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Expression of a flt-4 (VEGFR3) splicing variant in primary human prostate tumors. VEGF D and flt-4t$_{\Delta 773–1081}$ overexpression is diagnostic for sentinel lymph node metastasis

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Utilizing a cDNA expression library established from human prostate PC-3ML tumor cells, we have cloned a truncated flt-4 gene, termed flt-4t$_{\Delta 773–1081}$. We have then utilized RNase protection and ELISA to measure the relative levels of VEGF B, C, D and flt-1, KDR, flt-4 and flt-4t$_{\Delta 773–1081}$ expression in freshly isolated benign prostatic hyperplasia or BPH tissue ($n=21$), primary prostate cancers ($n=82$) and matching sentinel lymph node metastases from stage T2a–T2b/T3 tumors ($n=52$). Comparisons of the primary tumors with BPH showed that there was a significant upregulation of VEGF-B ($P=0.003$), VEGF D ($P=0.005$), flt-1 ($P=0.003$), KDR ($P=0.002$), flt-4 ($P=0.007$), and flt-4t$_{\Delta 773–1081}$ ($P=0.001$), but not VEGF-C ($P=0.543$). There was no correlation between VEGF-B and its receptor flt-1 ($P=0.545$), or VEGF-C and flt-4 ($P=0.16$) and KDR ($P=0.23$) receptor expression in tumor specimens. Conversely, there was no significant relationship between VEGF-D and the flt-4t$_{\Delta 773–1081}$ receptor ($P=0.516$) expression. Statistical analysis further showed that there was no significant correlation between VEGF-B, VEGF-C, VEGF-D, flt-1, KDR, flt-4 and flt-4t$_{\Delta 773–1081}$ with patient age ($P>0.10$), stage ($P>0.10$), PSA value ($P>0.15$) or tumor size ($P>0.15$). Likewise, there was no significant correlation between VEGF-B, VEGF-C, flt-1, KDR, and flt-4 with Gleason score ($P>0.15$). In comparison, flt-4t$_{\Delta 773–1081}$ levels clearly increased significantly in Gleason score 7 and Gleason score 8–10 tumors as well as in stage T2a–T2b/T3 tumors. The studies were extended to compare gene expression profiles in T2a–T2b and T3 tumors with ($n=26$) and without ($n=26$) matching sentinel lymph node metastases. The data showed that VEGF D and flt-4t$_{\Delta 773–1081}$ expression levels were significantly elevated in primary tumors with sentinel lymph node involvement compared to those lacking lymph node involvement ($P>0.0022$ and 0.006, respectively). These data suggest that targeting VEGF D and flt-4t$_{\Delta 773–1081}$ receptors may be particularly effective in the prevention of lymph node metastases.
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A problem in the management of patients with prostate cancer is predicting tumor behavior. Stage is the most useful determinant, with other features, including tumor grade and histology, being of lesser value. As solid tumors are highly angiogenic, several studies have measured the molecules that regulate neovascularization such as VEGF A. For example, studies show that VEGF A mRNA and protein are significantly elevated in renal tumors compared with BPH tissues with some evidence to suggest a relationship to microvessel density.[1–4] Furthermore, serum VEGF-A protein has been related to grade and stage and may be useful in predicting prognosis in nonoperable renal cancer patients.[5–7] Other studies in breast cancers in which up to seven angiogenic factors are expressed,[8] suggest that multiple angiogenic factors might provide useful clinical information for patient management. Recently, several new members of the VEGF family have been identified including VEGF-B[9–10] and VEGF-C,[11–12] which may help to develop this approach. The studies of renal cancer indicate both factors and their respective receptors are expressed at elevated levels in renal cancer.[13]

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These angiogenic factors may play an important role in tumor progression and metastasis. For example, VEGF-B binds to flt-1 (VEGF R1), receptor activation subsequently results in upregulation of urokinase plasminogen activator and its inhibitor plasminogen activator inhibitor-1 (PAI-1). Indirectly, these results suggest a role for VEGF B and flt-1 in endothelial cell migration and matrix remodeling.[14] Similarly, VEGF-C stimulates endothelial cell migration and proliferation in addition to increasing vascular permeability.[15] These processes are mediated through KDR (VEGF R2) and another related tyrosine kinase receptor flt-4 (VEGF R3).[16] The latter receptor appears to be largely restricted to lymphatic endothelium cells, but is also present on tumor neovasculature, which suggests a role in both angiogenesis and lymphatic spread and lymphangiogenesis.[17–20] Unlike the numerous studies demonstrating the importance of VEGF-A in breast or renal cancers, there are no data available for angiogenic factors and their receptors in prostate cancer.

We have cloned a truncated flt-4 gene, termed flt-4t$_{\Delta 773-1081}$, from human prostate PC-3ML tumor cells and examined the expression of this gene in human prostate cancer. Utilizing RNase protection assays, we have measured and compared the mRNA level of VEGF-B and VEGF-C together with their receptors, KDR, flt-4 and the flt-4t$_{\Delta 773-1081}$ gene in 21 BPH and 82 prostate cancers samples. 'RNase Protection Assays' and ELISAs were also employed to evaluate and compare mRNA/antigen expression profiles in primary (ie T2a–T2b and T3 tumors) with ($n=26$) and without ($n=26$) lymph node metastases. The data showed that VEGF D and flt-4t$_{\Delta 773-1081}$ expression levels were significantly elevated in both the primary tumors and sentinel lymph nodes compared to those lacking metastatic lesions, suggesting that targeting these two genes may be effective in the prevention of lymph node metastasis.

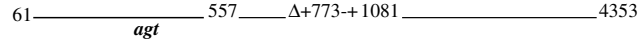
Materials and methods

Cloning the flt-4t$_{\Delta 773-1081}$ Gene

mRNA was isolated from PC-3 ML cells using a mRNA purification kit (PolyATtract system 1000′, Promega). A phage vector ZAP-express vector (Stratagene) was used to construct a cDNA library. Using the FASTA search algorithm carried out computer searches for flt-4 related sequences. The expressed sequence tag (EST) encoding the C-terminal region of flt-4 (GenBank accession no.XM 044114) had been isolated by the Integrated Molecular Analysis of Genome Expression Consortium as part of the Washington University-Merck EST Project. The EST was obtained from the American Type Culture Collection and was used as hybridization probe for isolation of cDNA from a cDNA library of the PC-3ML human prostate cells. The flt-4t$_{\Delta 773-1081}$ gene was cloned with a ZAP express vector (Stratagene Inc., CA, USA) in three separate experiments for comparison. The pBK-CMV phagemid was excised *in vivo* from the ZAP express vector according to Stratagenes protocol using a ZAP express vector kit. The pBK-CMV phagemid was utilized for DNA sequencing. The protein sequence was deduced from the cDNA sequence using a GCG program.

A truncated flt-4 gene was cloned with a base pair deletion between +773 and +1081 bp downstream of the agt translational start site at 557 bp.

Flt-4t$_{\Delta 773-1081}$ Gene showing Region Deleted from Parent flt-4 Gene



Tumors and Patients

Prostate carcinomas ($n=82$) and histological BPH tissues ($n=21$) taken from uninvolved prostate tissue distant from tumor were derived from patients undergoing surgery. The clinical and pathological characteristics of the patient series are contained in Table 1.

RNase Protection Assay Protocol

Tissue was snap frozen after surgical resection and total RNA was prepared by either the method of Chomczynski and Sacchi or the guanidinium thiocyanate lysis or cesium chloride-gradient method.[21] In all, 200 ng of each cDNA template was linearized

Table 1 Clinical data for prostate cancers studied

Gleason score	*Grade*	*PSA (ng/ml)*	*Tumor size (mm)*	*Patient age*
&5 ($n=13$)	T1a	10–19 (15)[b]	10–15 (12)[b]	50–56 (54)[b]
&6 ($n=17$)	T1c	14–33 (23)[b]	12–22 (18)[b]	54–65 (61)[b]
7[a] ($n=12$)	T2a–T2b	11–44 (33)[b]	14–29 (25)[b]	53–67 (58)[b]
8–10[a] ($n=14$)	T3	10–56 (28)[b]	15–38 (29)[b]	55–65 (61)[b]
&7 ($n=15$)	T2a–T2b	6–24 (18)[b]	10–20 (15)[b]	50–60 (54)[b]
&8–10 ($n=11$)	T3	7–36 (20)[b]	10–22 (19)[b]	45–60 (55)[b]

[a]Lymph node metastasis & no lymph node involvement; [b]Average PSA, tumor size and patient age for the respective category ($n=82$ total).

and antisense ^{32}P-RNA probes generated using a Riboprobe Combination System T3/T7 kit (Promega, Madison, WI, USA). The probes were designed to generate different sized protected fragments to allow several factors to be assayed within the same total RNA sample, thereby avoiding the problem of interassay variability. These comprised a 471-bp VEGF-A (positive control for cell line experiments), 340-bp VEGF-B probes, 415-bp VEGF-C and 350 bp VEGF-D probes together with a 218-bp flt-1, a 350-bp KDR, a 273-bp flt-4 probe bridging the deleted region observed in the flt-4t$_{\Delta 773-1081}$ gene[22] and a 250-bp flt-4t$_{\Delta 773-1081}$ probe bridging the region deleted from the flt-4 gene. The DNA template was removed with DNase1, and the reaction was purified using a mini Quick Spin RNA Column (Boehringer Mannheim Corp.). An amount of 20 μg of RNA samples were resuspended in hybridization buffer containing the respective labeled RNA probes and the samples were denatured and incubated overnight at 45°C. The RNA remaining after hybridization was digested with RNase A and RNase T1 (Boerhinger Mannheim GmbH, Mannheim, Germany). RNases were inactivated using Proteinase K and proteins removed by phenol/chloroform/isoamyl alcohol extraction. Hybridized RNA was ethanol precipitated and size separated on an 8% polyacrylamide gel. Gels were vacuum dried (Bio-Rad model 583) and exposed to X-ray film (Eastman Kodak Co., Rochester, NY, USA) between intensifying films at −80°C overnight. Aliquots of native labeled probes and labeled probes subjected to RNase digestion were included in all of the assays to determine probe integrity and efficacy of RNase digestion.[23] To minimize variable results from assay to assay, 20 μg of total RNA was loaded (for each sample) which contained known amounts of transcribed GAPDH sense probe. Scanning laser densitometry was used to quantify mRNA levels, and mRNA signal was standardized against the sense GAPDH control spike signal. An amount of 20 μg of tRNA was used as a negative control and 20 μg of PC-3 ML cell mRNA was used as a positive control for each experiment. Note that GAPDH was expressed by BPH and the different tumors at equivalent levels and is a good standard for these studies.

Statistical Analysis

The Spearman rank correlation coefficients were used for studying the association between continuous variables. Tests of hypotheses on the location parameter (median) were done using rank statistics (Mann–Whitney, Kruskall–Wallis, and adjusted Kruskall–Wallis for ordered groups). The χ^2-test was used to test for independence of categorical variables including categorized continuous variables and logistic regression/multivariate analysis to confirm any significant statistical associations. All of the tests were performed using the Stata package release 5.0 (Stata Corporation, College Station, TX, USA).

Results

VEGF-B, VEGF-C, VEGF D, FLT-1, KDR, FLT-4 and FLT-4t$_{\Delta 773-1081}$ Expression in BPH and Malignant Prostate Tissues

VEGF-B and its receptor flt-1 mRNA expression as well as VEGF-C and its receptors KDR and flt-4 mRNA expression were compared in BPH and malignant prostate samples by RNase Protection Assay. Representative cases are shown in Figures 1 and 2. VEGF-D and flt-4t$_{\Delta 773-1081}$ mRNA expression were also assayed in BPH and malignant prostate samples by 'RNase Protection Assay'. Representative cases are shown in Figure 3. All the BPH samples ($n = 21$) were positive for VEGF-B, VEGF-C, flt-1, and KDR. None of the BPH were positive for either VEGF-D or flt-4t$_{\Delta 773-1081}$ expression (ie the levels were at background levels of detection). In the tumor samples ($n = 82$), the number of specimens positive for VEGF-B, VEGF-C, VEGF-D were 76/82, 78/82 and 77/82, respectively; the number positive for flt-1, KDR, flt-4 and flt-4t$_{\Delta 773-1081}$ were 76/82, 82/82, 75/82 and 78/82, respectively.

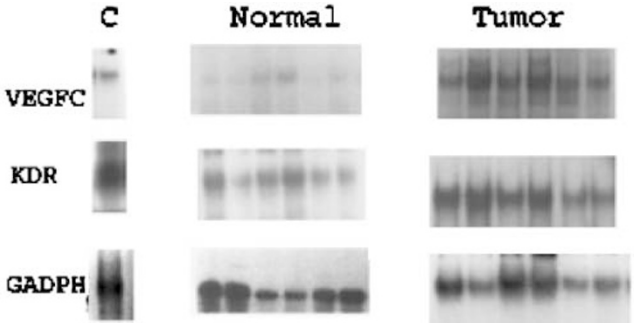


Figure 1 'RNase Protection Assay' showing VEGF-B and its receptor flt-1 expression in BPH (lanes 1–6) and matched prostate tumor (lanes 7–12) samples. GAPDH was used as an internal control. RNA from PC-3 ML cells was used as a positive control.

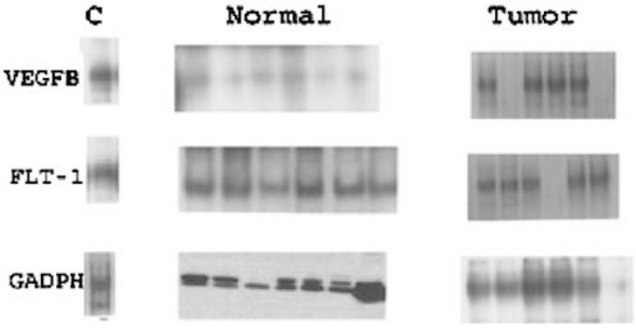


Figure 2 'RNase Protection Assay' showing VEGF-C and its receptors KDR and flt-4 in BPH (lanes 1–6) and matched prostate tumor (lanes 7–12) samples. GAPDH expression was used as an internal control. RNA from PC-3 ML cells was used as a positive control.

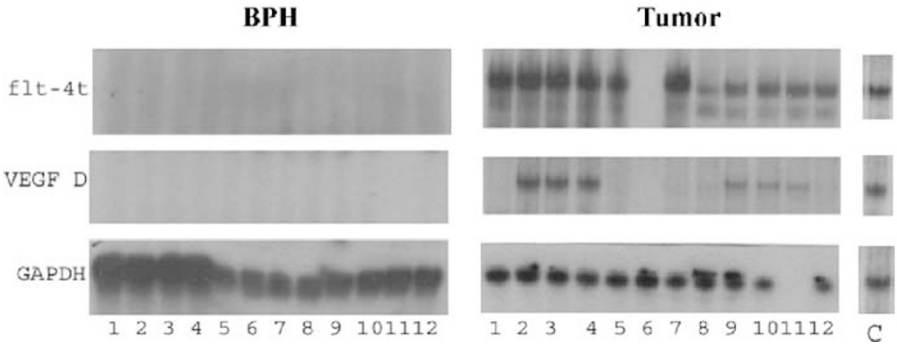


Figure 3 'RNase Protection Assay' showing VEGF-D and flt-4t$_{\Delta 773-1081}$ in BPH (lanes 1–12) and matched prostate tumor (lanes 1–12) specimens. RNA from PC-3 ML cells was used as a positive control. GAPDH was used as an internal control.

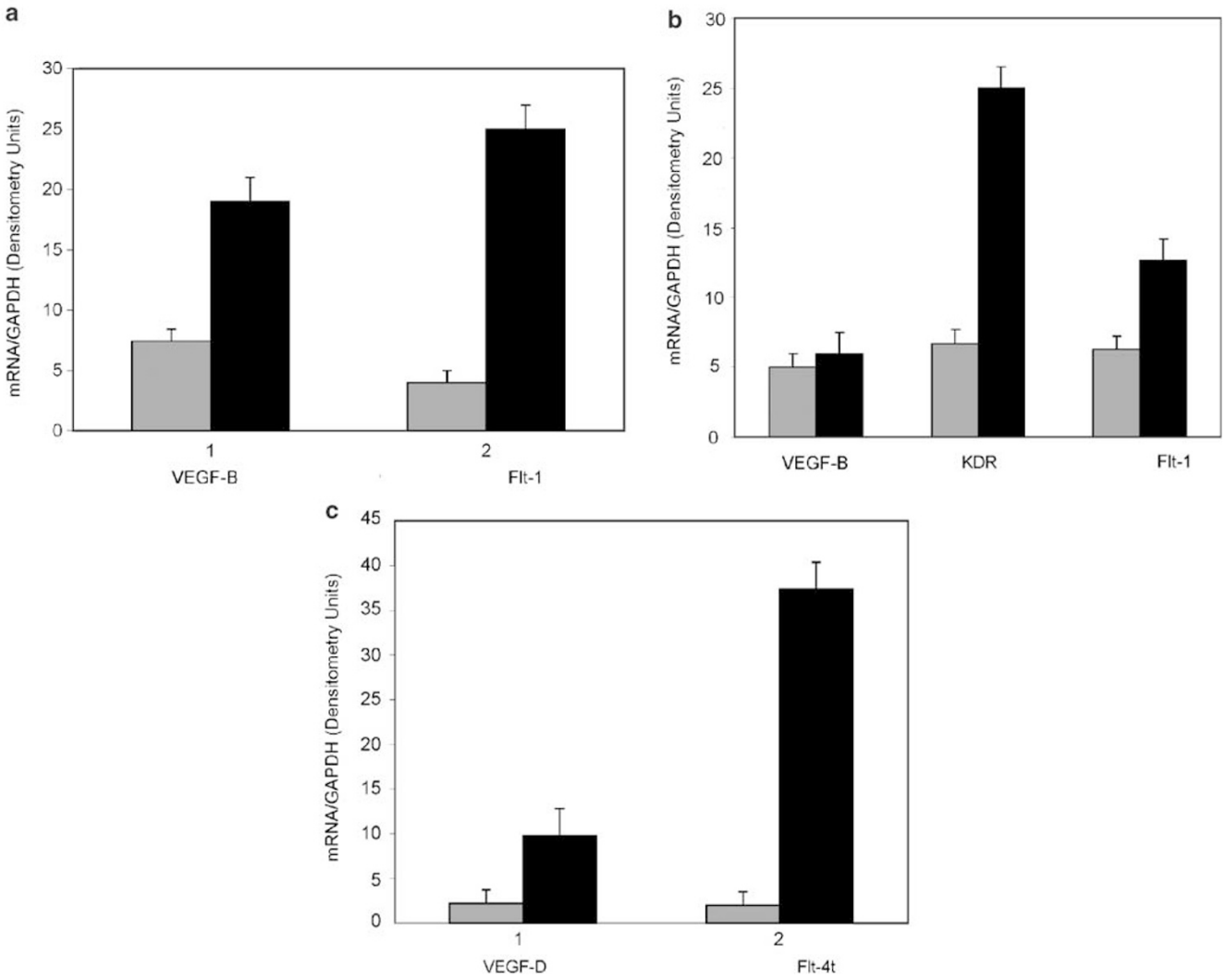


Figure 4 'RNase Protection Assay' showing the levels of: (**a**) VEGF-B and flt-1; (**b**) VEGF-C, KDR, and flt-4; and (**c**) VEGF D and flt-4t$_{\Delta 773-1081}$ mRNA expression in BPH (gray bars) and malignant (black bars) prostate samples dissected from the same prostate ($n = 21$, GS 7 tumors). Data from densitometry scans of the gels (A600 nm) was normalized relative to GAPDH mRNA levels and expressed as the mean $\pm$ s.d.

The overall differences observed for VEGF-B, VEGF-C, VEGF-D, flt-1, KDR, flt-4 and flt-4t$_{\Delta 773-1081}$ expression in BPH samples and tumor samples by RNase Protection Assay and densitometry are shown in Figure 4a–c. VEGF-B and flt-1 mRNA were significantly increased in tumor compared with BPH tissue ($P = 0.003$) and ($P = 0.003$), respectively (Figure 4a). Surprisingly, no significant

upregulation in VEGF-C ($P=0.543$) was observed, albeit significant differences in the KDR receptor ($P=0.002$) and flt-4 ($P=0.007$) mRNA were observed (Figure 4b). Also, there was no significant relationship between VEGF-B and its receptor flt-1 ($P=0.545$), VEGF-C and its receptors KDR ($P=0.23$) and flt-4 ($P=0.16$). Finally, VEGF D ($P=0.005$) and flt-4t$_{\Delta 773-1081}$ ($P=0.001$) were significantly upregulated in tumor compared with BPH tissue (Figure 4c). Again, there was no significant relationship between VEGF C ($P=0.342$) and the flt-4t$_{\Delta 773-1081}$ receptor, and between VEGF D ($P=0.516$) and the flt-4t$_{\Delta 773-1081}$ receptor.

Pathology and Clinical Parameters

The histograms (left to right for each gene) show the levels of mRNA expression for each gene for Gleason score (GS) 5–7, and 8–10 tumors (Figure 5a) and clinical stage T1a, T1c, T2a–T2b, and T3 tumors (Figure 5b), respectively. The data in Figure 5a and b showed that there was no significant difference in VEGF-B, VEGF-C and VEGF-D or flt-1, KDR, and flt-4 expression as a function of the Gleason score or the clinical stage of the tumor specimen. Statistical analysis further showed that there was no significant correlation between VEGF-B, VEGF-C, VEGF-D, flt-1, KDR, flt-4 and flt-4t$_{\Delta 773-1081}$ with patient age ($P>0.10$), stage ($P>0.10$), PSA value ($P>0.15$), or tumor size ($P>0.15$). Likewise, there was no significant correlation between VEGF-B, VEGF-C, flt-1, KDR, and flt-4 with Gleason score ($P>0.15$). In comparison, flt-4t$_{\Delta 773-1081}$ levels ($P<0.005$) clearly increased in Gleason score 7 and Gleason score 8–10 tumors (Figure 5a) as well as in stage T2a–T2b/T3 tumors when compared with the lower stage tumors (Figure 5b).

Comparison of Primary Tumors *With* and *Without* Lymph Node Involvement

Measurements of mRNA expression in primary tumors *without* (black and dotted bars) and *with* lymph node metastases (checkered and stripped bars) show that only VEGF-D ($P>0.0022$), and flt-4t$_{\Delta 773-1081}$ ($P>0.006$) were expressed at significantly elevated levels in T2a–T2b/T3 tumors *with* lymph node involvement (checkered and stripped bars) when compared with primary tumors *without* lymph node involvement (black and dotted bars), respectively (Figure 6). No significant difference in the expression level of the other genes (ie VEGF-C, VEGF-B, flt-1, KDR, and flt-4) was observed as a function of lymph node involvement ($P<0.3$, 0.5, 0.6, 0.2, 0.1, respectively) (Figure 6). ELISAs verified that antigen expression levels closely corresponded with the mRNA levels observed (Figure 7).

We then evaluated the matching sentinel lymph node tumors of the T2a–T2b and T3 tumors ($n=12$ and 14, respectively). RNase Protection assays (Figure 8) and ELISAs (Figure 9) showed that VEGF-B and VEGF-C levels were relatively low ($P>0.345$), but VEGF-D was elevated in the metastatic lesions ($P=0.0045$) compared to BPH. In addition, 'RNase Protection Assays' (Figure 8) and ELISAs (Figure 9) showed that flt-1, KDR, flt-4 and flt-4t$_{\Delta 773-1081}$ were expressed at relatively elevated levels in the sentinel lymph node metastases from the T2a–T2b and T3 tumors compared to BPH, $P<0.08$, 0.09, 0.07 and 0.1, respectively (Figures 8 and 9). However, flt-4t$_{\Delta 773-1081}$ levels were significantly higher than the other receptors ($P=0.0068$). Taken together, these data indicate that the primary tumors and matching sentinel lymph nodes consistently overexpress VEGF D and flt-4t. Note that ELISAs with GAPDH antibodies indicated GAPDH levels ranged from 65 to 85 ng/ml in the crude protein extracts assayed in Figures 7 and 9, indicating the quality of the protein extracts was not a contributing factor towards the differences in VEGF D and flt-4t levels observed among samples.

Discussion

We have examined VEGF B-D expression in benign and malignant prostate cancer utilizing 'RNase Protection Assays' and ELISAs. The data showed that VEGF-B and to a much lesser extent VEGF-C mRNA are expressed by BPH, indicating VEGF B (and or VEGF-C) may play a role in the development and repair of the microvasculature.[24,25] VEGF D is not expressed to any significant degree in BPH tissue, indicating that this ligand is not required. Interestingly, all three ligands are expressed in tumor tissues and are likely to play a role in the neovascularization of prostate cancer. In comparison to BPH, we observed a significant increase in VEGF-B, VEGF-D, flt-1, flt-4, and flt-4t$_{\Delta 773-1081}$ in tumor tissues. Perhaps more importantly, no difference in VEGF-C and its receptors was observed between BPH and tumor tissues. This suggests VEGF-B and VEGF-D might play a prominent role in the neovascularization of malignant tissues. However, since several of the tumors failed to express VEGF-D, flt-4 or flt-4t$_{\Delta 773-1081}$, and since VEGF-C and its receptors were expressed in some tumors, other unknown factors play contribute to the angiogenesis of tumor tissue. That is, different tumors might deploy distinct (yet redundant) angiogenic pathways to establish a neovasculature. For example, tumors unable to deploy VEGF-A, might express VEGF-B and/or VEGF-D or VEGF-C.[26]

Additional studies in this paper compared the expression levels of each of the genes/proteins of interest in the primary tumors and matching sentinel lymph node metastases. Measurements of mRNA expression in lymph node metastases of the T2a–T2b and T3 tumors showed that relatively low levels of VEGF-B and VEGF-C mRNA were expressed in the primary tumors and matching lymph

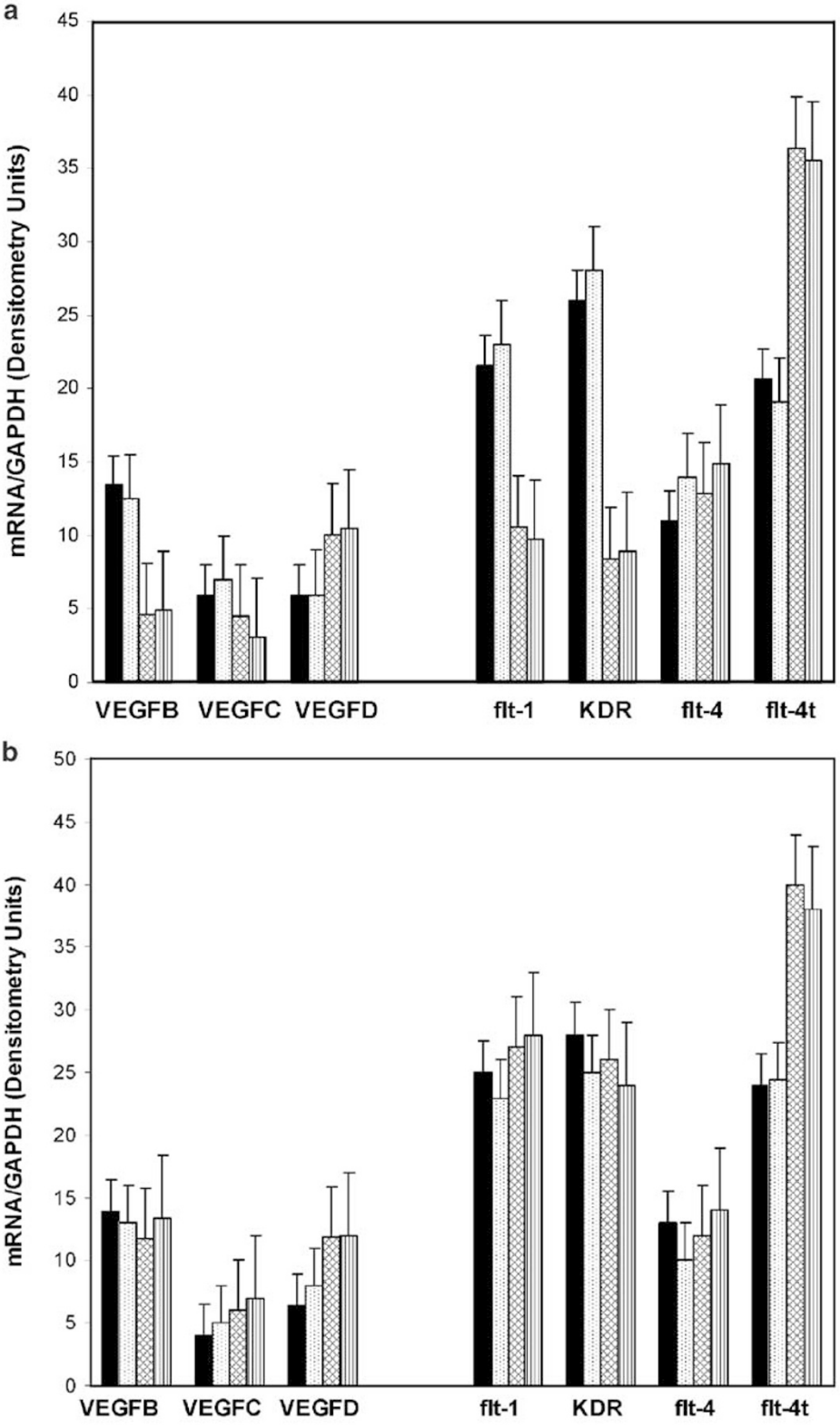


Figure 5 'RNase Protection Assays' showing the levels of mRNA expression relative to the: (**a**) Gleason Score (GS) of the tumors. Histograms show data for GS 5 (black bars), GS 6 (dotted bars), GS 7 (checkered bars), and GS 8–10 (stripped bars) tumors (data for each GS shown from left to right for each gene). (**b**) Clinical stage of the tumors. Histograms show data for stage T1a (black bars), T1c (dotted bars), T2a–T2b (checkered bars), and T3 (stripped bars) tumors (data for each stage shown from left to right for each gene). Data from densitometry scans of the gels (A600 nm) was normalized relative to GAPDH mRNA levels and expressed as the mean $\pm$ s.d.

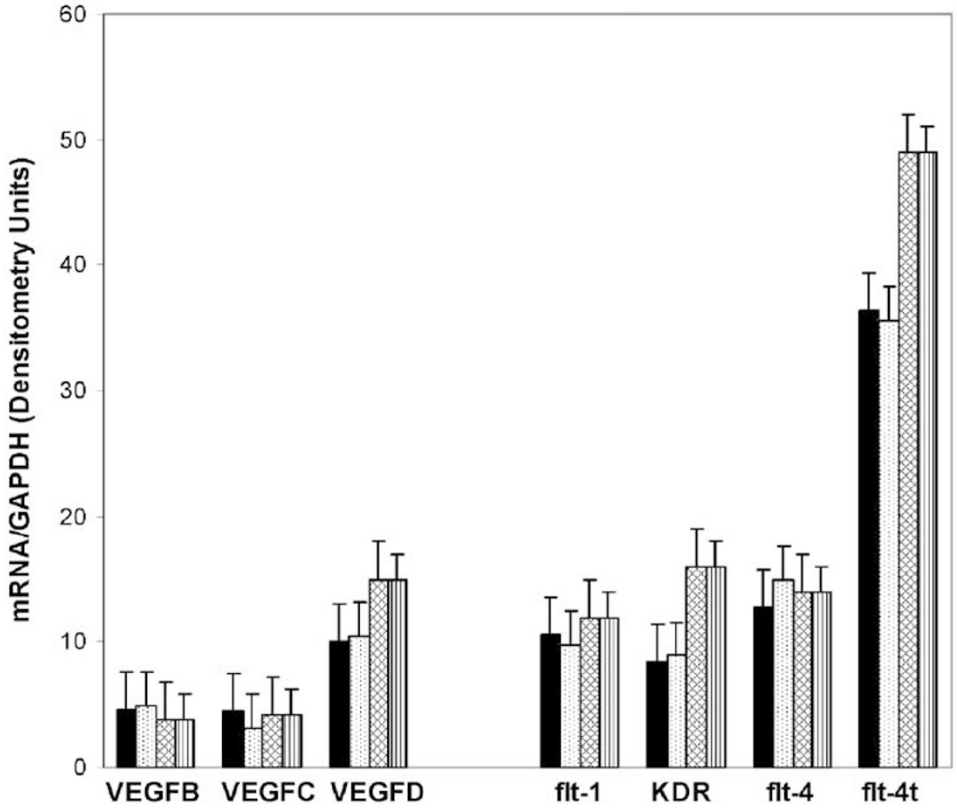


Figure 6 'RNase Protection Assays' showing the levels of mRNA expression of T2a–T2b (black, green bars) and T3 (red and pink bars) primary tumors *without* (black and red) and *with* (green and pink bars) lymph node involvement. Data from densitometry scans of the gels (A600 nm) was normalized relative to GAPDH mRNA levels and expressed as the mean ± s.d.

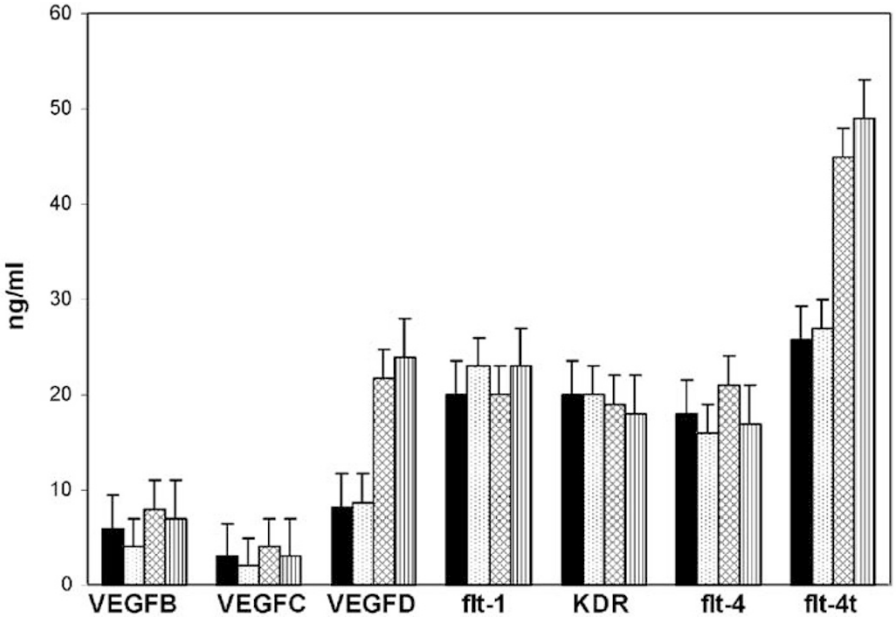


Figure 7 ELISA assays of antigen expression in T2a–T2b (black, checkered bars) and T3 (dotted and stripped bars) primary tumors *without* (black and dotted bars) and *with* (checkered and stripped bars) lymph node involvement. Data represents the mean ± 1 s.d.

node metastases. In contrast, VEGF-D was abundant in the primary tumors as well as the matching lymph nodes, indicating a causal association with tumor progression and lymphangiogenesis. Likewise, flt-1, KDR, and flt-4 receptors were expressed at relatively elevated levels by the primary tumors and the sentinel lymph node tumors, albeit not as prominent as VEGF D and flt-4t$_{\Delta 773-1081}$.

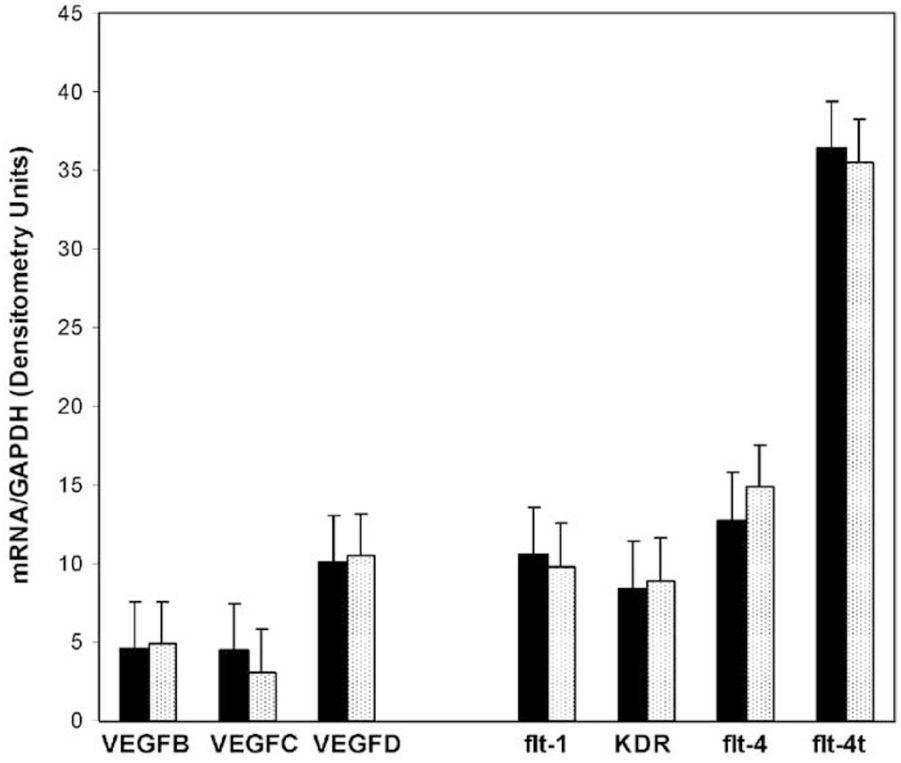


Figure 8 Measurements of mRNA expression by RNase Protection Assays in lymph node metastases of the T2a–T2b (black bars) and T3 (dotted bars) tumors. Data from densitometry scans of the gels (A600 nm) was normalized relative to GAPDH mRNA levels and expressed as the mean ± s.d.

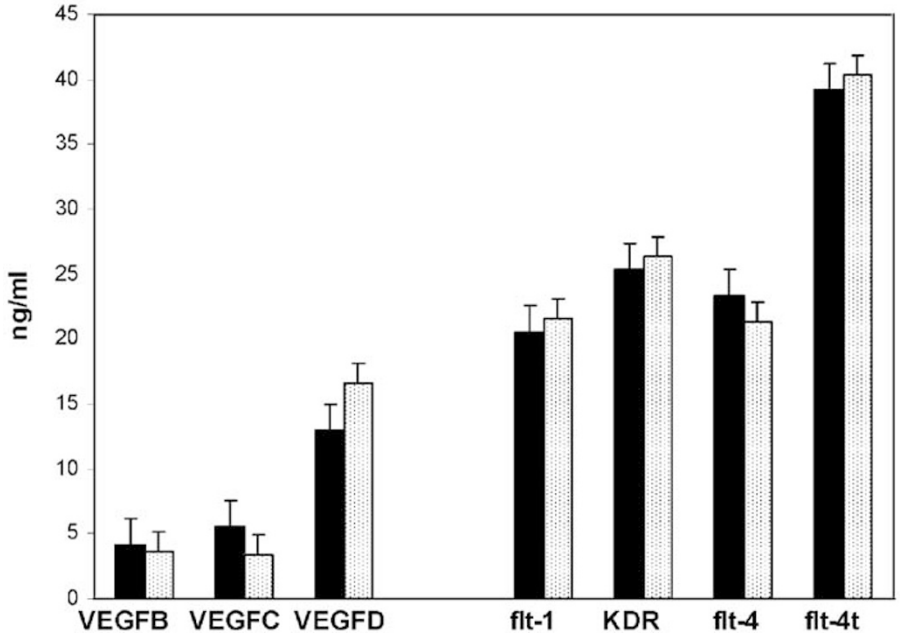


Figure 9 ELISA measurements of antigen expression in crude protein extracts (10 mg/ml) from lymph node metastases of the T2a–T2b (black bars) and T3 (dotted bars) tumors. Data represents the mean ± 1 s.d.

Perhaps more importantly, the truncated form of flt-4, termed flt-4$t_{\Delta 773-1081}$ was expressed in the majority of tumors examined ($n = 80/82$), and was not expressed in BPH tissue. Thus, the combined expression of VEGF D, flt-4$t_{\Delta 773-1081}$, flt-1, KDR, flt-4 and flt-4$t_{\Delta 773-1081}$ may be critical for tumor growth, lymphangiogenesis and metastases.

Unfortunately, there was no significant relationship between VEGF D ($P=0.516$) and flt-4t$_{\Delta 773-1081}$ receptor expression, indicating the combined expression of these genes is not associated with tumor progression. It may turn out that there is a simplistic explanation for this discrepancy such as a decline in VEGF D levels with the development of lymphatic networks at tumor surfaces.

A number of the primary T2a–T2b ($n=15$) and T3 ($n=11$) tumors were devoid of any lymph node involvement. RNase Protection Assays and ELISA data on the gene/protein expression profiles exhibited by these tumors revealed that VEGF B, and VEGF C were expressed at somewhat lower levels compared to the tumors with lymph node involvement ($P=0.102$, and 0.155, respectively). Flt-1 and KDR levels, in comparison, were similar to the values observed for the tumors with lymph node involvement ($P<0.04$ and 0.03, respectively). In contrast, VEGF D, and flt-4t$_{\Delta 773-1081}$ levels were significantly diminished in the primary tumors *without* lymph node involvement compared to that of the primary tumors *with* lymph node involvement ($P>0.0052$ and 0.006, respectively). The ELISA data closely mirrored the measurements of mRNA expression and the implication is that elevated VEGF D and flt-4t$_{\Delta 773-1081}$ expression levels are important for metastases to the lymph nodes (ie sentinel lymph node involvement).

Surprisingly, no significant correlation was observed between expression of VEGF-B, VEGF-C or VEGF-D and flt-1, KDR, flt-4 or flt-4t$_{\Delta 773-1081}$ and important clinical parameters examined (eg serum PSA, size of tumor or patient age). Likewise, there was no significant correlation between VEGF-B, VEGF-C, flt-1, KDR, and flt-4 with Gleason score of the tumors. This is in agreement with the reported literature in other tumor types in which associations to clinical–pathological parameters such as nodal metastasis have been conflicting.[27–30] For example, in studies of renal cancer, no significant correlation was observed between expression of either VEGF-B or VEGF-C and tumor stage, grade, size or patient sex and age.[13] Still, this may not be a critical issue, since solid cancers are known to express several angiogenic factors[8] that may act synergistically (eg VEGF-C and VEGF-A)[31] to form a tumor blood supply and lymphatic network.

In contrast to the other VEGF receptors, flt-4t$_{\Delta 773-1081}$ levels clearly increased significantly in Gleason score 7 and Gleason score 8–10 tumors as well as in stage T2a–T2b/T3 tumors when compared with the lower stage tumors, suggesting that the truncated form of flt-4t may be associated with tumor progression.

In this regard, Flt-4 or VEGFR-3 is the first lymphatic endothelial cell-specific receptor identified.[32] It was cloned as an orphan receptor from human erythroleukemia cell and placental cDNA libraries[33,34] and the flt-4 gene was located to the chromosomal segment *5q33–q35*.[34,35] Subsequently, VEGF-C and VEGF-D were found to bind to and to activate flt-4.[36–38] VEGF-C mRNA is expressed in close proximity to its receptor flt-4 during mouse development.[39] Accordingly, VEGF-C is capable of inducing hyperplasia of lymphatic vessels in transgenic mice and in chick chorioallantoic membrane.[40,41] These results suggest that VEGF-C, VEGF-D and flt-4 play a role in the generation of the lymphatic system and their maintenance.

Caution needs to be exercised in the interpretation of the results, as is difficult to claim that flt-4 expression (or flt-4t$_{\Delta 773-1081}$) is specific for lymphatic endothelial cells. Flt-4 is relatively specific for lymphatics in adults, but it is also expressed in some fenestrated blood endothelial cells and angiogenic blood vessels in tumors[42,43] and may play a role in angiogenesis as well. In any event, truncated polymorphisms of flt-4 may play a critical role in the development of lymphatic networks surrounding tumors. Recently, Iljin *et al*[44] cloned and analyzed the flt-4 gene and characterized its structure as well as the promoter region responsible for its endothelial cell-specific expression. The coding sequence of the flt-4 gene is organized into 31 exons that closely correspond to the genomic organization of the mouse flt-1 and human KDR genes.[45,46] They also identified many polymorphisms of the VEGFR3 sequence and have recently demonstrated that flt-4 mis-sense mutations are a cause of familial early onset lymphedema,[47,48] indicating several flt-4 mutations and or deletions may arise in lymphedema families and or tumors. The flt-4t$_{\Delta 773-1081}$ gene identified here appears to be a truncated version of flt-4 and to be expressed in advanced tumors with a high propensity for metastases to the lymph nodes. Future studies evaluating ligand binding and signaling should provide valuable insights as to the mechanistic aspects favoring tumorigenesis and metastasis.

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References

1 Takahashi A, Sasaki H, Kim SJ, *et al.* Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. Cancer Res 1994;54:4233–4237.
2 Nicol D, Hii SI, Walsh M, *et al.* Vascular endothelial growth factor expression is increased in renal cell carcinoma. J Urol 1997;157:1482–1486.
3 Nakagawa M, Emoto A, Hanada T, *et al.* Tubulogenesis by microvascular endothelial cells is mediated by vascular endothelial growth factor (VEGF) in renal cell carcinoma. Br J Urol 1997;79:681–687.
4 Tomisawa M, Tokunaga T, Oshika Y, *et al.* Expression pattern of vascular endothelial growth factor isoform is

closely correlated with tumour stage and vascularisation in renal cell carcinoma. Eur J Cancer 1999;35: 133–137.
5 Jacobsen J, Rasmuson T, Grankvist K, *et al.* Vascular endothelial growth factor as prognostic factor in renal cell carcinoma. J Urol 2000;163:343–347.
6 Dosquet C, Coudert MC, Lepage E, *et al.* Are angiogenic factors, cytokines, and soluble adhesion molecules prognostic factors in patients with renal cell carcinoma? Clin Cancer Res 1997;3:2451–2458.
7 O'Byrne KJ, Dobbs N, Propper D, *et al.* Vascular endothelial growth factor platelet counts and prognosis in renal cancer. Lancet 1999;353:1494–1495.
8 Relf M, LeJeune S, Scott PA, *et al.* Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor β-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res 1997;57: 963–969.
9 Grimmond S, Lagercrantz J, Drinkwater C, *et al.* Cloning and characterization of a novel human gene related to vascular endothelial growth factor. Genome Res 1996;6:124–131.
10 Olofsson B, Pajusola K, Kaipainen A, *et al.* Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA 1996;93:2576–2581.
11 Joukov V, Pajusola K, Kaipainen A, *et al.* A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinase. EMBO J 1996;15:290–298.
12 Lee J, Gray A, Yuan J, *et al.* Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. Proc Natl Acad Sci USA 1996;93:1988–1992.
13 Gunningham S, Currie M, Cheng H, *et al.* Vascular endothelial growth factor-B and vascular endothelial growth factor-C expression in renal cell carcinomas. Regulation by the von Hippel–Lindau gene and hypoxia. Cancer Res 2001;61:3206–3211.
14 Olofsson B, Korpelainen E, Pepper MS, *et al.* Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc Natl Acad Sci USA 1998;95:11709–11711.
15 Witzenbichler B, Asahara T, Murohara T, *et al.* Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. Am J Pathol 1998;153:381–394.
16 Joukov V, Sorsa T, Kumar V, *et al.* Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 1997;16:3898–3911.
17 Jeltsch M, Kaipainen A, Joukov V, *et al.* Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science (Washington DC) 1997;276:1423–1425.
18 Joukov V, Kaipainen A, Jeltsch M, *et al.* Vascular endothelial growth factors VEGF-B and VEGF-C. J Cell Physiol 1997;173:211–215.
19 Kaipainen A, Korhonen J, Mustonen T, *et al.* Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 1995;92:3566–3570.
20 Valtola R, Salven P, Heikkil P, *et al.* VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. Am J Pathol 1999;154:1381–1390.
21 Sambrook J, Fritsch EF, Maniatis T, (eds). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989, pp 7.6–7.11.
22 Gunningham S, Currie M, Cheng H, Scott RB, *et al.* The short form of the alternatively spliced flt-4 but not its ligand VEGF-C is related to lymph node metastasis in human breast cancers. Clin Cancer Res 2000;6: 4278–4286.
23 Scott PAE, Smith K, Bicknell R, *et al.* A reliable external control for ribonuclease protection assays. Nucleic Acid Res 1997;25:1305–1306.
24 Takahashi T, Huynh-Do U, Daniel TO. Renal microvascular assembly and repair: power and promise of molecular definition. Kidney Int 1998;53: 826–835.
25 Robert ST, John PL, Abrahamson DR. Direct visualization of renal vascular morphogenesis in Flk1 heterozygous mutant mice. Am J Physiol 1998;275: F164–F172.
26 Nicosia RF. What is the role of vascular endothelial growth factor-related molecules in tumor angiogenesis? Am J Pathol 1998;153:11–16.
27 Yonemura Y, Endo Y, Fujita H, *et al.* Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. Clin Cancer Res 1999;5:1823–1829.
28 Tsurusaki T, Kanda S, Sakai H, *et al.* Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer 1999;80:309–313.
29 Andre T, Kotelevets L, Vaillant JC, *et al.* VEGF, VEGF-B, VEGF-C, and their receptors, K. D. R., FLT-1 and FLT-4 during the malignant progression of human colonic mucosa. Int J Cancer 2000;86:174–181.
30 Niki T, Iba S, Tokunou M, *et al.* Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. Clin Cancer Res 2000;6: 2431–2439.
31 Pepper MS, Mandriota SJ, Jeltsch M, *et al.* Vascular endothelial growth factor (VEGF)-C synergizes with basic fibroblast growth factor and VEGF in the induction of angiogenesis *in vitro* and alters endothelial cell extracellular proteolytic activity. J Cell Physiol 1998;177:439–452.
32 Shibuya M, Yamaguchi S, Yamane A, *et al.* Nucleotide sequence and expression of a novel human receptor type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 1990;5:519–524.
33 Aprelikova O, Pajusola K, Partanen J, *et al.* FLT4, a novel class III receptor tyrosine kinase in chromosome 5q33-qter. Cancer Res 1992;52:746–748.
34 Galland F, Karamysheva A, Mattei M-G, *et al.* Chromosomal localization of FLT4, a novel receptor-type tyrosine kinase gene. Genomics 1992;13:475–478.
35 Armstrong E, Kastury K, Aprelikova O, *et al.* FLT4 receptor tyrosine kinase gene mapping to chromosome band 5q35 in relation to the t(2;5), t(5;6), and t(3;5) translocations. Genes Chromosomes Cancer 1993;7: 144–151.
36 Joukov V, Pajusola K, Kaipainen A, *et al.* A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996;15:290–298.
37 Achen MG, Jeltsch M, Kukk E, *et al.* Vascular endothelial growth factor D (VEGF-D) is a ligand for

the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 1998;95: 548–553.
38 Lee J, Gray A, Yuan J, *et al.* Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. Proc Natl Acad Sci USA 1996;93:1988–1992.
39 Kukk E, Lymboussaki A, Taira S, *et al.* VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development 1996;122:3829–3837.
40 Jeltsch M, Kaipainen A, Joukov V, *et al.* Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 1997;276:1423–1425.
41 Oh S-J, Jeltsch MM, Birkenhager R, *et al.* VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. Dev Biol 1997;188:96–109.
42 Partanen T, Arola J, Saaristo A, *et al.* VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. FASEB J 2000;14:2087–2096.
43 Valtola R, Salven P, Heikkila P, *et al.* VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. Am J Pathol 1999;154:1381–1390.
44 Iljin K, Karkkainen MJ, Lawrence ME, *et al.* VEGFR3 gene structure, regulatory region, and sequence polymorphisms. FASEB J 2001;15:1028–1036.
45 Kondo K, Hiratsuka S, Subbalakshmi E, *et al.* Genomic organization of the flt-1 gene encoding for vascular endothelial growth factor (VEGF) receptor-1 suggests an intimate evolutionary relationship between the 7-Ig and the 5-Ig tyrosine kinase receptors. Gene 1998; 208:297–305.
46 Yin LY, Wu Y, Ballinger CA, *et al.* Genomic structure of the human KDR/flk-1 gene. Mammal Genome 1998; 9:408–410.
47 Karkkainen MJ, Ferrell RE, Lawrence EC, *et al.* Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. Nat Genet 2000;25:153–159.
48 Irrthum A, Karkkainen MJ, Devriendt K, *et al.* Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. Am J Hum Genet 2000;67:295–301.