5.8 in Stage III A, 28.7 ± 7.5 in Stage III B, 23.0 ± 7.5 in Stage III C and 21.2 ± 5.6 in Stage IV. Statistically significant differences were detected only between IDC and DCIS ($P < 0.001$) with no significant differences among the different stages of IDC.

**Correlation between vasohibin-1 and histological grades of breast carcinoma cells.** The number of vasohibin-1-positive vessels among different groups of carcinoma cases and histological grade was 18.4 ± 7.5 in grade I, 20.8 ± 7.0 in grade II and 28.0 ± 8.0 in grade III. There were statistically significant differences of vasohibin-1 density between grade I and III, and grade II and III cases ($P < 0.001$) with no significant difference between grade I and II cases ($P = 0.14684$).

**Correlation between vasohibin-1 and overall survival or DFS in breast carcinoma patients.** Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more. The 10-year overall survival rates were 0.932203 and 0.72549 among these two groups, respectively. (The total 10-year overall survival rate in this cohort of patients was 0.838836.) Statistically significant differences in the 0–20 and 21 or more groups was $P = 0.004$ (Fig. 4A). The 10-year DFS were 0.92736 and 0.708333, respectively, in these two groups. Statistically significant differences were also detected in the 0–20 and 21 or more groups was at $P \leq 0.001$. (The total 10-year DFS rate was 0.81777; Fig. 4B.) The following variables were included in the multivariate analysis of OS: vasohibin-1, MVD, VEGF-A and Ki-67. This multivariate analysis demonstrated that vasohibin-1 was associated with VEGF-A ($P = 0.038$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.083$). The multivariate analysis of

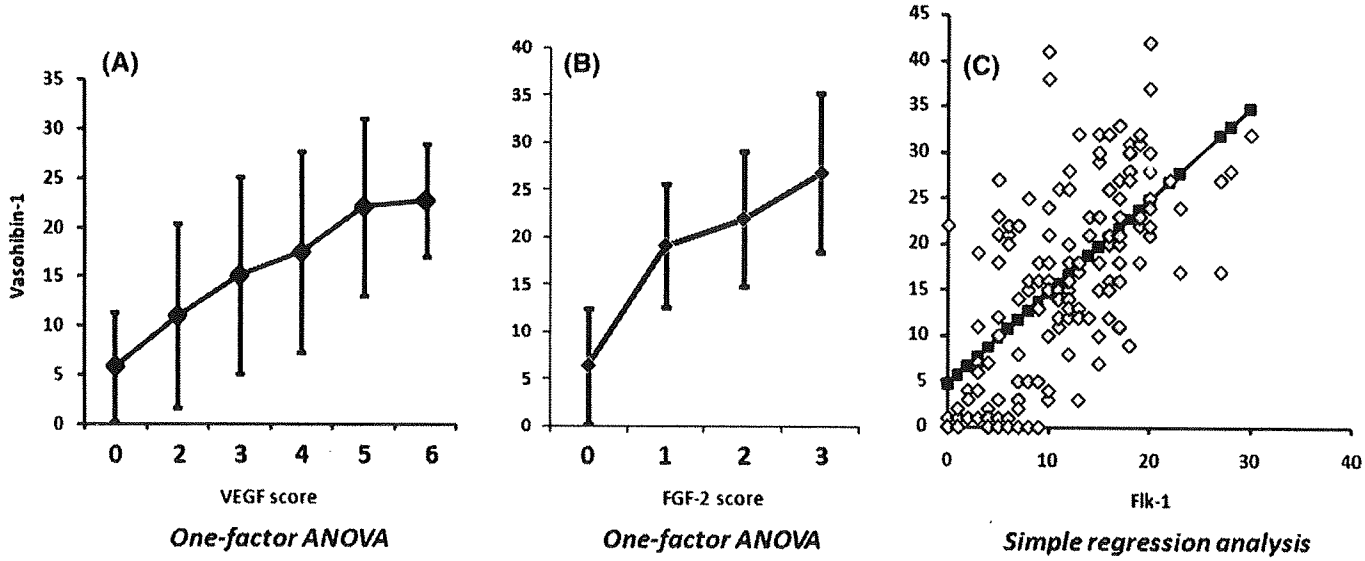
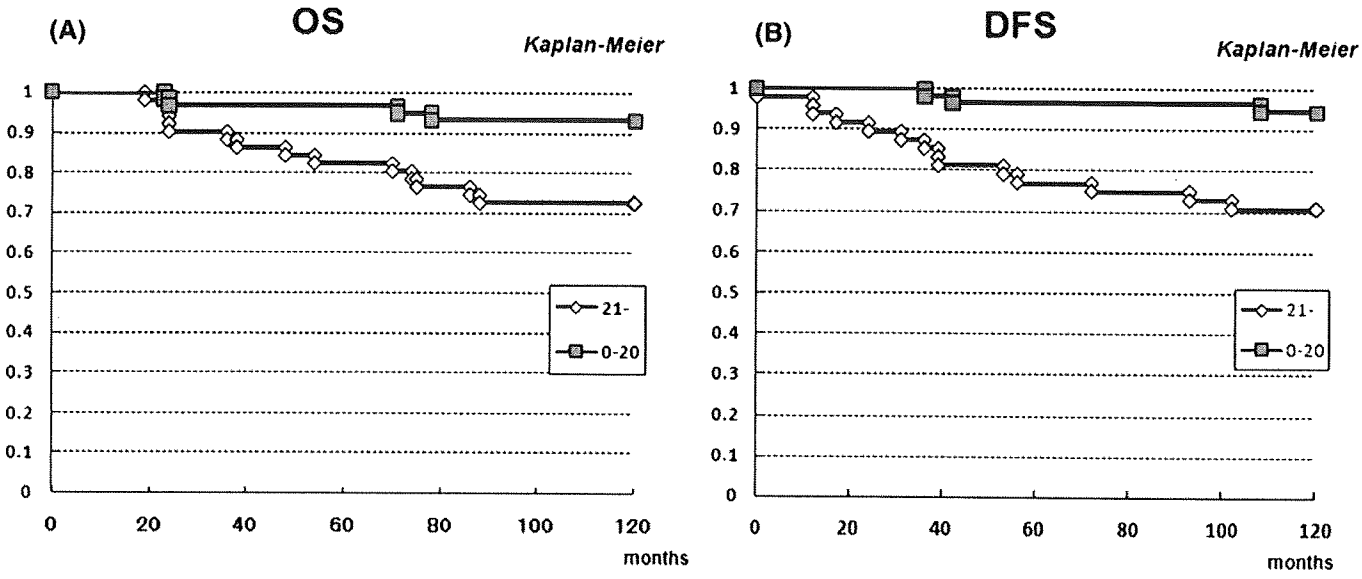



Fig. 3. (A) Result of the correlation between vasohibin-1-positive vessels and vascular endothelial growth factor (VEGF)-A expression in the tumor cells. (B) Result of the correlation between vasohibin-1-positive vessels and fibroblastic growth factor (FGF)-2 expression in the tumor cells. (C) Correlation between vasohibin-1 and Flk-1 in the 'hot spot'.




| Vasohibin-1 positive vessels | 10 year OS |
|---|---|
| 0-20 | 0.932203 |
| 21- | 0.72549 |

| Vasohibin-1 positive vessels | 10 year DFS |
|---|---|
| 0-20 | 0.927356 |
| 21- | 0.708333 |

Fig. 4. Summary of analysis of (A) overall survival and (B) disease free survival in relation to the status of vasohibin-1 expression. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more.

DFS also revealed that vasohibin-1 was associated with VEGF-A ($P = 0.004$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.081$).

**Double immunostaining with Ki-67 in microvessels.** Ki-67/vasohibin-1 double immunostaining analysis demonstrated that Ki-67 labeling index of vasohibin-1-positive vessels was 46.5% (33.3–62.5%), whereas that of CD31-positive vessels was 23.5% (12.7–37.5%) (Fig. 5A,B).

## Discussion

One of the most important functions of vasculature in general is to supply nutrients the distal organs. Three major types of regulation occur in the maintenance of vasculature: (i) vasodilation; (ii) changes in capillary permeability; and (iii) growth and development of new vessels, also known as angiogenesis.[24–26] Angiogenesis is a pivotal event in various biological processes



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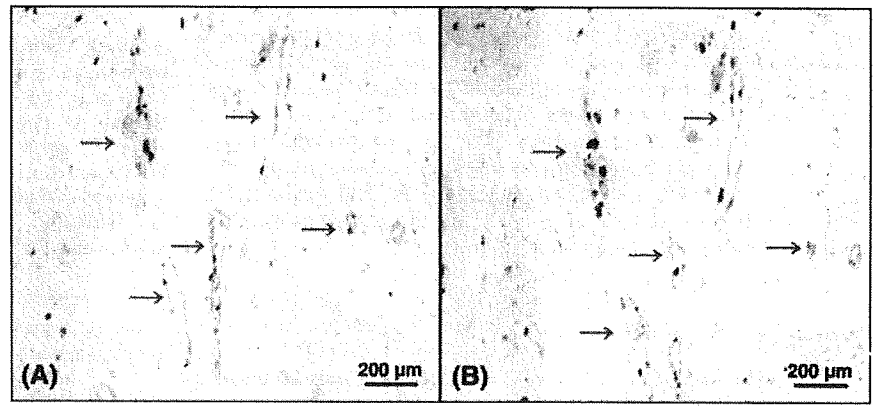


**Fig. 5.** Representative illustrations of double immunostaining for determining proliferating endothelial cells. (A) CD31/Ki-67 double staining; (B) vasohibin-1/Ki-67 double staining (arrow). (A) CD31 and (B) vasohibin-1 were colored blue, and Ki-67 was colored brown.

under both physiological and pathological conditions. Physiological conditions include embryonic development, reproduction and wound healing, and pathological conditions include cancers and inflammatory conditions.[2] *In situ* balance between angiogenesis stimulators such as VEGF and bFGF and inhibitors such as thrombospondin-1 (TSP-1) and pigment epithelium derived factor (PEDF) is generally considered to regulate the process of angiogenesis.[1] Negative feedback regulation is considered one of the most important physiological mechanisms with which bodies are endowed, and has been demonstrated to be involved in a wide range of biological phenomena.[27] This regulation is most effectively performed through the factors produced in endothelial cells but the endothelium-derived negative feedback regulators of angiogenesis have not been elucidated. Vasohibin-1 is therefore the first secretory anti-angiogenic factor from endothelial cells themselves induced by VEGF in EC.[2–4,28] The other anti-angiogenic regulator has been very recently identified and termed vasohibin-2 but this factor lacks the property of VEGF-A or bFGF inducibility in contrast to vasohibin-1.[28] Vasohibin-1 immunoreactivity was exclusively detected in endothelial cells in the present study, which is also consistent with results of previous studies of endometrial carcinoma[9] in lung carcinoma[3] and ischemic retina.[29] This is the first study to examine the status of vasohibin-1 in human breast disease in which angiogenesis also plays important roles in both physiological and pathological conditions.

Breast cancer has also been considered an angiogenic-dependent disease as in other human malignancies and angiogenesis has been demonstrated to play an essential role in breast cancer development, invasion and metastasis.[30–32] MVD assessed by CD31, CD34 and Factor VIII is generally considered as a gold-standard surrogate marker of tumor angiogenesis and has been also proposed by some investigators to identify patients at high risk of recurrence more precisely than classical indicators.[10,11]

In this study, we first examined how the vasohibin-1 expression was correlated to the MVD status. Vasohibin-1 immunodensity tended to be concordant with MVD in human breast tissues but they were not always parallel. The vasohibin-1 immunodensity was significantly higher in IDC than in DCIS but there was no difference of MVD between these two lesions. In addition, results of double immunostaining analysis which could simultaneously demonstrate two different proteins in the same cells, demonstrated the significant positive correlation between Ki-67-positive proliferating vascular endothelial cells, which may represent neovascular formation[16,17] and vasohibin-1-positive endothelial cells. Indeed, the Ki-67 labeling index among vasohibin-1-positive endothelial cells was significantly higher than Ki-67 in all CD31-positive endothelial cells. These results will clearly indicate that vasohibin-1 is considered a more appropriate biomarker for intratumoral neovascularization compared to CD31, which may detect all the vasculature including both resting and proliferating endothelial cells.

Results of our study also demonstrated the positive correlation between vasohibin-1 and VEGF-A or bFGF in carcinoma cells or Flk-1 in intratumoral endothelial cells, which also suggest that the vasohibin-1 in vasculature in human breast carcinoma is induced by VEGF-A, bFGF/Flk-1 signaling pathway. PKCδ was reported to play an important role in an induction of vasohibin-1 in endothelial cells.[4] Therefore, vasohibin-1 is supposed to be induced in the downstream of VEGF-A, bFGF/Flk-1 signaling pathway. Further investigations are necessary to reach the final conclusion.

The expression of vasohibin-1 in EC was proposed to be regulated either positively or negatively by certain factors at the transcriptional level, and this may influence the process of angiogenesis.[4] Another *in vivo* study also demonstrated the significantly positive correlation between vasohibin-1 and Flk-1 expression in vasculature of human endometrial carcinoma.[9] Significantly higher vasohibin-1 immunodensity in IDC than DCIS in our present study of human breast also indicate that the anti-angiogenic compensatory mechanism may be operational in invasive breast carcinoma, possibly in response to induction of angiogenesis by various factors related to carcinoma invasion into the surrounding stroma.

Results of several recent studies demonstrated the possible correlation between VEGF status in carcinoma cells and clinical outcome in breast cancer patients. VEGF was proposed to be correlated with worse DFS and overall survival rates especially in the patients with early-stage breast cancer.[33] VEGF expression in carcinoma cells was also reported as an independent prognostic marker in both node-positive and node-negative breast cancers.[34] Many previous immunohistochemical studies of MVD assessed by CD31, CD34 or Factor VIII antigen in human breast cancer demonstrated that high MVD in invasive ductal carcinoma is usually correlated with a greater likelihood of metastatic disease,[10] shorter relapse-free intervals and reduced overall survival in patients with node-negative breast cancer.[11] We therefore examined whether vasohibin-1 immunoreactivity is correlated with OS and DFS of the patients. Results of our study demonstrated that the cases with a higher number of vasohibin-1-positive vessels tended to be associated with better and statistically significant OS. In addition, a statistically negative or inverse correlation was detected between vasohibin-1 immunodensity and DFS. These results all suggest that an evaluation of the number of vasohibin-1-positive vessels may become one of the prognostic markers for metastasis and prognosis but it awaits further investigations to establish this approach as a surrogate marker such as MVD.

Recently, newer targeted therapies toward the control of tumor neovascularization such as anti-VEGF therapy have been developed in phase II and III clinical trials and demonstrated the clinical effects such as reduction of tumor angiogenesis and inhibition of solid tumors proliferation, either alone or in combination with chemotherapy.[35–38] In our present study, vasohibin-1 immunohistochemical staining was demonstrated to reasonably reflect the status of angiogenesis, and vasohibin-1 itself may be considered for anti-VEGF and anti-angiogenesis drugs to control tumor angiogenesis in future.


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# New Developments in Intracrinology of Human Breast Cancer

## Estrogen Sulfatase and Sulfotransferase

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**Steroid sulfatase (STS) hydrolyses biologically inactive estrogen sulfates to active estrogens, while estrogen sulfotransferase (EST) sulfonates estrogens to estrogen sulfates. Information regarding the expression of STS in human breast carcinoma tissues is still very limited compared to that of aromatase or 17β-hydroxysteroid dehydrogenases (17β-HSDs). In our study, EST and STS immunoreactivity was detected in carcinoma cells in 50 and 84 out of 113 breast carcinomas (44.2% and 74.3%, respectively), which was also associated with mRNA levels determined by RT/real-time PCR. Using microdissection/RT-PCR analyses, EST mRNA was localized to both carcinoma and intratumoral stromal cells, whereas STS was detected only in carcinoma or parenchymal cells. STS immunoreactivity was positively associated with tumor size. EST immunoreactivity was inversely correlated with tumor size or lymph node status and was significantly associated with a decreased risk of recurrence and improved prognosis. These data suggest that both EST and STS play important roles in the regulation of *in situ* estrogen production in human breast cancer. In addition, EST is an independent prognostic factor in human breast carcinoma and loss of EST may result in altered estrogen metabolism in hormone-dependent breast cancer cells. An inhibition of intratumoral STS in the patients with estrogen-dependent breast carcinoma is also considered to provide more clinical benefits, especially to the patients in which primary source of an availability of intratumoral estrogen is through STS rather than aromatase.**

*Key words:* **estrogen; breast cancer; sulfatase (STS); sulfotransferase (EST); intracrinology**

## Development of Intracrinology in Human Breast Carcinoma

Estrogens, especially estradiol, or E2, a biologically potent estrogen, play pivotal roles in the proliferation and development of hormone-dependent breast carcinoma cells.[1] Estradiol originates from different sources of the body before and after the menopause in women. In premenopausal women the ovary is the principle source of circulating estrogens.[2,3] However, estrogens cease to be produced in the circulating ovaries following menopause. In these postmenopausal women, circulating levels of estrogens could still be detected by routine hormone assays in clinical laboratories because of their production primarily through conversion of androgens of both adrenal and ovarian origins, especially those produced in the adrenal cortex, such as androstenedione, which is abundantly present in circulation.[4] Therefore, in postmenopausal women, the levels of circulating estrogens are markedly low compared to those in premenopausal women, but their substrates, that is, adrenal androgens are abundantly present despite slight progressive reduction throughout the ages in women.

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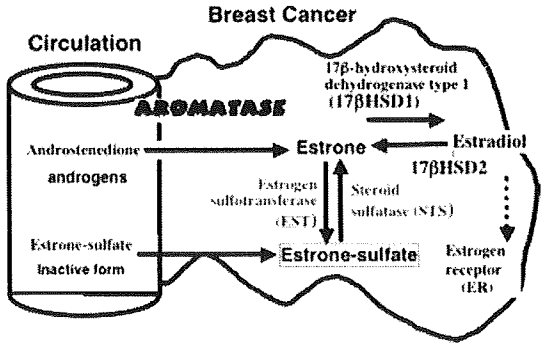


**Figure 1.** Summary of intratumoral estrogen metabolism and synthesis in human breast carcinoma.

These conversions of circulating androgens to estrogens, especially to estrone, occur principally in various peripheral tissues, such as skin,[5] muscle,[5] fat,[6] bone,[7] and others in postmenopausal women. This conversion from androgens to estrogens has been demonstrated to be catalyzed by the aromatase enzyme complex.[1,4]

It is also important to note that the great majority of estrone in circulation is present as sulfated form or estrone sulfate (E1-S), and steroid sulfatase (STS) hydrolyzes circulating E1-S to E1 in various human tissues *in situ*, which confers potent estrogenic actions.[8,9] Estrogen sulfotransferase (EST) (*SULT 1E1* or *STE* gene) is a member of the superfamily of steroid-sulfotransferases, and it sulfonates estrogens to biologically inactive estrogen sulfates.[10–12] Therefore, EST and STS, especially the balance of these two enzymes *in situ*, also play very important roles in maintaining an availability of biologically active estrogens in the tissues, including estrogen-dependent tissues in which estrogen receptors are present.

## STS and EST in Intracrinology of Human Breast Carcinoma

Both STS and EST play important roles in providing intratumoral estrogen in hormone-dependent human breast carcinoma, as summarized in Figure 1.

We evaluated the status of STS and EST in 113 cases of human breast invasive ductal carcinoma using immunohistochemistry and reverse transcription–polymerase chain reaction (RT-PCR).[13] EST and STS immunoreactivity was detected in carcinoma cells in 50 and 84 out of 113 carcinomas (44.2% and 74.3%, respectively), which was also associated with mRNA levels determined by RT/real-time PCR.

EST enzymatic activity has also been reported in human breast carcinoma and normal breast tissue.[14,15] In addition, the concentration of E1-S in breast cancer tissues has been reported to be significantly higher than plasma levels.[16] Results from our reported study are in good agreement with previous findings and suggest that EST plays an important role in the inactivation of local estrogens in human breast cancer tissues. E1-S is the most abundant estrogen in peripheral blood.[17] Enzymatic activity for STS has been reported to be higher in breast cancer tissues than that in normal breast tissues. In addition, Saeki *et al.*[18] reported STS immunopositivity in carcinoma cells in 22 out of 25 cases (88.0%). Utsumi *et al.*[19] reported that patients with high mRNA levels for STS were associated with an increased risk of recurrence. The enzymatic activity of STS is detected in the great majority of human breast tumors, which is considerably higher than tumor aromatase activity.[20,21] Evans *et al.*[21] reported no significant association between STS activity and time to recurrence or overall survival time in breast cancer patients. STS activity was reported to be correlated with STS mRNA expression level in human breast cancer cells.[22] Reed *et al.*[23] proposed that the sulfatase pathway may be more important than the aromatase route for intratumoral estrogen synthesis in breast cancers, because aromatase mRNA expression was reported to have no significant prognostic value in postmenopausal patients with breast cancer; but, this hypothesis should be confirmed by further study. Based on these findings STS is playing an important role in the *in situ* activation of E1 from E1-S, thereby contributing to the increment

of estrogenic actions in human breast cancer tissues.

Using microdissection/RT-PCR analyses, EST mRNA was localized to both carcinoma and intratumoral stromal cells, whereas STS was detected only in carcinoma or parenchymal cells. STS immunoreactivity was positively associated with tumor size. EST immunoreactivity was inversely correlated with tumor size or lymph node status and was significantly associated with a decreased risk of recurrence and improved prognosis. These data suggest that both EST and STS play important roles in the regulation of *in situ* estrogen production. In addition, EST is an independent prognostic factor in human breast carcinoma, and loss of EST may result in altered estrogen metabolism in hormone-dependent breast cancer cells.

## Potential Inhibition of Sulfatase as an Endocrine Treatment of Estrogen Receptor Positive Breast Carcinoma

Inhibitors that suppress intratumoral STS activity and/or expression have been developed by various investigators.[24–27] Some of these inhibitors demonstrated excellent *in vitro* efficacy with reasonable specificity; they are actually being used in clinical studies of postmenopausal patients with breast carcinoma without severe side effects. Such inhibitors include the dual inhibitor of aromatase and STS.[24–27] It is also important to note that this enzyme is regulated by the mechanisms that are different from those of aromatase and intratumoral aromatase, and that STSs are not correlated with aromatase in actual human breast carcinoma tissues, suggesting that some patients are associated with overexpression of STS but not aromatase in their tumor tissues. Therefore, the potential inhibitors against STS are considered to be promising as a new mode of endocrine therapy targeted against the reduction of intratumoral estrogens based on the concept of intracrinology. However, STS inhibitors could provide therapeutic effects only on the ER positive patients with overexpression of the enzyme in the tumor tissues. Therefore, the status of STS in breast cancer tissues must be examined in order to achieve any therapeutic effects. Immunohistochemistry of STS in resected breast cancer specimens could contribute greatly in this regard, and intratumoral STS immunoreactivity could be important surrogate marker of the treatment. It will then become very important to standardize the method of detection of this intratumoral enzyme when STS inhibitors may be used in clinical settings and confer the maximum benefits on the selected patients with breast cancer using newly developed inhibitors. In addition, it will be also important to clarify which patients could benefit from STS inhibitor therapy compared to aromatase inhibitors and the potential long term side effects of STS inhibitors by further studies, especially because this agent is expected to be used for a relatively long duration possibly as an adjuvant treatment.

### Conflicts of Interest

The authors declare no conflicts of interest.

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# Type 5 17β-Hydroxysteroid Dehydrogenase (AKR1C3) Contributes to Testosterone Production in the Adrenal Reticularis

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**Context:** The human adrenal gland produces small amounts of testosterone that are increased under pathological conditions. However, the mechanisms through which the adrenal gland produces testosterone are poorly defined.

**Objective:** Our objective was to define the role of type 5 17β-hydroxysteroid dehydrogenase (AKR1C3) in human adrenal production of testosterone.

**Design and Methods:** Adrenal vein sampling was used to confirm ACTH stimulation of adrenal testosterone production. Adrenal expression of AKR1C3 was studied using microarray, quantitative real-time RT-PCR, and immunohistochemical analyses. AKR1C3 knockdown was accomplished in cultured adrenal cells (H295R) using small interfering RNA, followed by measurement of testosterone production.

**Results:** Acute ACTH administration significantly increased adrenal vein testosterone levels. Examination of the enzymes required for the conversion of androstenedione to testosterone using microarray analysis, quantitative real-time RT-PCR, and immunohistochemistry demonstrated that AKR1C3 was present in the adrenal gland and predominantly expressed in the zona reticularis. Decreasing adrenal cell expression of AKR1C3 mRNA and protein inhibited testosterone production in the H295R adrenal cell line.

**Conclusions:** The human adrenal gland directly secretes small, but significant, amounts of testosterone that increases in diseases of androgen excess. AKR1C3 is expressed in the human adrenal gland, with higher levels in the zona reticularis than in the zona fasciculata. AKR1C3, through its ability to convert androstenedione to testosterone, is likely responsible for adrenal testosterone production. ***(J Clin Endocrinol Metab* 94: 2192–2198, 2009)**

The human adrenal zona reticularis (ZR) produces the so-called adrenal androgens dehydroepiandrosterone (DHEA) and DHEA-sulfate (1, 2). Although the factors leading to the production of adrenal androgen production are unclear, ACTH does play a key role in regulating DHEA production (2). Testosterone, a more active androgen, is primarily produced in the testis and, to some degree, the ovary, under the control of LH (2). However, several studies have shown that the adrenal gland contributes to the circulating pool of testosterone by direct secretion as well as by peripheral conversion of adrenal-derived precursor (3–5). Adrenal synthesis of testosterone is also elevated in some women with androgen excess associated with hirsutism and

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Abbreviations: AKR1C3, Aldo-keto reductase family 1 member C3; AVS, adrenal vein sampling; CYB5, cytochrome b5; CV, coefficient of variation; DHEA, dehydroepiandrosterone; HSD17B3, type 3 17β-hydroxysteroid dehydrogenase; LC-MS-MS, liquid chromatography/tandem mass spectrometry; LLQ, limit of quantification; qPCR, quantitative real-time RT-PCR; T-d5, testosterone-2,2,4,6,6-d5; ZF, zona fasciculata; ZR, zona reticularis.

polycystic ovarian syndrome (6, 7). Despite the potential importance of adrenal testosterone as a source of androgen in female androgen excess, the adrenal enzymes involved in testosterone synthesis remain poorly defined.

The high expression of type 3 17β-hydroxysteroid dehydrogenase (HSD17B3), which converts androstenedione to testosterone, is necessary for the level of testosterone produced in the testis. However, expression of this enzyme is highly restricted to the testis, and therefore, testosterone production in other tissues has recently been attributed to type 5 17β-hydroxysteroid dehydrogenase, also called aldo-keto reductase family 1 member C3 (AKR1C3) (6, 8–10). AKR1C3 is expressed in many tissues, including skeletal muscle, liver, adrenal gland, and ovary (11). However, to date, there have been no studies of human adrenal AKR1C3 expression. Herein, we show that the adrenal cortex expresses AKR1C3, with highest levels seen in the ZR. In addition, we show that decreasing levels of AKR1C3 in adrenocortical cells inhibits production of testosterone. These findings support a role for AKR1C3 in human adrenal production of testosterone and suggest that further studies are warranted to define the role of adrenal AKR1C3 as it relates to androgen excess.

## Materials and Methods

### Human tissue preparation

Whole human adult adrenal gland, liver, testis,[1] and pre- and postmenopausal ovaries were obtained through the Cooperative Human Tissue Network (Philadelphia, PA), Clontech (Palo Alto, CA), and Tohoku University School of Medicine. The use of these tissues was approved by the Institutional Review Boards of the Medical College of Georgia and Tohoku University School of Medicine. The tissues were kept frozen for subsequent RNA extraction or for cell isolation. Adult adrenal tissues were also fixed with 10% formaldehyde for immunohistochemistry. The method of RNA extraction was previously reported in detail (12). In addition, for microarray analysis, isolated RNA from human adrenal zona fasciculata (ZF) (n = 3) and ZR (n = 3) was prepared by microdissection of the adrenal gland as previously described (13).

### Microarray and quantitative real-time RT-PCR (qPCR) analyses for adrenal ZF and ZR cells

ZF and ZR cell RNA was analyzed using an Affymetrix human HG-U133 + 2 oligonucleotide microarray set containing 54,675 probe sets, representing approximately 40,500 independent human genes. The arrays were scanned at high resolution using an Affymetrix GeneChip Scanner 3000 located at Medical College of Georgia Microarray Core Facility. Results between arrays were studied with GeneSpring GX 7.3 software (Silicon Genetics, Redwood City, CA) using a gene list of steroidogenic enzymes that included 44 genes. To confirm the result of microarray analysis, qPCR analysis was performed using ZF and ZR cell RNA for AKR1C3, type 2 3β-hydroxysteroid dehydrogenase (HSD3B2), and cytochrome b5 (CYB5). The sequences for primers and probes for HSD3B2 and CYB5 were previously described in detail (14, 15). The protocol of cDNA synthesis was previously described in detail (12). qPCR were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Quantitative normalization of cDNA in each tissue-derived sample was performed using expression of 18S rRNA as an internal control. After quantitative normalization, the expression of each gene was compared as previously reported (12).

### qPCR for aldo-keto reductase and hydroxysteroid dehydrogenase family members

As well as AKR1C3, it is reported that there are several enzymes that are currently known to have the ability to catalyze the conversion of androstenedione to testosterone, specifically type 1 17β-hydroxysteroid dehydrogenase (HSD17B1), HSD17B3, aldo-keto reductase family 1 member C1 (AKR1C1), aldo-keto reductase family 1 member C2 (AKR1C2), and aldo-keto reductase family 1 member C4 (AKR1C4) (2, 9, 16). Therefore, the mRNA levels of AKR1C3 and these enzymes among different tissues were measured by qPCR analysis as described above. The primer/probe set for human AKR1C3 was designed as published previously (17). The primer set for AKR1C1 was designed as follows: forward, 5′-AAA GCC AGG TGA GGA AGT GA-3′, and reverse, 5′-CAT GTG GCA CAG AGA TCC AC-3′. HSD17B1, HSD17B3, AKR1C2, and AKR1C4 mRNA levels were measured using primers and probes from TaqMan Gene Expression Assays (Applied Biosystems).

### Immunohistochemistry

The method for immunohistochemistry was previously reported in detail (12). A monoclonal antihuman AKR1C3 antibody was obtained from Sigma-Aldrich (St. Louis, MO). It was previously reported that this monoclonal antibody does not cross-react with human AKR1C1, AKR1C2, or AKR1C4 (18). The polyclonal antibody for HSD3B2 was kindly provided by Dr. Mason (University of Edinburgh, Edinburgh, UK). Negative control staining, in which primary antibody was replaced with PBS, was also performed, and no specific immunoreactivity was detected in these tissue sections (data not shown).

### Transfection of AKR1C3 small interfering RNA (siRNA) and testosterone measurement in a human adrenocortical cell line (H295R)

The human adrenocortical cell line H295R was used for all transfection experiments and was routinely cultured in Dulbecco's modified Eagles/Ham F12 (DMF/F12) medium (Life Technologies, Inc., Carlsbad, CA) supplemented with 2.5% Ultroser G (Life Sciences, Cergy, France), 1% ITS plus Universal Culture Supplement Premix (BD Biosciences, Bedford, MA), and antibiotics, including 1% penicillin/ streptomycin solution (Life Technologies, Inc.) and 0.1% gentamicin solution (Sigma-Aldrich). siRNA of AKR1C3 was commercially obtained from Dharmacon (Chicago, IL). As a negative control, Stealth RNAi Negative Control Duplexes were also used (Invitrogen, Carlsbad, CA). Electrical transfection assays were performed using the Nucleofector System (AMAXA, Gaithersburg, MD). Briefly, cells were cultured to 80–90% confluence in growth medium and then trypsinized and resuspended in Nucleofector Solution R (AMAXA) at a ratio of 5 million cells per 100 μl solution. Indicated amounts of AKR1C3 siRNA or Stealth RNAi Negative Control Duplexes (10 nM at final concentration) were added to the solution, and the mixture was run under program T20 in the Nucleofection System. Cells were allowed to recover for 48 h before treatment. For treatment, H295R cells were then incubated for 48 h in a low-serum medium containing 0.1% Cosmic Calf serum (CCS) either under basal conditions or with forskolin (10 μM). To examine the potential role of AKR1C3 using a pharmacological approach, H295R cells were incubated for 48 h in a low-serum medium containing 0.1% Cosmic Calf serum either with or without indomethacin (10 μM; Sigma-Aldrich), which is known to inhibit AKR1C enzymes (19). The medium was collected, and testosterone was measured using RIA (Siemens, Tarrytown, NY). For testosterone measurement, 50 μl of each sample was incubated with [$^{125}$I]testosterone in antibody-coated tubes for 3 h at 37 C and counted for 1 min in a γ-counter. The limit of quantification (LLQ) was 0.14 nmol/liter, and the coefficient of variation (CV) ranged from 6–12%. Both RNA and protein were isolated from the cells for qPCR and Western analysis. For Western analysis, a monoclonal human anti-AKR1C3 (Sigma-Aldrich) and a monoclonal human anti-β-actin antibody (Sigma-Aldrich) were used.

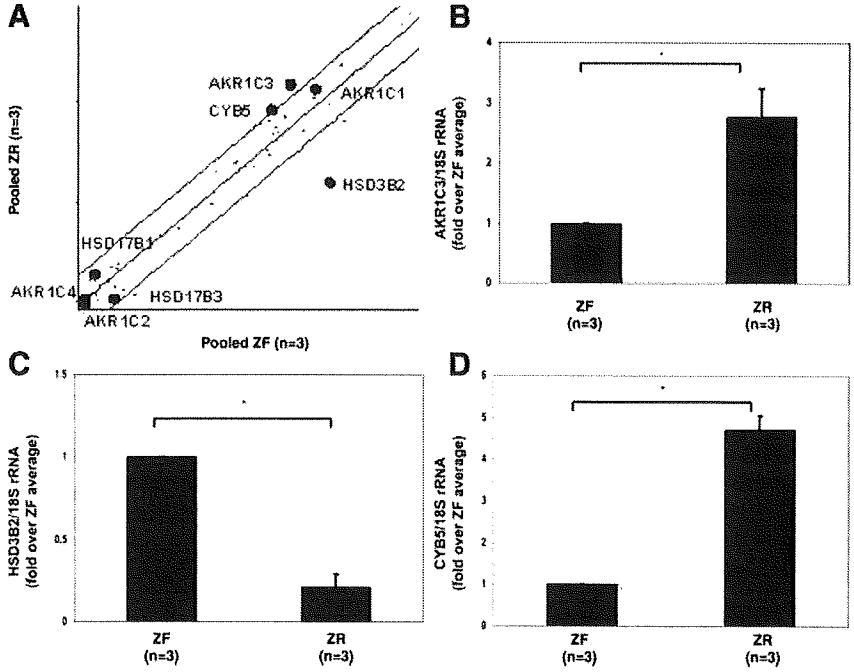


**FIG. 1.** A, Scatter plot from microarray analysis comparing the mRNA normalized signal intensity for steroidogenic enzymes between human adrenal ZR (n = 3) and ZF cells (n = 3). Each spot represents a unique transcript, with a total of 44 steroid-metabolizing transcripts examined. Transcripts with the highest signal variation, AKR1C3, CYB5, and HSD3B2 transcripts are labeled. Microarray analysis was confirmed using qPCR specific for AKR1C3 (B), HSD3B2 (C), and CYB5 (D). qPCR was used to quantify the transcripts for these genes in the ZR (n = 3) and ZF cells (n = 3), as described in *Materials and Methods*. Data are expressed as the fold over the average expression levels seen in the liver. mRNA levels were normalized to 18S rRNA. *, $P < 0.05$.

### Adrenal vein sampling (AVS) and measurement of serum testosterone

The premenopausal (n = 4) and postmenopausal female (n = 4) patients with primary aldosteronism had samples taken at Tohoku University Hospital from 2007–2008 via AVS as has previously been reported (20). Informed consent was also obtained from all the patients in AVS. During AVS, blood was collected from the bilateral adrenal veins and the iliac vein. In addition, simultaneous bilateral blood collection 15 min after 0.25 mg (10 IU) ACTH stimulation was also performed. Successful adrenal venous cannulation was confirmed based on the cortisol level after ACTH stimulation in the adrenal venous sample, which was more than five times higher than that in the vena cava samples (20). For our study, we used iliac and adrenal vein plasma from the normal adrenal opposite to the aldosterone-producing tumor side. The testosterone content of patient plasma was determined using a liquid chromatography/tandem mass spectrometry (LC-MS-MS) analysis. Plasma aldosterone and cortisol were measured by SPAC-S Aldosterone Kit (TFB Inc., Tokyo, Japan) and fluorescence polarization immunoassay (Abbott Japan Co., Chiba, Japan), respectively. For aldosterone measurement, the LLQ was 2.5 ng/dl, and the CV ranged from 4.5–4.7%. For cortisol measurement, the LLQ was 0.7 μg/dl, and the CV was 20%.

### LC-MS-MS sample preparation and analysis condition

Samples to be analyzed (200 μl) were processed by liquid/liquid extraction using 4 ml methyl tertiary butyl ether. Testosterone-2,2,4,6,6-d5 (T-d5) was used as the internal standard. The organic phase was evaporated to dryness and reconstituted in 100 μl water/acetonitrile (80:20), and 20 μl was analyzed on a Waters Xterra C18 (2.1 × 250 mm, 5 μm) column by reversed-phase HPLC (Agilent, Palo Alto, CA, and Leap Technologies, Carrboro, NC) coupled with a tandem quadrupole mass spectrometer (Waters, Beverly, MA) run in electrospray-positive mode. The column temperature was held at 40 C. The mobile phase was a gradient of 0.5% formic acid in water (vol/vol) and 0.5% formic acid in acetonitrile (vol/vol). The retention time for testosterone and T-d5 was 10.2 min. The LC-MS-MS transition for testosterone was 289.0–96.9 atomic mass units, and the transition for T-d5 was 294.0–99.8 atomic mass units. Calibration curves prepared in water and quality control (QC) samples prepared in human plasma from normal donors were analyzed in parallel with the samples. Sample responses were acquired, and concentrations were determined based on the calibration curve using Masslynx analysis software (Waters). The concentration of analyte in the QC samples was calculated by subtracting the mean endogenous concentration determined in normal donor plasma from the total concentration found in the QC sample.

### Data analysis and statistical methods

Results are given as mean ± SEM where appropriate. Statistical analyses were done by unpaired *t* test or one-way ANOVA, followed by *post hoc* test for comparisons between two groups dependent on the data types. Significance was accepted at the 0–0.05 level of probability ($P < 0.05$).

## Results

### Microarray and qPCR analysis using ZF and ZR cell RNA

Four transcripts were found to have greater than a 2-fold difference in expression between ZR and ZF among 44 steroidogenic enzymes and related proteins (21). Among the transcripts that were preferentially expressed in the ZR were AKR1C3 and CYB5, which are known to be involved in androgen synthesis (Fig. 1A). AKR1C3 has many enzymatic activities, one of which is the ability to convert androstenedione to testosterone. HSD3B2, which is needed for glucocorticoid production, was expressed at levels 7-fold higher in ZF *vs.* ZR (Fig. 1A). These transcript differences were confirmed by qPCR using three different ZF and ZR sample pairs (Fig. 1, B–D).

AKR1C1 transcripts were also abundant in both the ZF and ZR; however, the expression levels were not significantly different between the ZF and ZR (Fig. 1A). Four other enzymes, HSD17B1, HSD17B3, AKR1C2, and AKR1C4, which have been reported to have the ability for androstenedione to testosterone conversion, showed very low expression signals in both the ZR and ZF (Fig. 1A).

### qPCR analysis using several human tissues

qPCR was used to compare mRNA levels for HSD17B1, HSD17B3, AKR1C1, AKR1C2, AKR1C3, and AKR1C4 among different human tissues. AKR1C1 and AKR1C3 mRNA was easily detectable in all tissues examined, but levels were lower in whole adrenal gland, compared with the liver ($P < 0.05$) (Fig. 2). The levels of HSD17B1 and HSD17B3 mRNA were significantly higher in the testis compared with other tissues we studied (Fig. 2) ($P < 0.05$). HSD17B1 mRNA was also high in the ovary (Fig. 2). On the other hand, HSD17B1 and HSD17B3 transcripts were

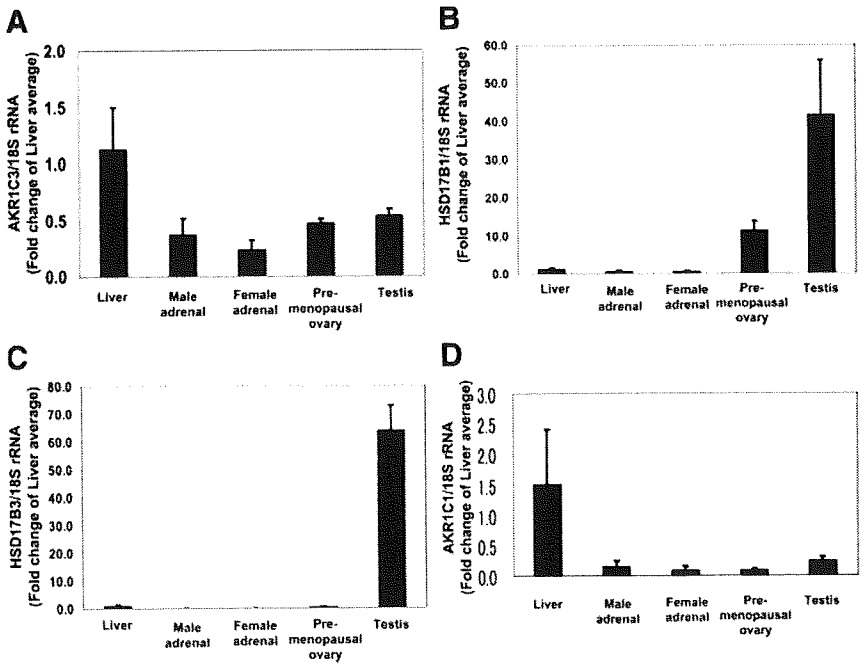


**FIG. 2.** Quantification of AKR1C3 (A), HSD17B1 (B), HSD17B3 (C), and AKR1C1 (D) transcript levels in the human male and female adrenal (n = 6 each), liver, testis, and pre- and postmenopausal ovary (n = 3 each). qPCR was performed to quantify the level of the mRNA, as described in *Materials and Methods*. Data are expressed as the fold over the average expression levels seen in the liver. mRNA levels were normalized to 18S rRNA. *, $P < 0.05$.

low in the adrenal gland (Fig. 2). These data support a broad expression pattern for AKR1C1 and AKR1C3 and a highly restricted pattern of expression for HSD17B1 and HSD17B3. The expression of AKR1C2 and AKR1C4 mRNA was very low or undetectable in human adrenal glands (data not shown).

### Immunohistochemistry

Immunohistochemical staining of adrenal sections was performed to localize the expression of AKR1C3 and HSD3B2 within the human adrenal (Fig. 3). AKR1C3 immunoreactivity was predominantly detected in cytoplasm of the ZR in human adrenal gland (Fig. 3B). We also confirmed that HSD3B2 immunoreactivity was highly detectable in the ZF but not the ZR in human adrenals (Fig. 3C). The pattern of AKR1C3 immunoreactivity showed no significant difference related to sex or age (data not shown). No staining was observed in the absence of AKR1C3 antibody (data not shown).

### Testosterone production in H295R adrenal cells after AKR1C3 siRNA transfection

The H295R adrenal cell line produces a variety of androgens, including testosterone (22). After transfection of H295R cells with AKR1C3-specific siRNA, the levels of AKR1C3 mRNA dropped by 87% and protein level dropped by 40%, compared with control cells (Fig. 4A). Forskolin (used to activate cAMP-dependent pathways) increased H295R testosterone production by approximately 2-fold in cells transfected with scrambled (control) or siRNA directed at AKR1C3 (Fig. 4A). However, testosterone production was significantly lower (by 40%) in both AKR1C3 siRNA-transfected cells when compared with control cells (Fig. 4B). To examine the contribution of AKR1C3 to testosterone using a pharmacological strategy, H295R cells were incubated for 48 h in the absence and presence of indomethacin (10 $\mu$M). Testosterone production was significantly inhibited (by 35%) by indomethacin treatment compared with control cells (Fig. 4C).

### Steroid levels in human adrenal and iliac vein

Adrenal testosterone production was examined in eight independent adrenal vein samples taken before and after ACTH administration (15 min). Testosterone levels in adrenal vein plasma increased by average 6.3-fold after ACTH administration (Fig. 5A and Table 1). The increase in testosterone production was not different in pre- or postmenopausal women (data not shown). The increase in testosterone did not result from peripheral conversion of adrenal androgen precursor, because there was no change in iliac vein plasma after 15 min ACTH administration (Fig. 5B and Table 1).

## Discussion

In this study, we used microarray analysis to compare the expression of steroidogenic enzymes between the human adrenal ZF and ZR. As was previously demonstrated, HSD3B2, steroid sulfotransferase (SULT2A1), and CYB5 are differentially expressed between the zones in a manner that supports the production of cortisol by the ZF and DHEA-sulfate by the ZR (1). However, this analysis also demonstrated that AKR1C3 was predominantly expressed in the ZR in the human adult adrenal gland. The ability of this enzyme to convert androstenedione to testosterone provides a potential mechanism for adrenal production of testosterone in normal physiology and in diseases associated with androgen excess.

There are several enzymes that are currently known to have the ability to catalyze the conversion of androstenedione to testosterone, specifically HSD17B1, AKR1C3, HSD17B3, AKR1C1, AKR1C2, and AKR1C4 (2, 9, 16). Within the testis, the enzyme HSD17B3 is localized to the Leydig cells, where it

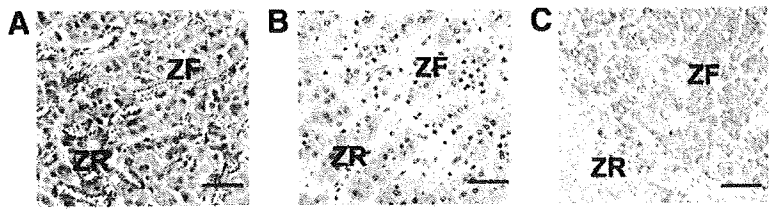


**FIG. 3.** Hematoxylin-eosin staining (A) and immunohistochemical localization of AKR1C3 (B) and HSD3B2 (C) in the human adult adrenal gland. *Bar*, 10 $\mu$m. These are representative photomicrographs from the examination of 10 adrenals.