25-31	RAPKRLK	c-Jun	RKRKL
380-87	KVKFMKSK	SV-40	PKKKRKV
163-171	VLLFTILNPI	PKI	LALKLAGLDI
		Rev	LQLPPLERLTL

FIGURE 9 – NLS-like and NES-like sequences in hnRNP L. There are 2 NLS-like sequences that resemble the NLS sequences of c-Jun and SV-40 large T antigen and one NES-like sequence that resembles the NES sequences of PKI and Rev.

3 RRMs, hnRNP L was distributed throughout the cell. These results indicate that the N-terminal portion of each RRM is required for determination of the intracellular localization of hnRNP L (Fig. 8a, *arrows*). We then searched the sequence of hnRNP L and found 2 sequences that were rich in alkaline amino acids (residues 25–31, 380–387) and a sequence that was rich in hydrophobic amino acids (residue 163–171, Fig. 9). The sequences rich in alkaline amino acids showed high homology with the NLS sequences of c-Jun and SV40 large T antigen,[13,14] and the sequence rich in hydrophobic amino acids showed high homology with the NES sequences of PKI[15] and Rev[16] respectively. The N-terminal portion of RRM1 and RRM3 contain the NLS-like sequences, residue 25–31 and residue 380–387, respectively, and the N-terminal portion of RRM2 contains the NES-like sequence, residue 163–171.

DISCUSSION

There are approximately 20 major hnRNPs, and some of them have been reported to be highly expressed in cancer tissues. Sueoka *et al.*[3] demonstrated elevated expression of hnRNP B1 mRNA in human lung cancer tissue, and hnRNP I and hnRNP K mRNA have been reported in malignant glioblastoma and breast cancer, respectively.[2,17] We demonstrated expression of hnRNP L in human lung cancer cell lines and high expression of hnRNP L is presumably present in lung cancer tissue.

We reported previously that a nuclear protein in human cancer cells binds to the DUM-DNA adduct. The protein, DARP, preferentially bound to the DNA damage induced by DNA-alkylating minor groove binders such as DUMs and CC-1065. Because the amino acid sequence of DARP was identical to hnRNP L, hnRNP L is a candidate protein that binds to the DNA damage induced by DUM. A water-soluble derivative of DUM, KW-2189, exhibits broad spectrum antitumor activity in a series of experimental tumor models and entered clinical trials. KW-2189 was designed as a prodrug to generate active species, DU86, in tumor cells and DARP bounds to the DNA induced by DU86 (unpublished results). Although KW-2189 alkylates DNA *in vitro*, only the DU86-DNA adduct was detected in the human cells treated with KW-2189.[18,19] The transfection study demonstrated that hnRNP L enhanced the cellular sensitivity to KW2189. As described previously, DARP did not recognize the DNA adducts of cisplatin and mitomycin C *in vitro*.[18] We show that when we examined the transfectants for sensitivity to other DNA-damaging agents, *i.e.*, the major groove binders mitomycin C and cisplatin (data not shown), ectopic hnRNP L expression had no affect on cell sensitivity to them. These results suggest that DARP could be hnRNP L and it acts specifically on DNA damage induced by the minor groove binder.

Other possible mechanisms of increased sensitivity to KW-2189 are: 1) that hnRNP L facilitates transportation of the drug to the nucleus, and 2) that hnRNP L increases the stability of the drug-DNA adduct in a sequence-specific manner.

We have described the difference in intracellular localization of hnRNP L in human lung cancer cell lines. Although there is a report claiming that hnRNP L localized in the nucleoplasm in HeLa cells transfected with hnRNP L,[12] we showed that the intracellular localization of hnRNP L differs among human lung cancer cell lines.

There was a report that hnRNP A2 is located in the cytoplasm in post-mitotic phase.[20] In this study, few mitotic cells were observed in the culture condition indicating that mitosis was not correlated with hnRNP L distribution. We speculate that in the case of hnRNP A2 a different mechanism might be involved in the intracellular localization of hnRNP L. Nevertheless, synchronization experiments must be examined.

SBC-3 and PC-14 cells grow faster than H69 cells. Even though cell growths of SBC-3 and PC-14 cells were equal in our culture condition, distribution of hnRNP L in these cells were different. This result indicate that the distribution depends on the cell type rather than difference of the cell growth.

To determine whether the localization of hnRNP L is altered by drug exposure, we examined the immunofluorescent staining of hnRNP L in lung cancer cells exposed to KW-2189 for 24 hr. An increased population of cells in which hnRNP L was localized in the nucleus was observed after exposure of a small cell lung cancer (SBC-3) cell line to KW-2189 (data not shown). Although this result was not observed in the rest two cell lines, it can support the hypothesis that hnRNP L helps drugs to transport into nuclear and involves in cell sensitivity mentioned above.

To test the hypothesis that the differences in intracellular localization in lung cancer cells are due to gene alterations, we compared the hnRNP L cDNA sequences in these cell lines. No mutations were detected in any of the lines (data not shown), suggesting that hnRNP L might be co-localized with other proteins. Interaction between hnRNPs has been reported and hnRNP L is known to have a binding domain for interaction with other hnRNPs (*e.g.*, hnRNP I and hnRNP K),[21] which are recognized to have NLS. Based on this evidence, the differences in localization of hnRNP L in these cell lines might be due to changes in the molecules that interact with hnRNP L, such as hnRNP I or K. In addition, the putative sites for regulation of localization signal in hnRNP L that we found (25–31, 380–387 and 163–171) would be involved in these interactions. Further studies should extend the potential use of hnRNP L as a factor to assess sensitivity to chemotherapy and candidate molecules for drug development. In addition, expression of hnRNP L needs to be investigated in tissue from lung cancer patients for therapeutic exploitation.

In summary, we have demonstrated the expression of hnRNP L with different intracellular localization in human lung cancer cell lines and that ectopic hnRNP L expression increases cellular sensitivity to a minor groove binder.

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Featured Article

Her2/neu Expression Predicts the Response to Antiaromatase Neoadjuvant Therapy in Primary Breast Cancer: Subgroup Analysis from Celecoxib Antiaromatase Neoadjuvant Trial

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ABSTRACT

Purpose: Many studies suggest that Her2/neu play an important role in neoadjuvant endocrine therapy. This study aimed to determine whether the level of Her2/neu expression in advanced breast cancer changes after antiaromatase neoadjuvant treatment, as well as to identify the relationship between Her2/neu expression and response to this kind of therapy.

Experimental Design: Thirty-six postmenopausal patients with hormonal receptor-positive primary breast cancer were included in a study of three monthly cycles of neoadjuvant endocrine therapy with either Aromasin (25 mg daily) or Femara (2.5 mg daily). Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) for Her2/neu were conducted both on pretreatment biopsies and surgical tumors.

Results: Using IHC, 5 of 36 (13.9%) of the patients had a Her2/neu overexpression after treatment, as compared with 16 of 36 (44.4%) before. Meanwhile, there was no change in 21 (58.3%) patients, and through FISH, there was a change from amplification to no amplification in 15 (41.7%) patients. The response rate to the treatment was 75% for Her2/neu (+) tumors and 35% for Her2/neu (−) tumors ($P = 0.017$) while FISH was performed. The response rate was also significantly affected by the decrease in Her2/neu status after the treatment, with 73% of the tumors showing decreased Her2/neu expression and with 38% of the tumors showing no change of Her2/neu expression ($P = 0.037$).

Conclusions: Using both IHC and FISH, advanced breast cancers show statistical evidence of decreasing incidence of Her2/neu expression after antiaromatase neoadjuvant treatment. Our data also suggest that Her2/neu expression and its change during the treatment might be predictive markers for this kind of therapy.

INTRODUCTION

Human epidermal growth factor receptor-2 is a proto-oncogene encoding a cell-surface glycoprotein designated the Her2 or c-erbB-2 receptor that belongs to the tyrosine kinase receptor family. The *Her2/neu* gene is amplified and/or its protein is overexpressed in 15–25% of breast cancers (1–4). Her2/neu status is a prognostic marker for poor clinical outcome (2, 3) and possibly a predictive marker for tamoxifen resistance (5–9). Although experimental data suggest an important role for Her2/neu in primary and acquired resistance to endocrine therapy using tamoxifen, early data from the neoadjuvant setting indicate that response to aromatase inhibitors may be maintained in patients with Her2/neu overexpression (10–12).

The goal of neoadjuvant therapy is shrinkage of locally advanced and unresectable primary breast tumors, permitting their successful surgical removal (13, 14). It has been used more recently in patients with large operable breast cancers that would require mastectomy but in whom tumor shrinkage can permit breast-conserving surgery (15, 16). Agents used have been mainly limited to cytotoxins used in other forms of chemotherapy. However, endocrine treatment is becoming an attractive alternative in hormone receptor-positive postmenopausal women.

It has been widely demonstrated that various endocrine agents (including tamoxifen and the aromatase inhibitors) can reduce the tumor volume over a 3–4-month treatment in postmenopausal estrogen receptor (ER) (+) patients (10, 11, 17–20). Aromatase inhibitors have most recently been shown to be superior to tamoxifen as initial therapy and are being extensively tested in the neoadjuvant setting instead of tamoxifen. In this setting, aromatase inhibitors not only show enhanced efficacy but also overcome tamoxifen resistance (10, 11). Although studies as yet have failed to show any survival advantage in patients receiving neoadjuvant compared with adjuvant chemotherapy (16, 21), there could nevertheless be benefits from neoadjuvant endocrine therapy provided there was more appropriate patient selection. When selecting patients for endocrine treatment, ER (+) status and, to a lesser extent, progesterone receptor (+) status are important determinants of response (10).

We have studied the expression of Her2/neu before and after neoadjuvant endocrine therapy in patients with breast cancer in an attempt to obtain more information on the effect of endocrine treatment on these patients. Her2/neu status was assessed by both immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH). It was also examined in relation to clinicopathological variables and clinical response.

MATERIALS AND METHODS

Patients. Thirty-six pathologically proven, post-menopausal patients with hormonal receptor positive breast cancer were included between October 2001 and July 2003 as part of the Celecoxib Antiaromatase Neoadjuvant Trial (22). The third generation aromatase inhibitor either letrozole (Femara; Novartis Pharma AG, Basel, Switzerland) or exemestane (Aromasin; Pharmacia & Upjohn Company, Kalamazoo, MI) was given daily in a 2.5 or 25 mg dose over the monthly cycle after pathological confirmation from core biopsy of the primary tumor. Eighteen patients were admitted into each group. Each patient was treated for three cycles, and surgery was performed within 7 days after the last cycle.

Physical examination and ultrasound examination were repeated every cycle. Response categories were defined according to the standard Union International Contre Cancer criteria as complete remission, partial remission, no change, progressive disease and not assessable.

All core biopsy and surgically excised tumors of the above patients were fixed in formalin and embedded in paraffin wax. Her2/neu expression was determined using IHC and FISH simultaneously.

IHC. The Hercep Test (Dako Corp., Carpinteria, CA) was performed according to the approved protocol as described by the manufacturer. Tissue sections were cut, mounted on plus slides, heat-treated for antigen retrieval, and immunostained. The sections were counterstained with H&E and then mounted in Permount. Immunostaining was interpreted with a bright-field Olympus microscope according to the scoring system of the manufacturer as 0, 1+, 2+, and 3+ (Dako Corp.). Controls without primary antibody and positive control tissues were included in all experiments to ensure the quality of staining.

FISH. FISH was performed according to the PathVysion (Vysis, Inc., Downers Grove, IL) protocol, described in the package insert as approved by the United States Food and Drug Administration. In brief, the PathVysion protocol involves re-
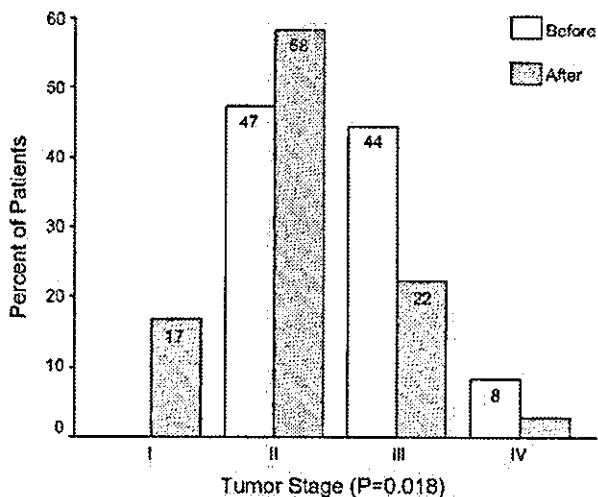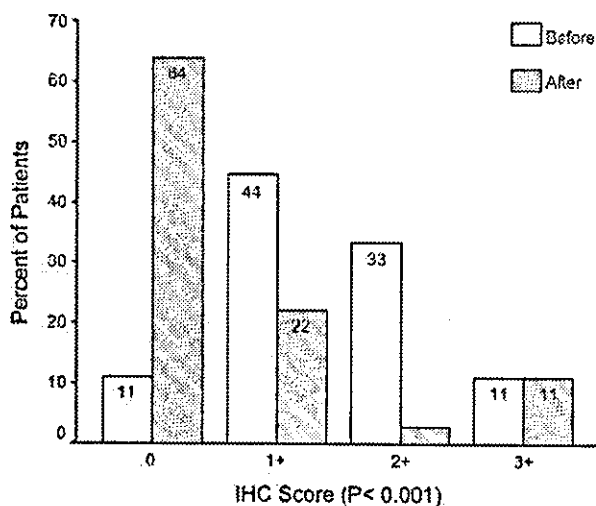

Fig. 2 The comparison of immunohistochemistry (IHC) scores of Her2/neu status before and after neoadjuvant endocrine therapy. The overexpression of Her2/neu are decreased significantly after the treatment ($P < 0.001$). □, before; ▓, after.

hydration of paraffin-embedded, 4-µm thick, multitumor tissue sections. The sections were air-dried, pretreated, and digested with protease before being hybridized with fluorescent-labeled probes for Her2/neu gene and α-satellite DNA for chromosome 17. The nuclei were routinely counterstained with an intercalating fluorescent counterstain, 4',6-diamidino-2-phenylindole. For each tumor, 20 tumor cell nuclei were identified and scored for both *Her2/neu* and chromosome 17 centromere numbers. *Her2/neu* gene amplification was defined as a Her2-to-chromosome 17 ratio > 2.0 as required by the manufacturer.

Statistical Methods. Statistical analysis was performed using the SPSS 11.0 (SPSS, Inc., Chicago, IL). Associations between clinicopathological variables and Her2/neu status before and after treatment were evaluated using the χ^2 test. The paired samples *t* test was performed to compare the *Her2/neu*-to-chromosome 17 ratios before and after treatment. All *P* values reported were two-sided with $P < 0.05$ considered to be statistically significant. κ was estimated to evaluate concordance among Her2/neu assay methods.

RESULTS

The median age of the patients at initial diagnosis was 66 years, ranging from 48 to 84 years. Histology was as follows: invasive ductal carcinoma in 31 (86%) patients; mixed invasive ductal carcinoma and mucinous in 2 (5.6%) patients; and invasive lobular carcinoma, ductal carcinoma *in situ*, and mucinous in 1 (2.8%) patient each. The invasive ductal carcinoma was calculated as grade 1 (Bloom and Richardson grade) in 11 (35.5%) patients, grade 2 in 15 (48.4%) patients, and grade 3 in 5 (16.1%) patients. There was no significant correlation between Her2/neu and any of the clinicopathological variables, including histological type, grade, or disease.

All 36 enrolled patients were fully assessable for response. The overall response rate in the intent-to-treat population of 36 patients was 53%, with 3 complete responses (8%) and 16

Fig. 1 The comparison of tumor stage before and after neoadjuvant endocrine therapy. Most of the tumors are down-staged significantly after the treatment ($P = 0.018$). □, before; ▣, after.

Fig. 3 Her2/neu overexpression (3+) determined by immunohistochemistry, original magnification, ×200.
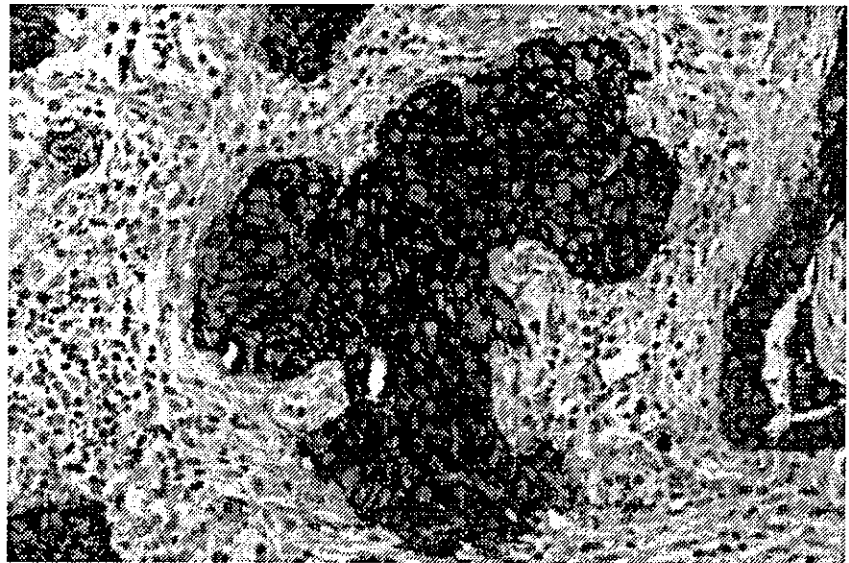
partial responses (45%). No changes were observed in 47% of assessable patients. Over the study period, none of the patients had direct disease progression without a period of stable disease. The percentage of patients with changes in tumor staging before and after the neoadjuvant therapy were compared (Fig. 1).

A total of 36 pairs of core biopsy and surgically excised tumors were available for IHC and FISH assays. Change in the distribution of Her2/neu IHC expression scores before and after therapy is shown in Fig. 2. Before therapy, 4 (11.1%), 16 (44.4%), 12 (33.4%), and 4 (11.1%) patients achieved scores of 0, 1+, 2+, 3+ (Fig. 3), respectively, yielding overexpression in 16 of the 36 patients (55.6%). In general, the IHC scores decreased after operation, except for 9 patients (Table 1). Thus, 5 of 36 (13.9%) of the patients had a Her2/neu expression of 2+ and 3+ after treatment, as compared with 16 of 36 (44.4%) before. Fig. 2 depicts the change in percentage of patients scored at each staining level before and after treatment by IHC assay. Using FISH, the Her2/neu-to-chromosome 17 ratios varied from 0.68 to 13.07. The ratios decreased significantly after the treatment. (t = 4.947, P < 0.001). Her2/neu amplification (Fig. 4) was determined in 20 patients (55.6%) before neoadjuvant therapy compared with 5 patients (13.9%) during surgery (Fig. 5). Concordance between the IHC and FISH results for the patients in whom data from both assays were available is listed in Table 2. The κ value of 0.875 suggests that there was excellent agreement between IHC and FISH in our population.

As shown in Table 3, the response rate to the treatment was significantly influenced by initial Her2/neu status, which was confirmed by FISH, with a response rate of 75% for Her2/neu (+) tumors and 35% for Her2/neu (−) tumors (P = 0.017). In addition, the response rate was also significantly affected by the decrease in Her2/neu status after the treatment, with a response rate of 73% for tumors showing decreased Her2/neu expression and 38% for tumors showing no change in Her2/neu expression (P = 0.037). There was no significant difference in either response rate (P = 0.52) or the change of Her2/neu expression (P = 0.50) between the Femara group and the Aromasin group.

DISCUSSION

Her2/neu positivity of breast cancer has been suggested that may be indicative of resistance to hormonal (predominantly tamoxifen) therapy, but the data are by no means conclusive (23–27). The heterogeneity of the published data may in part result from the ER status of the tumor not being considered. Much of the reported hormonal insensitivity of Her2/neu (+) tumors could result from ER (−) rather than Her2/neu (+) *per se* (27). So in this study, we had an attempt to solve this problem by selecting an entirely ER (+) group of patients.

Currently, no single assay is globally accepted as the gold standard for Her2/neu testing. Of a wide range of techniques, two technologies are now predominant in the routine clinical practice: determination of Her2/neu protein overexpression by IHC; and *Her2/neu* gene amplification by FISH (28). After directly comparing parallel IHC and FISH assessment of the same samples (29–37), some studies suggest the combination of these two assays provide comprehensive and valuable information on both Her2/neu protein concentrations and gene amplification (29–31). We also conducted both of these methods to help us make crucial management decisions. The variables produced by clonal selection using IHC can be overcome by detection of *Her2/neu* copy number. Although there is high

Table 1 Change of Her2/neu status before and after treatment

	Change of Her2/neu status	No. of patients (%)
IHC[a]	No change	8 (22.2)
	1+ → 0	15 (41.7)
	2+ → 0	4 (11.1)
	2+ → 1+	7 (19.4)
	2+ → 3+	1 (2.8)
	3+ → 2+	1 (2.8)
FISH	No change	21 (58.3)
	Amplification → nonamplification	15 (41.7)

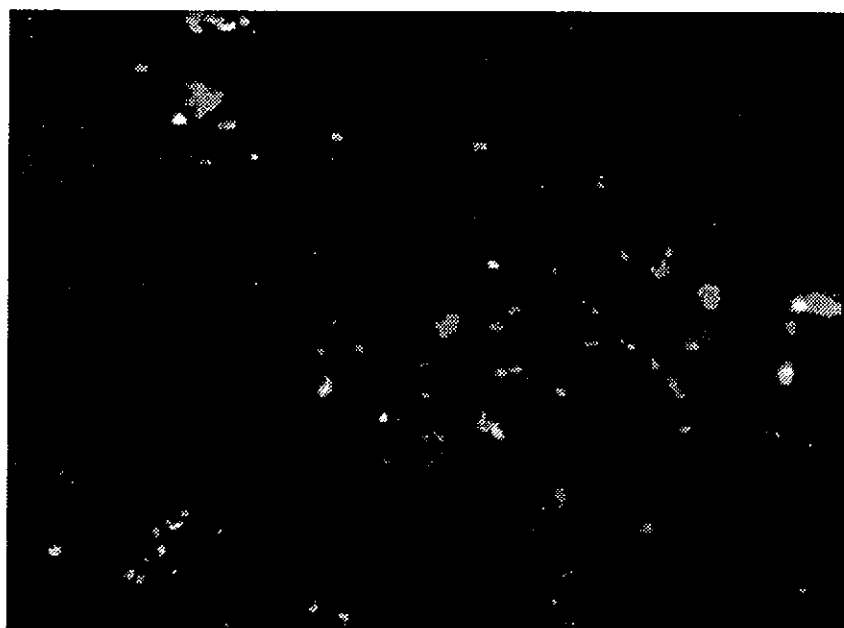[a] IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

Fig. 4 Her2/neu amplification determined by fluorescence *in situ* hybridization, ×400.

concordance of IHC and FISH results in our group (κ = 0.875), discrepancies are still known to occur because of the transcriptional or posttranscriptional regulation for increased surface receptor expression in the absence of gene amplification (32).

We have evaluated 36 primary breast cancers to determine whether Her2/neu status changes after the neoadjuvant endocrine therapy. Overexpression and amplification of Her2/neu was 44.4 and 55.6%, respectively. Our findings indicate a higher level of overexpression of Her2/neu in other groups with the primary diagnosis of breast cancers (1–4), which is possibly due

Table 2 Concordance of Her2/neu assay results: IHC *versus* FISH[a]

| IHC score | FISH (No. of patients) | | Total (no. of patients) |
	Nonamplified	Amplified	
0–1+	46	4	50
2+–3+	0	22	22
Total	46	26	72

Note. Kappa = 0.875.

[a] IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.
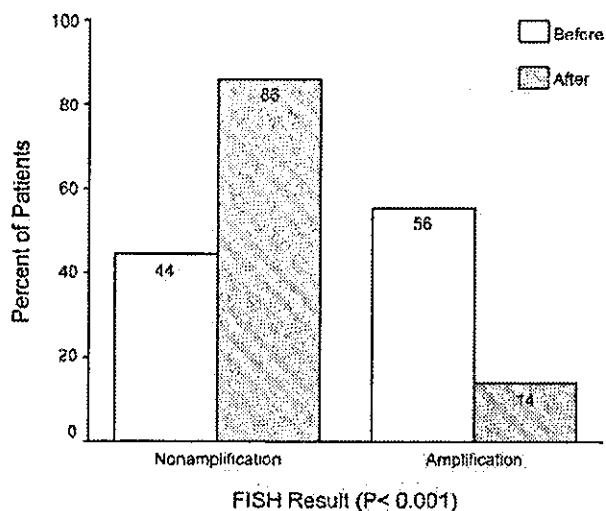

FISH Result (P< 0.001)

Fig. 5 The comparison of fluorescence *in situ* hybridization (FISH) results of Her2/neu status before and after neoadjuvant endocrine therapy. The amplification of Her2/neu are decreased significantly after the treatment (*P* < 0.001). □, before; ▨, after.

to the patients included in our group having developed more aggressive tumors (38, 39). Although we didn't find any significant correlation between Her2/neu positivity and clinicopathological factors, which might be due to the small size of our cohort, a statistically significant decrease in positivity for Her2/neu has been shown after neoadjuvant endocrine therapy.

To date, there have been few controlled studies of neoadjuvant endocrine therapy, especially for the use of the new selective third generation aromatase inhibitors. In appropriately selected patients, the Edinburgh group indicated that neoadjuvant endocrine therapy also produces significant responses compared with the preoperative chemotherapy (40). The overall response rates were 78–96%. The Duke group has shown that letrozole produces a superior response rate to tamoxifen (60 *versus* 48%), and the differences in response rates between letrozole and tamoxifen were most marked for Her2/neu (+) tumors (88 *versus* 21%), whereas Her2/neu (−) tumors did not show a statistically significantly higher response rate for letrozole compared with tamoxifen (54 *versus* 42%; Ref. 10). Although our study showed a slightly lower clinical response rate of 53%, we can confirm that Her2/neu (+) tumors show a significantly higher response rate than their Her2/neu (−)

Table 3 Clinical response according to initial and decrease of Her2/neu status

		No. of responders/Total	Response rate (%)	P
Initial Her2/neu status	(+)	12/16	75	0.017
(FISH)[a]	(−)	7/20	35	
Decrease of Her2/neu status	(+)	11/15	73	0.037
(before and after therapy)	(−)	8/21	38	

[a] FISH, fluorescence *in situ* hybridization.

counterparts. This remarkable finding suggests that the Her2/neu-activated second messenger pathway mediates estrogen-dependent growth through ER, presumably via ER phosphorylation. Preclinical modeling is consistent with the conclusion that ER (+) and Her2/neu (+) tumors are highly estrogen dependent (41). Additional data also indicate that ER-dependent transcriptional activity in a Her2/neu (+) breast cell line can be impeded by estrogen deprivation caused by aromatase inhibitors (42), which would suggest that a higher sensitivity to these agents might exist in breast cancer.

More interestingly, we found that the amplification of *Her2/neu* decreased in 41.7% of patients after the therapy by FISH. These results suggest that aromatase inhibitors might frequently repress the aggressive nature of breast cancer. The mechanism of aromatase inhibitor-induced Her2/neu down-regulation is unclear and extremely variable (43). A molecular explanation for these findings might be related to inactivation of signal transduction of Her2/neu through the mitogen-activated protein kinase pathway. Additional exploration of the molecular mechanism underlying this phenomenon may prove very useful in explaining and in controlling breast cancer progression in the future. Most importantly, we found that tumors, which show decreasing Her2/neu expression during the treatment, also show a significantly higher response rate than tumors, which show no change of Her2/neu expression. These remarkable observations suggest that positive Her2/neu status and a decrease in Her2/neu expression became significantly sensitive markers for the neo-adjuvant endocrine therapy based on aromatase inhibitors. Because it generally takes longer for endocrine therapy than chemotherapy to act, it seems essential to identify nonresponse early in the course of treatment so that the patient can be transferred to alternative therapies. Defining the best way to monitor response is therefore of fundamental importance. The determination of Her2/neu status by repeated biopsy during the therapy may be carried out in the future study. Nevertheless, our study has attempted to explore the role of a decrease in Her2/neu expression as a predictive marker for neoadjuvant endocrine therapy.

In conclusion, we have shown that Her2/neu gene amplification and protein expression decrease after neoadjuvant therapy using aromatase inhibitors. Despite the limited size of the cohort and immature survival data, our findings that both the positive Her2/neu expression and a decrease in Her2/neu expression have a predictive value with respect to the treatment could be clinically relevant.

ACKNOWLEDGMENTS

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IMMUNODETECTION AND QUANTIFICATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-3 IN HUMAN MALIGNANT TUMOR TISSUES

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Vascular endothelial growth factor recpetor-3 (VEGFR-3) and its ligands, vascular endothelial growth factor-C (VEGF-C) and –D (VEGF-D), are the major molecules involved in developmental and pathological lymphangiogenesis. Here we describe for the first time the development of a specific indirect enzyme-linked immunosorbent assay (ELISA) for the quantification of VEGFR-3 in different human cell and tissue lysates. A combination of the goat polyclonal anti-VEGFR-3 antibody and the mouse monoclonal anti-human VEGFR-3 antibody was used. The assay was highly sensitive and reproducible with a detection range of 0.2–25 ng/ml. The assay was specific for VEGFR-3, with no cross-reactivity to VEGFR-1 or VEGFR-2. Complex formation with VEGF-C and VEGF-D had no effect on the sensitivity of the assay. The VEGFR-3 concentration in the lysates of cultured human dermal microvascular endothelial cells was 14-fold higher than in the lysates from human umbilical vein endothelial cells. In human kidney, breast, colon, gastric and lung cancer tissues the protein levels of VEGFR-3 were in the range of 0.6–16.7 ng/mg protein. Importantly, the level of VEGFR-3 protein detected in the ELISA correlated significantly with the number of VEGFR-3 positive vessels observed in histochemical sections, suggesting that the ELISA assay may be a reliable surrogate of measuring VEGFR-3-positive vessel density. The protein levels of VEGFR-3 in 27 renal cell carcinoma samples had a significant correlation with the levels of VEGF-C ($p < 0.001$), or biological active, free VEGF-A ($p < 0.0001$), but not with VEGFR-1 or total VEGF-A. This assay provides a useful tool for the investigations of the expression levels of VEGFR-3 in physiological and pathological processes, particular in cancer and in lymphangiogenesis-related disease.
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Key words: *VEGFR-3; sandwich-ELISA; lymphangiogenesis; growth factors*

The development and maintenance of blood or lymph vessels is dependent on a number of molecular systems. A major group of molecules is represented by vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs). In man, there are 5 known members of VEGF family: VEGFs A–D and placenta growth factor (PlGF).[1] These factors interact with 3 membrane receptors, VEGFR-1 (Flt1), VEGFR-2 (KDR or flk-1 in mouse) and VEGFR-3 (Flt4), which belongs to the subfamily of receptor protein tyrosine kinase. They are characterized by the presence of 7 immunoglobulin-like domains in the extracellular domain and an intracellular domain with homology to the platelet-derived growth factor receptor (PDGFR) subfamily.[2] Two alternatively spliced isoforms of VEGFR-3 have been reported,[3] though in contrast to VEGFR-1, there is no evidence so far that a naturally occurring soluble receptor form exists for VEGFR-3.[4]

The growth of lymphatic vessels is called lymphangiogenesis and occurs after tissue injury, obstruction or damage of lymphatic vessels.[5] VEGFR-3 is a receptor for VEGF-C and -D.[1] VEGFR-3 has an essential role in the development of the embryonic cardiovascular system before the emergence of the lymphatic vessels,

and the embryonic lymphatic vessels sprout from the endothelial cells of the venous-sac-like structure, called the mesonephric vein and anterior cardinal vein, where VEGFR-3 is expressed.[6,7] In the adult, VEGFR-3 and its ligands are known to play key roles in the molecular regulation of lymphangiogenesis.[8] After binding to VEGF-C and VEGF-D, VEGFR-3 is capable of transducing signals triggering the proliferation of VEGFR-3-expressing cells *in vitro* and *in vivo*.[9–11] Blocking of VEGFR-3 activation inhibits the formation of lymphatic vessels in the developing embryo.[10]

VEGFR-3 and its ligands may play an important role in several pathological conditions where lymphangiogenesis occurs or the function of the lymphatics is involved. More recent studies suggest that patients with mutations in the VEGFR-3 gene develop lymphedema.[12,13] Currently there is little direct evidence of VEGF-C/VEGFR-3 in physiological or disease systems, especially in relation to progression or metastasis of carcinoma. However, in several experimental models VEGF-C overexpression enhances mestastatic spread to regional lymph nodes.[7]

Very recently, we have established a sandwich ELISA for the measurement of mature and partially processed VEGF-C protein in human tissue and in animal models.[14] However, such a reliable and sensitive assay for VEGFR-3 has not been available up to now. The aim of our work was therefore to develop a highly sensitive and reproducible assay format for the detection and quantification of VEGFR-3 in human cellular models and tissues. This goal was achieved by establishing a sandwich ELISA for VEGFR-3. VEGFR-3 amounts in the higher picogram range can be measured with our new ELISA, and we present here quantitative data for VEGFR-3 in relation to VEGF-C, VEGFR-1 and VEGF-A in human tissue samples for the first time.

MATERIAL AND METHODS

Antibodies and recombinant proteins

Mouse monoclonal anti-human VEGFR-3 antibody 9D9F9 was produced as described earlier.[15] Goat polyclonal anti-human VEGFR-3 antibody AF349 was purchased from R&D Systems (Abingdon, Oxfordshire, U.K.). A polyclonal anti-human VEGFR-3 antibody 5149 against recombinant VEGFR-3 was developed in rabbits. The procedure was similar to that described previously for the polyclonal sVEGFR-2 antibody.[16] Briefly, a total amount of 1.2 mg soluble VEGFR-3 protein, comprising the whole extracellular domain[17] (amino acid 26 to 725) purified from baculovirus infected insect cells, was used for immunization of 2 New Zealand white rabbits. Total IgG from rabbit serum was isolated using HiTrap Protein-A Sepharose columns (Amersham Bioscience, Freiburg) and then antigen-affinity purification was performed by 0.5 mg recombinant soluble VEGFR-3 on a NHS-activated HiTrap column (Amersham Bioscience, Freiburg). Mouse monoclonal anti-soluble VEGFR-1 antibody Flt-11 and anti-soluble VEGFR-2 antibody KDR-2 was produced as described.[18] The recombinant human proteins VEGFR-1, VEGFR-2, VEGF-C and VEGF-D were produced in insect cells and purified from supernatants as previously described.[19,20] Recombinant VEGFR-3 was produced and purified very similarly to the procedure described.[6]

Cell lines, tissues and sample preparation

Primary human dermal microvascular endothelial cells (HDMEC; Bio Whittaker, MD) and primary human umbilical vein endothelial cells (HUVEC; Bio Whittaker, MD) were cultured in EBM medium (Bio Whittaker, MD) containing 5% FCS and growth factor supplements according to the manufacturer's instructions. Both cell types were grown at 37°C in a humidified incubator with 5% CO_2. The cells were grown to 80% confluence in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark).

Tissue samples from different human tumors were from Tokyo Metropolitan Komagome Hospital, the Department of Pathology at the University of Kiel, or from the Department of Pathology at DKFZ, Heidelberg. All Patients signed an informed consent according to a protocol approved by the ethics committee of the institute. Tumor tissues were snap frozen in liquid nitrogen. Tissue samples or cultured cells were homogenized in RIPA buffer (0.1% SDS, 1% TRITON X100, 0.5% Na-deoxycholate, protease-inhibitor cocktail in phosphate-buffered saline). Protein concentrations were estimated according to standard protocols (BCA assay, Perbio, Rockford).

Immunoprecipitation

To reveal the VEGFR-3 expression in the samples, immunoprecipitation and immunoblotting were performed using HUVEC and HDMEC. Four micrograms of rabbit-anti-human VEGFR-3 antibody 5149 or mouse monoclonal antibody 9D9F9 was added to cell lysates containing 1 mg of protein and incubated overnight at 4°C. Following addition of 10–20 µl protein A-agarose or protein G-sepharose (Sigma-Aldrich, St. Louis, MO), incubation continued for 4 hr at 4°C. Immunoprecipitates were isolated by centrifugation and washed twice with RIPA buffer. The supernatants were discarded and reducing sample buffer was added to the tube and boiled for 10 min followed by cooling on ice.

Immunoblotting

All samples were loaded onto a 5 or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and separated under reducing conditions. Immunoblotting onto a PVDF membrane (Millipore, Bedford, MA) was performed for 20 to 60 min at 15 V in a semi-dry blotting chamber (Biorad, Munich, Germany). The membrane was saturated with 20% nonfat milk in TBS (20 nM Tris-HCl and 150 mM NaCl, pH 8.0) for 1 hr at room temperature. Monoclonal antibody 9D9F9 (0.5 µg/ml), polyclonal antibody against human VEGFR-3 (0.1 µg/ml), KDR-2 (1 µg/ml) or Flt-11 (1 µg/ml) was applied in TBS containing 10% nonfat milk for 1 hr at room temperature. The secondary antibody and the ECL detection kit were used according to the manufacturer's method (Amersham Bioscience, Freiburg).

VEGFR-3 Sandwich-ELISA

The development of a highly sensitive and specific sandwich ELISA for VEGFR-3 was achieved by using standard methods.[21] The detection antibody used was mouse monoclonal anti-human VEGFR-3 antibody 9D9F9. A standard curve was created using the recombinant VEGFR-3 protein containing only the extracellular domain of the receptor (IgG domain 1–7). The procedure was as follows: 96-well microtiter plates were coated with 50 µl/well of goat anti-human VEGFR-3 antibody diluted to a final concentration of 1 µg/ml in 100 mM $NaHCO_3$ (pH 8.0). The coated plates were incubated at 4°C overnight then blocked with 200 µl/well of 1% BSA, 25% of low fat milk in PBS and 0.1% Tween 20 (Sigma-Aldrich; PBS-T) for 2 hr at room temperature. Dilution buffer was 75% PBS-T with 1% BSA and 25% diluent MOD-U-CYTE IV (Serological Proteins, Inc., Kankakee, IL). One hundred microliters of test samples, diluted 1:2 or 1:4 in dilution buffer, were added to the plates in duplicate. Recombinant VEGFR-3 was diluted serially to generate a standard curve with concentrations ranging from 25 to 0.2 ng/ml. Fifty microliters of mouse monoclonal anti-VEGFR-3 antibody 9D9F9 diluted to a final concentration of 0.16 µg/ml was added to each well. After 2 hr incubation at room temperature, amplification of signal was achieved by the addition of 100 µl/well of biotinylated goat-anti-mouse IgG (Dianova, Hamburg, Germany) at 1:20,000 dilution in dilution buffer and incubated with shaking at room temperature for 1 hr. For visualization of the detector, streptavidin-enzyme conjugate was used (Endogen, Woburn, MA) followed by the addition of TMB (tetra-methyl-benzidine; Roche Mannheim, Germany). After stopping the reaction with 1 M H_2SO_4, the absorbance was measured at 450 and 620 nm with an ELISA plate reader (Labsystems, Finland). Each tumor sample has been split in 2 parts and used for both, ELISA measurements and for immunohistochemistry (see below). From the tumor material homogenized (2–3 g) only 150–250 µg protein was used for ELISA. Generally, the tumor and other samples were analyzed in different dilutions, measuring each dilution in duplicates.

Intra- and inter-assay variation and specificity of the ELISA system.

To evaluate the reproducibility of the assay, the intra-assay variation was analyzed using repetitive measurement of the cell lysates from HUVEC and HDMEC or tumor lysates on a single plate. The inter-assay variation was also measured using the same samples across 8 separate experiments. The specificity of the system for detection of VEGFR-3 was determined by the substitution of recombinant VEGFR-3 protein for known concentrations of recombinant soluble VEGFR-1 and VEGFR-2 protein to assess for any evidence of cross-reactivity. The intra-and inter-assay coefficients of variation (CV) were calculated as CV= (standard deviation/mean) × 100.

Measurement of total-VEGF-A, free-VEGF-A, VEGF-C and VEGFR-1

The protein levels of the other VEGF family members, total-VEGF-A, free-VEGF-A, VEGF-C and VEGFR-1 in the tumor extracts were measured by ELISA. All samples were lysed with RIPA buffer. The measurements were performed with standard methods.[21] Briefly, total-VEGF-A concentrations were measured by a colorimetric sandwich ELISA using a polyclonal, antigen-affinity purified antibody to human recombinant VEGF165 as capture and biotinylated detector antibody. The human recombinant VEGF165 isoform was used as a standard between 1.25 and 0.02 ng/ml by serial dilutions. Free, bioactive and uncomplexed VEGF-A was measured with a ligand receptor binding assay (BIOLISA, BenderMedSystems, Vienna). The ELISA plate coated with recombinant soluble VEGFR-1 D1-6 protein and bound mol-

ecules were detected with polyclonal antigen-affinity purified anti-human VEGF antibody. VEGF-C measurement was performed using our recently developed ELISA method.[14] The VEGFR-1 sandwich immunoassay used 1 monoclonal anti-human VEGFR-1 antibody and 1 polyclonal antibody.[22,23]

Immunohistochemistry

Freshly frozen 27 renal cell carcinoma tissue samples were obtained from the DKFZ, Heidelberg. The samples consisted of clear cell carcinoma ($n = 25$) and chromophilic carcinoma ($n = 2$). Seven of the clear cell carcinoma were well differentiated, 15 were moderately and 2 were poorly differentiated. One of the chromophilic carcinoma was well differentiated, and the another was poorly differentiated. All samples (approximately 2–3 g tumor material) had been frozen immediately after surgical excision in liquid nitrogen and stored at −70°C. Mouse monoclonal antibodies 9D9F9 against human VEGFR-3 were used for staining. CD31 staining was performed according to the protocol of the manufacturer (BD Bioscience, San Diego, CA). Five micrometer cryosections of the tissues were air-dried and fixed in cold acetone for 10 min. The sections were rehydrated in phosphate-buffered saline (PBS) and incubated for 30 min in 5% normal goat serum at room temperature. The sections were then incubated for 2 hr in a humid atmosphere at room temperature with the 9D9F9 at a concentration of 1.0 μg/ml. A subsequent incubation for 30 min in biotinylated anti-mouse serum was followed by a 60 min incubation using reagents of the Vectastain Elite Mouse IgG ABC kit (Vector Laboratories, Burlingame, CA). Peroxidase activity was developed with DAB substrate kit (DAKO Laboratories, Carpinteria, CA) until the desired color intensity was reached. Finally, the sections were stained with hematoxylin for 20 sec. The densities of VEGFR-3 positive vessels were calculated according to the method described by Gasparini and Harris.[24] The vessel density was counted per a ×400-magnification high-power field (hpf) in the areas with the highest vessel density. A minimum of 5 fields was counted per slide, after which the 3 highest counts were averaged.

Statistical analysis

All statistical calculations were carried out using StatView statistical software, version 5.0 (SAS Institute, Inc., Cary, NC). Unless specified, the data were expressed as median and range. The data were not parametrically distributed, consequently the significance of differences between groups was calculated by applying nonparametric tests. The Mann-Whitney U tests were used to analyze the difference of VEGFR-3 concentration between different types of tumor. The correlation between 2 factors was calculated using the Spearman's correlation coefficient by rank. Differences at $p<0.05$ were considered to be statistically significant.

RESULTS

Characterization of the anti-VEGFR-3 antibodies

The first approaches towards the development a VEGFR-3 ELISA were to search for the best combination of VEGFR-3 antibodies. Initial experiments showed that a goat polyclonal anti-human VEGFR-3 antibody from a commercially available source and a mouse monoclonal antibody were the best pair. Both of the antibodies are produced against extracellular domain of human VEGFR-3 protein. The monoclonal antibody is species specific. Western blot analysis with recombinant human VEGFR-3, VEGFR-1 and VEGFR-2 proteins revealed that both the monoclonal and polyclonal antibodies bind strongly to VEGFR-3 (Fig. 1). There was no cross-reactivity with human VEGFR-1 or VEGFR-2 protein (Fig. 1), the most closely related proteins to VEGFR-3.

Sensitivity, cross-reactivity and reproducibility of the sandwich ELISA for VEGFR-3

For the capture antibody, the polyclonal goat antibody was used and for detection, the monoclonal antibody 9D9F9 was used. This

combination resulted in the most sensitive results. A typical standard curve obtained with the ELISA is shown in Figure 2. The minimum detection limit estimated by serial dilution was about 0.2 ng/ml recombinant human VEGFR-3. Recombinant human
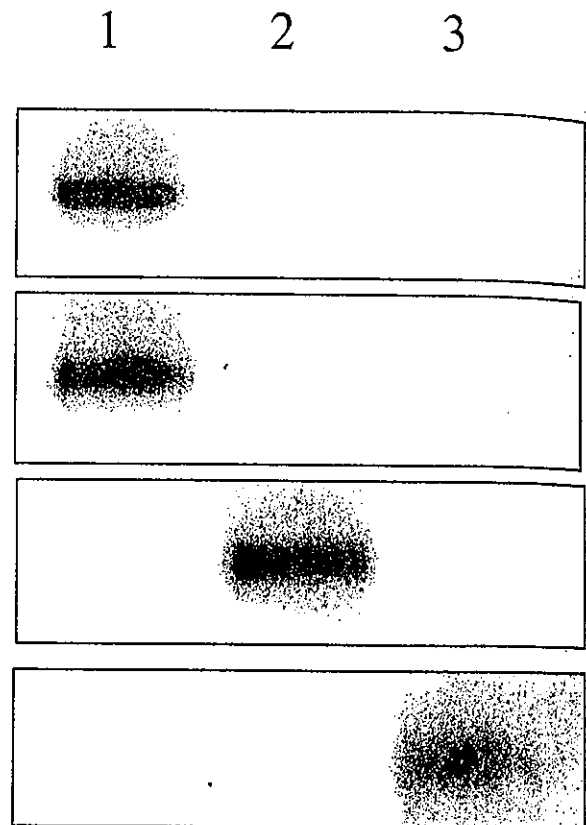

FIGURE 1 – Immunoblot with human VEGFR-3, VEGFR-2 and VEGFR-1 using the polyclonal anti-VEGFR3 antibody AF 349 (a), the mouse anti-VEGFR-3 monoclonal antibody 9D9F9 (b), the mouse anti-Flt1 antibody Flt11 (c) and the anti-KDR antibody KDR2 (d). Lane 1: 100 ng His-tagged human recombinant VEGFR-3; lane 2: 100 ng human VEGFR-1 and lane 3: 100 ng human VEGFR-2. Proteins were separated by 12% SDS-PAGE and blotted onto PVDF membranes as described. After saturation, the membrane was incubated for 1 hr with each antibody at 1–2 μg/ml in TBS containing 10% low-fat milk, followed by incubation with a alkaline horse radish peroxidase-conjugated secondary antibody.
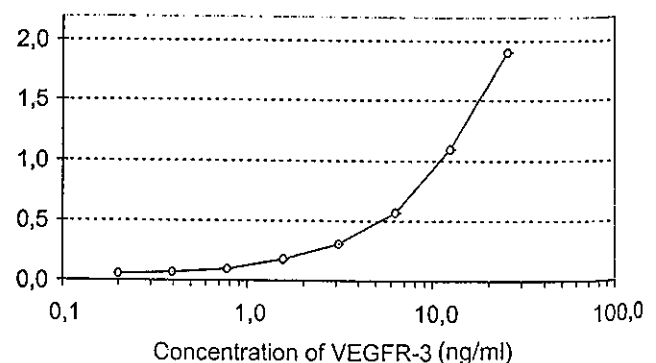

FIGURE 2 – Standard curve of VEGFR-3 concentration. Recombinant human soluble VEGFR-3 with His-tagged protein was serially diluted to each concentration from 25 ng/ml to 0.2 ng/ml, and the curve was generated.

VEGFR-1 or VEGFR-2 molecules were not recognized by this assay even at a very high concentration such as 250 ng/ml (data not shown).

In order to exclude interactions between VEGF-C or VEGF-D/ VEGFR-3 and VEGFR3/antibody binding, an increasing amount of the recombinant VEGF-C or VEGF-D protein was added to known concentrations of recombinant VEGFR-3- containing test samples. As demonstrated in Figure 3, an 18-fold molar excess of VEGF-C or -D protein did not interfere with the binding of 40 ng/ml VEGFR-3 to the capture antibody. This indicates that the capture antibody can detect receptor-ligand-complexed and free VEGFR-3 equally well (Fig. 3).

Intra-and inter-assay coefficients of variation

The precision of the assay was determined by calculating the intra-and inter-assay coefficients of variation for the ELISA system. The intra-and inter-assay coefficients of variation (CV) are obtained by measuring the variation within an individual assay and between different assays respectively. Four different samples, cell lysates from HUVEC and HDMEC, and renal cell carcinoma tissue lysates from 2 different cases were used. Typically, the intra-and inter-assay coefficients of variation for an ELISA should be less than 5% and 10%.[25] The intra-assay coefficients of variation were calculated from the results of 12 measurements from single plates, whereas the inter-assay coefficients of variation were calculated from the results of 8 individual assays for each ELISA developed. Intra-and inter-assay coefficients for the ELISA system are displayed in Table I. The intra-assay coefficients of variation

were below 4% and the inter-assay coefficient of variation was below 9%.

Expression of VEGFR-3 in vascular cell types

Only limited information has been published about the kinetics and the amounts of VEGFR-3 produced in human cells or in tumor tissues. HUVEC and HDMEC were used as a positive control.[26] HDMECs are commonly used as a source for the isolation of primary lymphatic endothelial cells.[27] In the ELISA measurement, the protein level of VEGFR-3 in HDMEC was more than 10-fold higher than in HUVEC. (Table I)

Immunoprecipitation followed by immunoblotting was applied to lysates of HUVEC and HDMEC cells. The combination of 9D9F9 or rabbit polyclonal antibody 5149 and goat polyclonal anti-VEGFR-3 antibody was used for immunoprecipitation and immunoblotting, respectively. The results of these experiments are shown in Figure 4 and demonstrate that the combination of 9D9F9 and goat polyclonal anti-VEGFR-3 antibody allows robust detection of VEGFR-3 protein expression and correspond with the protein concentration measured with the ELISA. The bands seen at molecular weight at 195 kDa and 125 kDa represent the uncleaved and proteolytically cleaved forms of VEGFR-3.[28] The band at 175 kDa is considered to be intracellular, unglycosylated precursor.

VEGFR-3 positive vessels in renal cell carcinoma

The mouse monoclonal antibody 9D9F9 against human VEGFR-3 extracellular domain was used to stain the renal cell carcinoma tissues. Strongly stained VEGFR-3-positive vessels were present in all renal cell carcinoma tissues studied (Fig. 5). Most of the VEGFR-3-positive small vessels were also positive for CD31 staining (Fig. 5b,c, arrowheads). On the other hand, larger vessels were generally negative for VEGFR-3 and positive for CD31 staining (Fig. 5b,c, arrows). The intratumoral
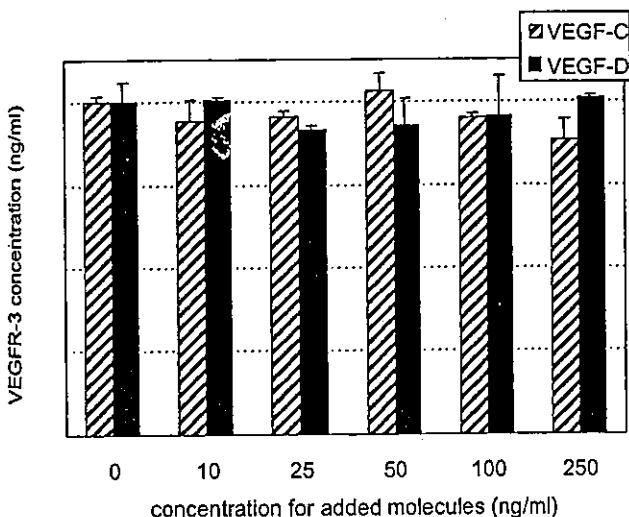

FIGURE 3 – The assay system detects VEGFR-3 independenly of receptor-ligand complex formation. VEGFR-3 (40 ng/ml) was added to each well that was coated with polyclonal antibody against VEGFR-3 in presence or absence of increasing concentrations of VEGF-C (stripe bars) and VEGF-D (gray bars). Monoclonal antibody 9D9F9 was used to detect and quantify VEGFR-3. All measurements were performed in duplicate.
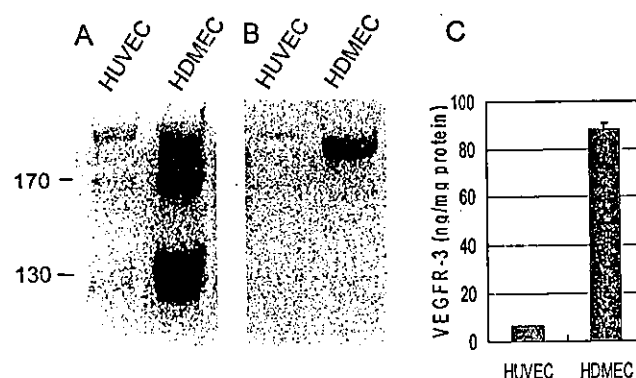

FIGURE 4 – Western analysis and quantification of VEGFR-3 protein expression by cultured HUVEC and HDMEC. (*a,b*) The equal amount (1 mg protein) of homogenized protein was immunoprecipitated with the mouse monoclonal anti-VEGFR-3 antibody 9D9F9 (*a*) or rabbit polyclonal anti-VEGFR-3 antibody 5149 (*b*) followed by gel electrophoresis and Western blotting with goat polyclonal anti-VEGFR-3 antibody. (*c*) Quantification of VEGFR-3 protein level of HUVEC and HDMEC lysates by ELISA.

TABLE I – INTRA- AND INTER-ASSAY COEFFICIENTS FOR VEGFR-3 ELISA[1]

	HUVEC	HDMEC	Kidney tumor-1	Kidney tumor-2
Intra-assay (*n* = 12)				
Mean ± SD (ng/mg protein)	6.281 ± 0.210	87.970 ± 2.763	2.169 ± 0.060	2.826 ± 0.077
CV(%)	3.33	3.14	2.759	2.715
Inter-assay (*n* = 8)				
Mean ± SD (ng/mg protein)	6.406 ± 0.337	86.400 ± 4.500	2.288 ± 0.198	2.914 ± 0.250
CV(%)	5.27	5.21	8.66	8.6

[1]*n* = 12 and *n* = 10 for the inter- and intra-assay coefficients of validation (CV), respectively. HUVEC, human umbilical vessel endocelial cells; HDMEC, Human dermal microvascular endothelial cells.
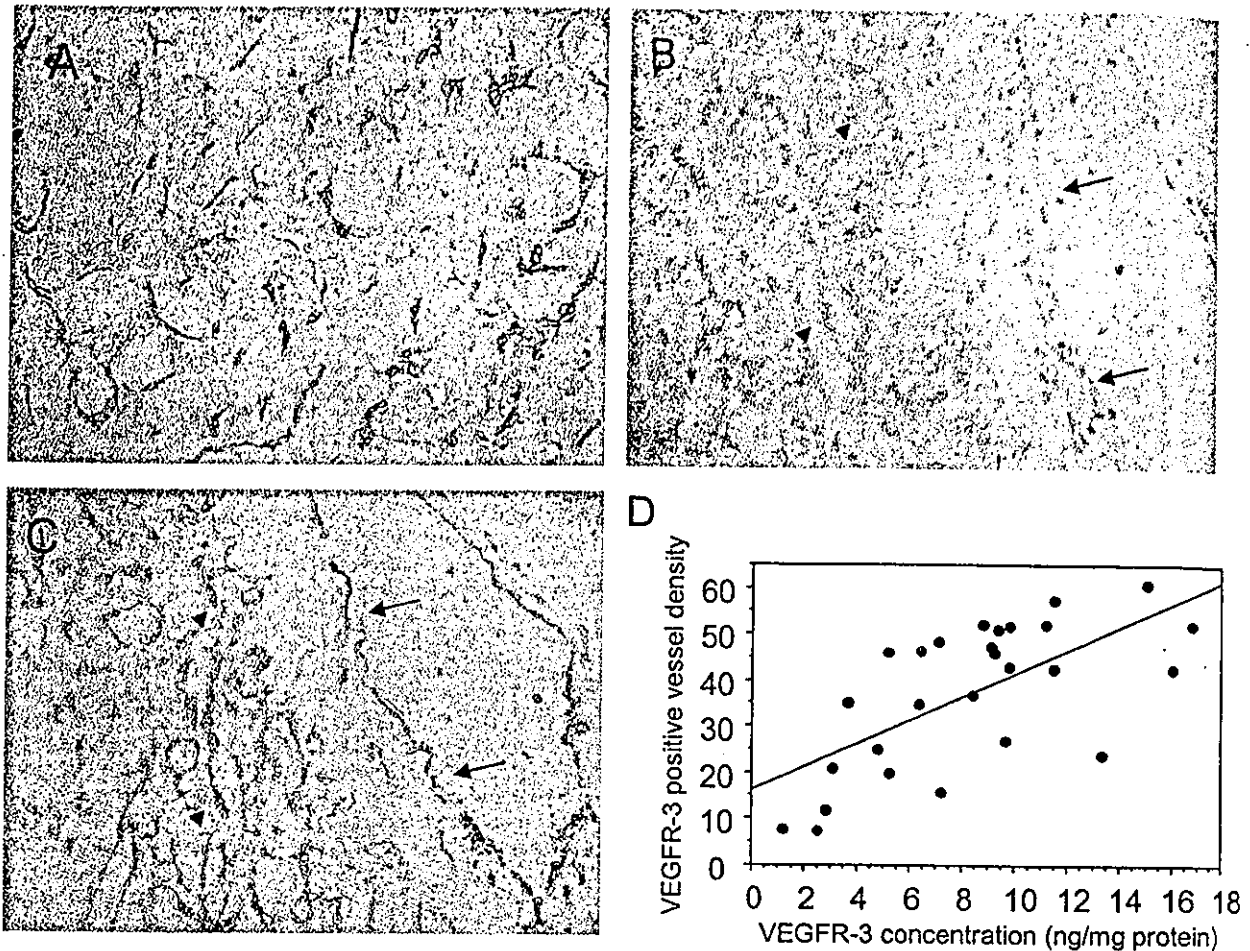
Figure 5 – Immunostaining for VEGFR-3 ($a+b$) and CD31 (c) in kidney tumor tissue sections. (a) A case with high count of VEGFR-3-positive microvessels in the stroma. ×300. (b,c) A set of sections was compared to staining of VEGFR-3 (b) and CD31 (c). Colocalization of VEGFR-3 with CD31 was found in capillaries in the stroma of the tumor tissues arrowheads. On the other hand, in large vessels, VEGFR-3 staining was mainly negative (arrows). ×200. (d) The VEGFR-3 positive microvessel count against the concentration of VEGFR-3 measured by ELISA in a simple scatterplot. A regression line is shown to illustrate the relationship.

VEGFR-3 positive vessel density (median 38, range 7 to 61 vessels pre hpf; $n = 27$) was significantly correlated with measured protein levels of VEGFR-3 by ELISA. ($p < 0.001$, Spearman's Rank Correlation test). Immunostaing for tumor cells were not detected. However, the observed correlation must be seen under the limitation that only a very limited number of sections have been used for vessel counting, which may not be representative of a tumor. Furthermore, vessel distribution in a tumor is not uniform and the highest vessel count will not necessarily correlate with the VEGFR-3 protein amount measured by ELISA.

*Correlation Analysis between VEGF family member factor in renal cell carcinoma tissues*

Protein levels of VEGF family members, total VEGF-A, free VEGF-A and VEGFR-1, were measured in 27 renal cell carcinoma samples by enzyme linked assay using the same tissue extracts. Spearman's nonparametric rank correlation tests showed that correlations exist between the expression of VEGFR-3 and the other VEGF family members (Fig. 6). Expression of VEGFR-3 had a significant positive correlation with expression of VEGF-C and free-VEGF-A ($p = 0.0005$ and $p = 0.0002$, respectively). On the other hand, the expression of total-VEGF-A as well as VEGFR-1 are not correlated with that of VEGFR-3.

Detection and quantification of VEGFR-3 from different human tumor tissues and correlation to VEGF-C data by using different detection methods

After the discovery of the VEGFR-3 ligand VEGF-C, several studies reported the expression of these genes or proteins in tissues and organs of human or murine origin.[6,29-31] All these studies used PCR, Northern blot analysis or immunohistochemistry for detection of VEGFR-3. Our optimized assay was employed to measure the levels of VEGFR-3 in tumor tissues from different source. The results shown in Figure 7 demonstrate that the levels of VEGFR-3 were significantly elevated in renal cell cancer tissues, 8.1 ng/mg protein (1.4–16.7 ng/mg protein, $n = 27$), compared to breast cancer tissues, 1.5 ng/mg protein (0.6–2.6, $n = 18$), colon cancer tissues, 1.3 ng/mg protein (0.7–1.9, $n = 10$), lung cancer tissues, 2.1 ng/mg protein (1.5–3.0, $n = 10$) and gastric cancer tissues 1.0 ng/mg protein (0.7–1.4, $n = 10$) ($p < 0.0001$, Mann-Whitney U-test). Among several kinds of tumors, renal cell carcinoma cells exhibit the highest expression level of VEGFR-3 protein, followed by lung cancer tissues. Gastric cancer tissues contained the lowest amount of VEGFR-3 protein ($p < 0.05$).

DISCUSSION

The present study describes for the first time the establishment of a quantitative sandwich ELISA human VEGFR-3. During the
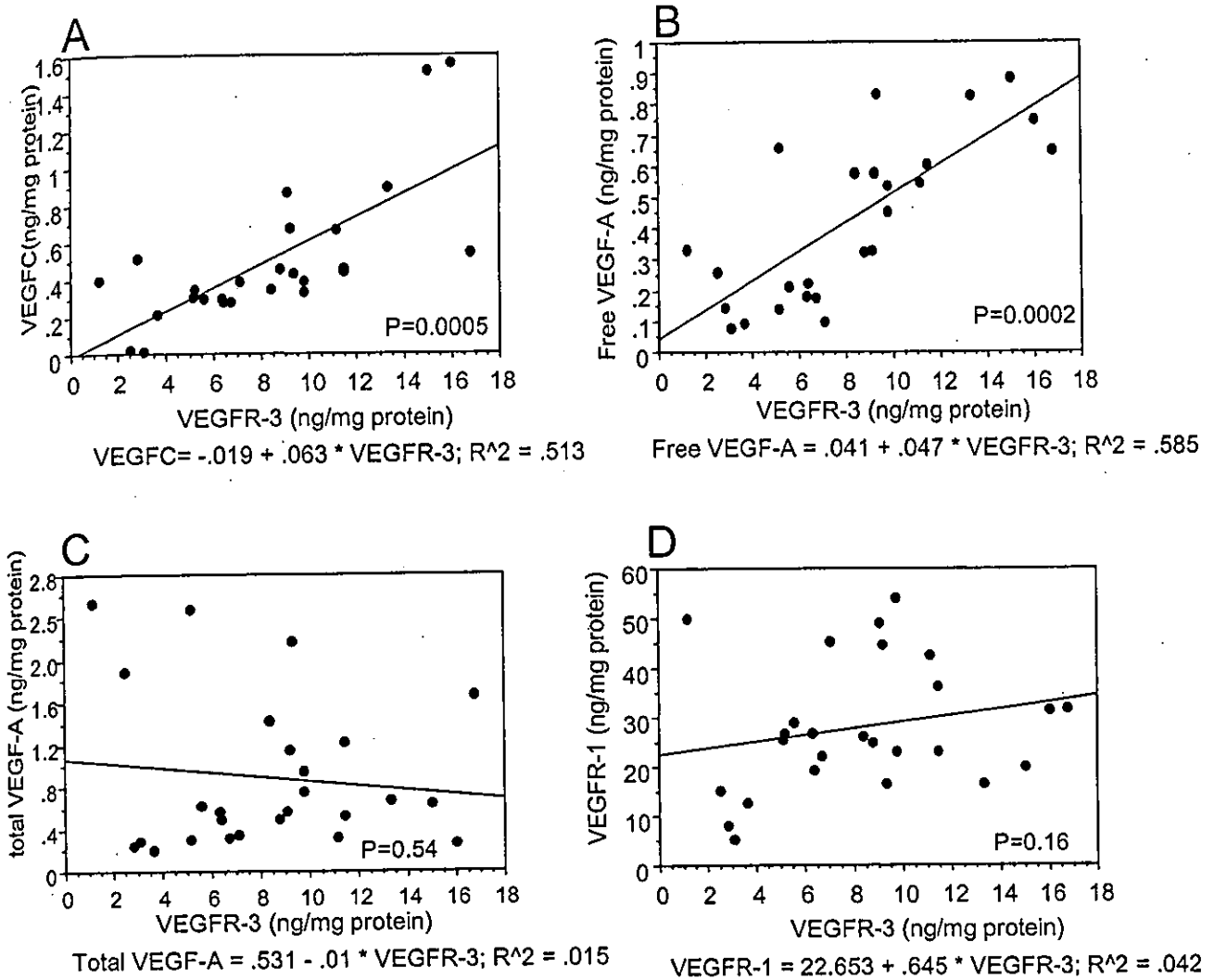
FIGURE 6 – Correlation analysis of VEGFR-3 protein levels with its related proteins VEGF-C (a), free VEGF-A (b), total VEGF-A (c) and VEGFR-1 (d). The protein levels of VEGFR-1, VEGFR-3, free VEGF-A, total VEGF-A and VEGF-C in kidney tumor extracts were determined by ELISA as described. VEGFR-3 expression significantly correlated those of VEGF-C(p = 0.0005) and free VEGF-A(p = 0.0002) but not with VEGFR-1 nor total VEGF-A (Spearman's correlation coefficient by rank).

development of this ELISA, several polyclonal or monoclonal antibodies for VEGFR-3 were produced, and the most sensitive and specific combination was the monoclonal antibody 9D9F9 together with a goat polyclonal anti-VEGFR-3 antibody. Intra-and inter-assay variations were minimal and the good reproducibility of the assay was achieved. Immunoprecipitation and immunoblotting analysis confirmed the presence and detection of VEGFR-3 in samples using the same 2 antibodies as used in the ELISA system. Significantly, the ELISA measurements for VEGFR-3 protein levels significantly correlated with VEGFR-3-positive vessel density in immunohistochemical sections, suggesting that the ELISA assay can be used as a convenient surrogate measure of the number of VEGFR-3-positive cells in biological samples.

VEGFR-3 has been reported to be expressed on a variety of cell types. The major site of expression in the adult organism is in the lymphatics, and it has been employed as a marker for lymphatic vessels in normal and pathological tissue samples[32] and has been used to demonstrate an apparent lymphatic origin of Kaposi's sarcoma cells.[15] VEGFR-3 is one of several markers for lymphatic endothelial cells. Others include LYVE1, podoplanin and prox-1.[33] In addition to being expressed on lymphatic endothelial cells, VEGFR-3 has also been detected on the cells of the hematopoietic

system, reflecting a common stem cell origin for hematopoietic and endothelial cells.[34] More recent data show the VEGFR-3 can also be expressed on blood vessel endothelium, for example, in fenestrated capillaries.[35] We and others also found that VEGFR-3 is expressed and upregulated on blood vessels during fetal development, in tumors and in chronic wounds, together with the corresponding ligand VEGF-C.[36,37] VEGFR-3 can also be weakly expressed in the capillary endothelium of normal breast tissue as well. Thus, when measuring VEGFR-3 levels in tissues, possible contributions from cell types in addition to lymphatic endothelial cells should be taken into consideration.

VEGFR-3 is not only an important lymphatic marker but is also a functional trigger and signaling molecule for angiogenesis, lymphangiogenesis and regional metastasis. Thus, blocking of VEGFR-3 signaling by receptor bodies suppressed tumor-induced lymphangiogenesis and regional lymph node metastasis in animal lung and breast cancer models.[38,39] A considerable body of literature suggests that VEGF-C and VEGF-D expression in human tumors correlates with metastasis to regional lymph nodes and poor prognosis (reviewed in reference 40). For example, in a study with gastric cancer samples, it could be demonstrated that VEGFR-3 expression is localized on endothelial cells of lymphatic
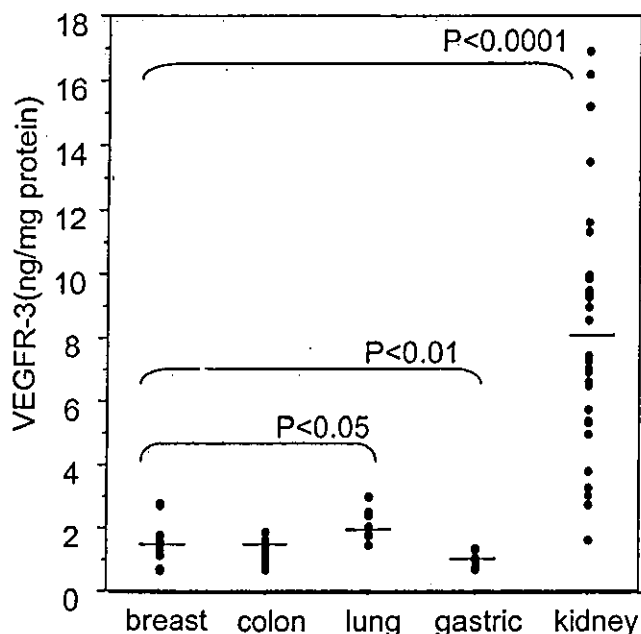
FIGURE 7 – Protein levels of VEGFR-3 in various cancer tissues measured by ELISA. Significant differences were found between kidney tumors ($n = 27$) and all the other kinds of cancer groups ($p<0.001$), between gastric cancer ($n = 10$) and the other groups ($p<0.01$), and between lung cancer ($n = 9$) and the other groups ($p < 0.05$), but not between breast ($n = 18$) and colon ($n = 10$) cancer (Mann-Whitney U test).

vessels and that VEGF-C secreted from cancer cells may directly induce the proliferation of lymphatic vessels in the stroma of primary gastric cancer; similar mechanisms have been proposed in thyroid tissue from patients with auto-immune disease.[41,42] Several reports have reported elevated levels of VEGFR-3 expression in tumors.[41,43,44] Nevertheless, a lack of correlation between density of a variety of lymphatic markers and lymphatic metastasis in cancer patients has also been reported.[45] The ELISA we report here should help to resolve these conflicting findings, especially as with the exception of a few studies using antibodies to detect VEGFR-3 by immunohistochemistry, most investigations used RT-PCR or Northern blot techniques. However, several limitations of protein expression analysis, like the spatial resolution in tissue samples, highest sensitivity of protein detection, expression of VEGFR-3 also on blood vessels cannot be resolved by the ELISA technique alone and need further investigations by using different techniques and model systems. A VEGF-C/VEGFR-3 autocrine loop could be verified in a subset of acute leukemias to promote

survival and proliferation of these cells.[46] The VEGF-C and VEGFR-3 ELISAs that we have developed could be useful for screening for the subset of leukemia patients with this autocrine loop.

Our first, though limited ELISA study of VEGFR-3 levels in breast, colon, gastric, lung and kidney tumor samples indicated that VEGFR-3 concentration varies among the different tumor types and is higher in kidney and in lung tumor than the other cancers. In some of our earlier studies with normal kidney tissue, we reported that VEGFR-1 and VEGFR-2 are both upregulated and, together with the abundant detectable VEGF-A, the function in the adult kidney may be independent of angiogenesis.[18] The upregulation of the receptors may be a consequence of microvascular permeability or seen in context with the fenestrated endothelium. Therefore we cannot exclude the possibility that the elevated VEGFR-3 concentration we measured in the tumor kidney samples may reflect endogenous constitutive expression in the adult kidney and less upregulation due to the microenviroment of the tumor.

In our immunohistochemical study, colocalization of CD31 with VEGFR-3 was found in capillaries but in most cases, VEGFR-3 staining was absent or very faint in large vessels. The result of the experiment was in good agreement with the report from Witmer *et al.*[31] that showed that in normal kidney tissues, VEGFR-1 is present in glomerular or other capillaries, venule or veins, and in basement membrane in tubuli, while VEGFR-3 was present on capillaries and in lymphatics but not on venules, veins or basement membrane of tubuli.

In kidney tumors, VEGFR-3 expression was compared to that of the other members of the VEGF family. The concentration of VEGFR-3 was significantly correlated with its ligand VEGF-C, as expected. Surprisingly, a stronger correlation was detected with free VEGF-A, which is not reported to be a ligand for VEGFR-3. The physiological significance of this unexpected finding is not clear at the moment, but it is also evident from other studies that VEGF-A and VEGFR-2 are often upregulated in tumor tissues, probably by different underlying mechanisms.[47,48] It can be speculated that VEGFR-2 and VEGFR-3 may be regulated by similar mechanism in tumors independent by hypoxia. It needs still to be discussed in which way VEGFR-3 can contribute to tumor lymphangiogenesis, angiogenesis, tumor progression or metastasis. To investigate the clinical significance of VEGFR-3 in tumor biology, we believe that our new ELISA for VEGFR-3 measurements will be very useful.

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Clinical Trial Note

Application of a Continual Reassessment Method to a Phase I Clinical Trial of Capecitabine in Combination with Cyclophosphamide and Epirubicin (CEX) for Inoperable or Recurrent Breast Cancer

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A phase I clinical trial was started in order to determine the recommended doses of capecitabine and epirubicin, when administered in combination with a fixed dose of cyclophosphamide (600 mg/m^2 day 1 q3 weeks) in patients with inoperable or recurrent breast cancer. This study consists of five dose levels with combinations of three levels of epirubicin (75, 90 and 100 mg/m^2 day 1 q3 weeks) and three levels of capecitabine (1255, 1657 and 1800 mg/m^2/day consecutive administration for 2 weeks followed by 1 week of rest). Dose escalation and de-escalation decisions are based on a continual reassessment method (CRM). We conducted a survey of the clinical oncologists participating in this trial to determine the dose escalation/ de-escalation rule, including a prior distribution for model parameters used in the CRM.

Key words: capecitabine – continual reassessment method – breast cancer – phase I trial

INTRODUCTION

Infusional 5-fluorouracil (5-FU) produces remarkable antitumor effects in breast cancer and has been widely used in combination chemotherapy with cyclophosphamide and adriamycin (CAF, C: cyclophosphamide, A: adriamycin, F: 5-FU) as a gold standard (1). However, the accumulation of adriamycin causes serious cardiotoxic effects. Thus, a phase I clinical trial of CEF chemotherapy in which adriamycin was replaced with epirubicin, was conducted to determine the recommended doses (RD) (2). Furthermore, infusional 5-FU has not been favored due to its cumbersome mode of administration by drip infusion via a peripheral venous infusion line. Thus, a research team from the European Organization for Research and Treatment of Cancer (EORTC) conducted a phase I clinical trial of the CEX regimen, a combination chemotherapy in which 5-FU was replaced by capecitabine (3).

Capecitabine is a novel oral fluoropyrimidine derivative known to be tolerated at high doses. It is specifically designed to be selectively converted to 5-FU at the tumor lesion through a three-step enzymatic metabolic process, following oral administration (4). Capecitabine monotherapy demonstrated high antitumor activities against metastatic breast cancer (5,6).

Although a phase I study of CEX chemotherapy was conducted by the EORTC research team, a concern was raised regarding the possible differences in the RD of CEX between Caucasians and Japanese. In order to resolve this concern, we decided to conduct a clinical trial to determine the RD for CEX treatment, i.e., combination chemotherapy of epirubicin and capecitabine with a fixed dose of cyclophosphamide, in Japanese breast cancer patients. Assessment of dose escalation and de-escalation of dosage is based on a continual reassessment method (CRM) (7,8). In order to obtain prior information for CRM, more specifically, the reference information used to determine the escalation/de-escalation rule regarding the initial dose and dosage, we conducted a survey of the participating clinical oncologists while setting up the study protocol.

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PROTOCOL DIGEST OF THE STUDY

PURPOSE

To determine the maximum tolerated dose (MTD) and recommended dose (RD) for a future phase II trial of combination therapy of capecitabine and epirubicin with a fixed dose of cyclophosphamide in patients with inoperable or recurrent breast cancer.

STUDY SETTING AND PROTOCOL REVIEW

Open-label, phase I clinical trial. The protocol was approved by the Protocol Review Committee of the Japan South West Oncology Group.

ENDPOINTS

Primarily, adverse events. Secondarily, objective tumor response and pharmacokinetics of capecitabine and epirubicin.

ELIGIBILITY CRITERIA

Patients with a histologically-confirmed diagnosis of inoperable or recurrent breast cancer were eligible.

INCLUSION CRITERIA

1. Inoperable (stage IIIB, excluding patients for whom radiation or surgery is not indicated or stage IV) or recurrent (with metastates or local recurrence observed after surgery) breast cancer
2. Histologically-confirmed breast cancer
3. Age ranging from 20–74 years
4. No effects of previous antitumor therapy
5. No radiation therapy at the targeted lesion prior to enrollment
6. ECOG performance status 0–1
7. Sufficient organ function
8. In patients with a history of anthracycline treatment, a left ventricular ejection fraction (LVEF) level confirmed to be within the normal range (not less than 50%) by echocardiography or radionuclide angiocardiography
9. Life expectancy longer than 12 weeks
10. Capable of ingestion
11. Measurable disease according to RECIST
12. Written informed consent

EXCLUSION CRITERIA

1. Women with ongoing pregnancy, breast feeding or contemplating pregnancy
2. History of solid organ or bone marrow transplantation
3. Allergy to fluoropyrimidine-based drugs and a history of severe adverse effects
4. CNS diseases which require clinical treatment
5. Mental disorders that may affect ability or willingness to provide informed consent or abide by the study protocol

6. HBs antigen positive or HCV antibody positive
7. Cardiovascular diseases with any clinical concerns (congestive heart failure, symptomatic coronary artery disease, arrhythmia uncontrolled by medication, etc.)
8. Patients with active multicancer
9. Evidence of pleural fluid/pericardial effusion, which needs medical attention
10. Evidence of active intestinal ulcer or hemorrhage
11. Complications with varicella
12. Any other cases for which the investigator disapproves of participation in this clinical trial

REGISTRATION

Participating investigators should send an eligibility criteria checking report via Fax to the ECRIN Data Center after confirmation of the above criteria. Patients are then registered. Information regarding the necessary follow-up examinations and recommended chemotherapy schedule is then sent from the Data Center.

TREATMENT METHODS

The five dose levels scheduled for epirubicin and capecitabine in combination with 600 mg/m^2 of cyclophosphamide are as follows: Level 0: 75 mg/m^2 and 1255 mg/m^2; Level 1 (starting dose): 75 mg/m^2 and 1657 mg/m^2; Level 2: 90 mg/m^2 and 1657 mg/m^2; Level 3: 90 mg/m^2 and 1800 mg/m^2; Level 4: 100 mg/m^2 and 1800 mg/m^2. These doses were established in light of data from previously conducted phase I trials (3,9,10). Capecitabine is administered orally twice daily for two weeks. In Japan, the approved dosage of capecitabine is 1657 mg/m^2/day, and the approved schedule is a three-week administration of this dose followed by one week of rest. In this study, the dose intensity of capecitabine at Levels 3 and 4 is milder than that of the approved regimen. Moreover, although the doses of epirubicin at Levels 2 to 4 (90 and 100 mg/m^2) are not approved in Japan, 100 mg/m^2 of epirubicin has been tested in a phase I trial (3). In this study, cyclophosphamide and epirubicin are administered intravenously on day 1 of each treatment cycle. In addition, based on the body surface area (BSA), the subjects are divided into three subgroups (BSA <1.31 m^2, 1.31 m^2 ≤BSA <1.64 m^2 and BSA ≤1.64 m^2) in order to adjust respective doses. One cycle consists of 3 weeks of consecutive administration and is repeated for two cycles. Toxicological effects are assessed after completion of two cycles. Thereafter, administration is continued for four cycles, if possible. For patients previously treated with adriamycin, extreme caution must be taken so as to not exceed a total dose of 900 mg/m^2 of epirubicin in each case. Patients should receive capecitabine for as long as possible even after completing the set dosage cycle. Discontinuation of therapy should be based on blood cell counts and hepatic and renal function prior to initiation of each treatment course. Drug doses should not be modified during the first and second cycles.

DEFINITION OF DOSE LIMITING TOXICITY

Dose limiting toxicity (DLT) is defined as the occurrence of any one of the following observed during the first and second cycles of treatment: (a) grade 4 leukopenia and neutropenia for 7 days or more, (b) grade 3 neutropenia along with fever for 3 days or more, (c) grade 4 thrombocytopenia, (d) grade 3 plantar-palmar erythrodysesthesia, (e) grade 3 or greater non-hematologic toxicity excluding alopecia and nausea/vomiting and (f) total treatment interruption lasting for more than 2 weeks.

FOLLOW-UP

Patients are examined by their physicians every week. Thoracic computed tomography or radiography, abdominal computed tomography and measurements of the tumor markers CEA and CA15-3 are performed after every two treatment cycles as well as at baseline. Blood tests and symptom checks are carried out before treatment and every week during treatment. When grade 3 neutropenia is observed, oral antibiotic drugs are prescribed, and the patient is also instructed to contact her or his physician as soon as she or he develops fever. Urinalysis, measurements of body weight and vital signs and assessment of ECOG performance status are done before treatment and at the end of each treatment cycle.

STUDY DESIGN AND STATISTICAL METHODS

In this study, a dose escalation/de-escalation decision is made using the CRM calculations (7,8). The target toxicity level is set at 33%. The RD of this study is determined to be the dose that is closest to the level at which 33% of patients would experience the DLT. The MTD of this trial is defined as the dose level that is one level higher than the final RD. The first included patient is treated at Level 1. After enrollment of the first patient, the CRM runs sequentially in three patients per cohort. Each cohort is treated at the dose level with an estimated probability of DLT closest to the target toxicity level (33%). Bypassing more than one dose level is not permissible in the CRM calculations.

We carried out a survey to determine a prior distribution for a model parameter employed in the CRM. This was done by asking eight breast cancer oncologists participating in this trial, to predict the RD based on their knowledge and experiences with respect to three chemotherapeutic drugs. We employed a gamma distribution as a prior distribution reflecting results of the survey. In addition, this study applies a stopping rule proposed by O'Quigley (2002) to bring the trial to an early halt before including the entire sample size of patients ($n = 22$) (11). After completing the toxicity assessment for each cohort,

we calculate the probability that a certain dose level administered to a cohort is the dose level recommended to all remaining patients in the study and is the final RD.

INTERIM ANALYSIS AND MONITORING

The Data and Safety Monitoring Committee (DSMC) independently reviews the interim analysis and can consider stopping the trial early. Protocol compliance, safety and on-schedule study progress are also monitored by the DSMC.

PARTICIPATING INSTITUTIONS

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