Angiogenesis Correlates with Vascular Endothelial Growth Factor Expression but not with Ki-ras Oncogene Activation in Non-Small Cell Lung Carcinoma

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ABSTRACT

A total of 195 non-small cell lung carcinoma (NSCLC) specimens were studied for the presence of mutations in their ras family genes, for tumor vascularity, and for their immunostaining pattern with an antibody to vascular endothelial growth factor (VEGF). ras mutation was found in 37 of 104 (34.6%) adenocarcinoma specimens, in 0 of 64 squamous cell carcinomas, and in 2 of 27 (7.4%) large cell undifferentiated carcinomas. All mutations were found on the Ki-ras gene, with 37 (95%) of them on codon 12 and the remaining 2 on codon 13. Thirty (77%) of the mutations were G to T transversions. There was a correlation between increasing tumor vascularity and VEGF immunostaining score, but there was no correlation between either of them with the activation of the ras oncogene. A study of VEGF mRNA expression in 14 NSCLC cell lines also demonstrated a lack of correlation between the constitutive expression levels of VEGF and the presence or absence of ras mutation in these cell lines. The results suggest that VEGF is a major angiogenesis factor in NSCLC but that other factors beside ras mutations may influence tumor vascularity in these tumors. The two parameters may potentially serve as independent prognostic factors in NSCLC.

INTRODUCTION

NSCLC represents approximately 75% of all lung cancers. Its overall prognosis is poor, with a 5-year survival rate of 15%.

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3 The abbreviations used are: NSCLC, non-small cell lung carcinoma; VEGF, vascular endothelial growth factor; ADC, adenocarcinoma; SQCC, squamous cell carcinoma; LCUC, large cell undifferentiated carcinoma; ASOH, allele-specific oligonucleotide hybridization; MGH, Montreal General Hospital.

Even for stage I disease, the survival rate after complete surgical resection remains at 60–70%. Patients whose resected lung tumor shows certain distinct genetic and biological features seem to be at even greater risk of relapse and death from this disease (reviewed in Refs. 1 and 2). Two of these genetic/biological prognostic factors include activation of the Ki-ras oncogene (3–5) and increased tumor vascularity (6–8). The prognostic significance of these markers seems to be independent of the clinical stage and conventional histopathological characteristics such as histological type, degree of differentiation, and proliferative activity of the tumor cells.

Recent reports (9, 10) suggest that the expression of an activated (mutant) ras oncogene results in the up-regulation of the expression of VEGF, which has been shown to be one of the potent angiogenic factors (11, 12). Because both Ki-ras mutation and angiogenesis have been reported to correlate with poor prognosis in early-stage lung cancers, we investigated the possible relationship between Ki-ras oncogene activation, VEGF expression, and tumor vascularity in a series of NSCLC cell lines and primary NSCLC tissue.

MATERIALS AND METHODS

Tissue Material. Frozen tissues and formalin-fixed paraffin blocks of primary NSCLC and corresponding normal lung parenchyma were obtained from lobectomy or pneumonectomy specimens. These were usually sampled immediately, but not later than 2 h after resection. The tissues were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Paraffin blocks were processed according to routine surgical pathological technique. A total of 195 NSCLC tumors were examined in this study: 125 were from specimens resected at MGH, and 70 cases were obtained from the Canadian Lung Tumor Bank established in association with the National Cancer Institute of Canada-Clinical Trial Group BR10 protocol. These included 104 ADCs, 64 SQCCs, and 27 LCUCs. Tumors were classified according to the WHO classification (13) and staged according to the tumor-node-metastasis staging system adopted by the American Joint Committee on Cancer and the Union International Contre Cancer (14).

Cell Lines. The establishment of the RVH-6849 and MGH series NSCLC cell lines has been reported previously (15, 16). These cell lines were cultured in either RPMI 1640 plus 10% fetal bovine serum (R10) or ACL4 media. The cell lines of the National Cancer Institute series and the A549 cell line were cultured in R10 medium. The ACL4 medium was prepared as reported previously (17), but without supplementation with epidermal growth factor.
ras Oncogene and Angiogenesis in Lung Cancers

RPMI 1640 was purchased from Life Technologies, Inc. (Grand Island, NY), and fetal bovine serum was from Wisant (St. Bruno, Quebec, Canada). The establishment of human bronchial epithelial cell line HBE135 has been reported previously (18). HBE135 is routinely cultured in keratinocyte serum-free medium (KSF) supplemented with epidermal growth factor and bovine pituitary extract (Life Technologies, Inc.).

Total RNA Extraction. Total cellular RNA of cell lines was isolated from confluent cultures using the method described previously (18). Northern hybridization was also performed as reported previously (15), using a 3.36-kb VEGF cDNA. The cDNA probe was labeled by [32P]dCTP using an oligolabeling kit from Pharmacia (Piscataway, NJ). The hybridized membranes were stringently washed twice for 30 min at 60°C in 0.2× SSC containing 0.1% SDS. Membranes were exposed to Kodak XAR-5 film at −80°C for 3–5 days.

ras Mutation Analysis. Genomic DNA was extracted from frozen normal lung and tumor tissues as described previously (15). The presence of mutations in Ha-ras, Ki-ras, and N-ras were identified using PCR followed by hybridization with allele (mutant)-specific oligonucleotide probes (the PCR-ASOH method). The PCR amplification primers (amplimers) for exons 1 and 2 of Ki-ras, Ha-ras, and N-ras genes were purchased from Clontech (Palo Alto, CA). A small portion of the PCR product was electrophoresed in a 12% polyacrylamide gel in 1 Tris-borate EDTA buffer at 200 V. The remaining PCR products were dot-blotted onto Hybond-N nylon filter membrane (Amersham Canada, Oakville, Ontario, Canada) using a blotting manifold (Life Technologies, Inc.). On each filter, each amplified product was dot-blotted at 1-, 2-, and 4-μl fractions, and six replicate dot-blot membranes were prepared for each set of products. The membranes were then cross-linked using a UV Stratalinker 1800 (Stratagene, La Jolla, CA).

Individual single-allele mutant probes. However, because we knew in which group of pooled mutant probes the positive signal was found, this second-round hybridization was restricted to the mutant probes belonging to the mixture group. The positive control using the corresponding wild-type probe was once again performed in parallel.

Confirmation of Mutation by Sequencing. Positive ras mutations were confirmed by sequencing. PCR amplification was repeated using the same amplification primers, and the product was separated by electrophoresis. The desired band was isolated and then blunt end-ligated into the pGEM-T vector by using the pGEM-T Easy Vector System (Promega, Wisconsin, IL). The DNA ligation reaction product was used to transform DH5α competent cells (Life Technologies, Inc.). Ten bacterial colonies were randomly selected and screened for the presence of mutant ras gene, using the PCR-ASOH method described above. Plasmid DNA from representative colonies with mutant ras was extracted and sequenced using the T7 Sequencing kit (Pharmacia).

Immunohistochemistry. For each case, 5-μm sections were prepared from a representative formalin-fixed paraffin-embedded tumor tissue of the surgical pathology archival blocks. Immunohistochemical stainings were performed using rabbit polyclonal antibodies to Von Willebrand factor VIII (DAKO, Carpenderia, CA) and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA). The VEGF (147) antibody is a rabbit polyclonal IgG recognizing the 165, 189, and 121 VEGF splice variants. Immunohistochemistry was performed using routine methodology. Before incubation with the primary antibodies, the sections were digested with an aqueous solution containing 0.4% pepsin at 42°C for 5 min. The factor VIII antibody was used at a 1:5000 dilution, and the VEGF antibody was used at a 1:800 dilution. Incubation was for 1 and 16 h, respectively, at room temperature. The secondary antibody was biotinylated goat antirabbit IgG (Zymed Laboratory, South San Francisco, CA) at a 1:200 dilution, which was followed by streptavidin-conjugated horseradish peroxidase (DAKO). The immunoreactivities were revealed by incubation for 10–20 min in a 0.2 μM acetate buffer (pH 5.2) containing 0.0125% of 3-aminio-9-ethyl-carbazole. The slides were then counterstained in Mayer’s hematoxylin and mounted with Crystal Mount (Biomeda, Foster City, CA).

Tumor Vascularity. Immunohistochemical staining of the endothelium for factor VIII was used to reveal blood vessels. The microvessel density of the tumor was assessed by three different methods. The first method was a qualitative assessment of the overall stromal vascularity in the tumor by two of us (M-S. T. and J. V.!). This was classified into three relative grades, with increasing grade representing increasing vascularity. The second method was that described by Weidner et al. (19). After scanning the section, a field of the tumor stroma that showed the highest vascularity was chosen. Care was taken to make sure that the area represented the invasive stroma of tumor and not the normal bronchial mucosa or alveolar septae that are located at the edge of the invasion front of the tumor cells. In the lung, these normal areas are usually more vascular than the tumor stroma. The number of discrete microvessels stained by factor VIII was counted at ×200 magnification. This was also performed simultaneously by two of us (M-S. T. and J. V.) using a multiheaded microscope, and the final count represents the average of our separate scores.
confirmed once again by sequencing. The initial positive hybridization results. All positive results were designed to confirm the prognostic value of Ki-ras gene mutation factor VIII. By comparing the bright-field microscopic image and segmentation image simultaneously, some manual editing was selected based on a positive immunostaining of the endothelium for smooth muscle cells of either the bronchial wall or blood vessels. In cases in which the blood vessel wall stained provided a convenient internal positive control for the quality of immunostaining. In cases in which the blood vessel wall stained normally, the microvessel density using an image analysis system. For each tumor section, four to six of the most vascular areas of the tumor were evaluated. These were usually located in the desmoplastic stroma where tumor cell invasion was most evident, and the same precaution as in method 2 was taken. The blood vessels were counted using a Zeiss Axioskop light microscope fitted with a JVC ky-17 series 3 charge-coupled device color video camera, which was interfaced to a SAMBA 4000 image analysis system with a Matrox 640 frame grabber board (Image Products International, Inc., Chantilly, VA). The offset and gain of the color camera and associated digital board were set with Kohler illumination. An achrostigmat ×10 objective lens was used to yield an image size of 640 × 480 mm or 0.31 mm²/frame. The threshold gray level to segment true blood vessels from background noise was visually selected based on a positive immunostaining of the endothelium for factor VIII. By comparing the bright-field microscopic image and the segmentation image simultaneously, some manual editing was performed to eliminate false signals generated by dust particles, which are commonly present in the lung parenchyma and even within the tumor stroma. The mean blood vessel count from four to six fields was calculated, and the values were expressed as the number of blood vessels/mm². The variation between fields was usually within 10–20%.

**RESULTS**

**Prevalence of ras Mutations.** We used the PCR-ASOH method for ras analysis because of its ease in evaluating possible mutations in three codons and in all three ras genes. Once labeled, the individual oligonucleotide probe or the probe mixtures could be aliquoted and stored for up to 2 months, thus reducing the cost of these ongoing analyses. This became necessary when the analyses were carried out immediately as tumor samples were collected in support of the BR10 clinical trial, a prospective study partially designed to confirm the prognostic value of Ki-ras gene mutation in early-stage NSCLC patients. Furthermore, the two rounds of hybridization, the second one for specific codons, served to confirm the initial positive hybridization results. All positive results were confirmed once again by sequencing. Among the 195 NSCLC tumor samples analyzed, 39 were found to harbor an activated ras oncogene (Table 1). Thirty-seven of these were found among the 104 ADCs analyzed, representing a prevalence of ras mutation in 34.6% of lung ADCs. In contrast, all 64 SQCCs analyzed demonstrated wild-type ras family genes. The prevalence of ras mutation among LCUCs was also low, being found in only 2 of 27 tumors analyzed. Among the ADCs, mutations were found in 39.7% of well-differentiated or moderately differentiated tumors but in only 27.8% of poorly differentiated tumors. This difference, however, is not statistically significant (P = 0.2838). There were also no significant differences in the prevalence among tumors of different T and N stages (Table 1).

All mutations found occurred on the Ki-ras gene, and the overwhelming majority (37 of 39) were on codon 12 (Table 2). Mutations on codon 13 were found in only two tumors, representing 5% of all ras mutations we detected. The most common mutations were G to T transversions (24 TGT and 5 GTT on codon 12 and 1 TGC on codon 13).  

**Tumor Angiogenesis.** An initial assessment to evaluate the method of choice for evaluating tumor stromal vascularity was performed using the 70 tumor samples of the BR10 protocol. We found a highly significant correlation between the subjectively scored vascular grade with the number of vessels counted directly under the microscope (Fig. 1A) and between the latter and the vessel density counted semiautomatically by the SAMBA image analysis system (Fig. 1B). We elected to use the SAMBA counting method for our subsequent analyses of the remaining tumors. This decision was partially based on our concern that counting the number of vessels in a single chosen field might introduce bias into the results. The measurements using the SAMBA system were performed on four to six of the most vascular fields, hence the results were more likely to be representative of the overall vascularity of the tumor. Among the BR10 tumor samples, ADCs were significantly more vascularized than SQCCs (Fig. 1C). There were too few cases of LCUC to make a meaningful comparison between them and ADCs or SQCCs. Sections from an additional 62 ADCs belonging to the MGH cases were also evaluated for their vascularity. Fig. 1D shows that when the ADC cases from both the BR10 and MGH specimens were analyzed, there was no significant difference in the vascularity of ADCs with or without Ki-ras oncogene activation (P = 0.667).

**VEGF Immunohistochemistry.** In normal lung, the smooth muscle cells of either the bronchial wall or blood vessels always stained very strongly for VEGF (Fig. 2, A and C), and this provided a convenient internal positive control for the quality of immunostaining. In cases in which the blood vessel wall stained

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<th>Table 1</th>
<th>The prevalence and clinicopathological characteristics of NSCLC with activated ras oncogene</th>
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<td>ras gene family</td>
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<td>ras</td>
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<td>All NSCLC</td>
<td>156</td>
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<td>ADC</td>
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<td>Well/moderate</td>
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* Fisher's exact test, two-tailed P values.

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<th>Table 2</th>
<th>The ras gene and type of point mutations found among 39 primary NSCLCs</th>
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negatively or weakly, the immunostaining was repeated or the case was rejected. The normal ciliated columnar epithelial cells of the bronchial or bronchiolar epithelium also stained positively but less intensely (Fig. 2A). The staining intensity of these smooth muscle cells and of the bronchial epithelium was used as internal standard to grade the VEGF immunoreactivity of the tumor cells. VEGF immunoreactivity in tumor cells tended to be diffusely cytoplasmic, although occasional tumors showed preferential staining of the basal cytoplasm facing the stroma. The specificity of the immunostaining was confirmed by a complete loss of staining when the antibody was preneutralized with the corresponding control antigen (catalogue number sc-4060WB) purchased from the same company (data not shown).

The immunostaining of VEGF in tumor cells was scored into three grades. Grade 1 (Fig. 2A) represented cases in which tumor cells showed no or focal (usually less than 20% of the tumor cells) positive staining, irrespective of the level of cytoplasmic staining intensity. Grade 2 represented cases that showed diffuse VEGF reactivity in most of the tumor cells, but with a staining intensity similar to that of the bronchial epithelium and distinctly less than that of the smooth muscle cells present in the same section (Fig. 2C). Grade 3 was assigned to cases in which the tumor cells diffusely showed intense positive staining at levels comparable to that in the smooth muscle cells (Fig. 2D). In addition to these categories, some tumors demonstrated very prominent and intense positive staining of the basal lamina and the stroma surrounding the infiltrating islands of tumor cells (Fig. 2B). We isolated these cases and analyzed them separately (designated as ecm+ cases) from tumors with similar VEGF cytoplasmic staining grade but without extracellular matrix staining (Fig. 2A).

The sections from all 70 BR10 tumors and 75 cases of MGH tumors (62 ADCs and 15 LCUCs) that were previously evaluated for their microvessel counts were also evaluated for VEGF immunostaining score. Fig. 2A shows the distribution of microvessel density among the various categories of VEGF score. Among tumors with absent or very focal VEGF immunoreactivity, those with prominent staining of the stromal/extracellular matrix (VEGF 1/ecm+) consistently demonstrated high vascularity, whereas those without stromal staining (VEGF 1) demonstrated the lowest mean microvessel count. Tumors with VEGF scores 2 and 3 showed graduated increases in their mean microvessel density. Concurrent extracellular matrix staining in these tumors (VEGF 2,3/ecm+) also correlated with high microvessel density. The results strongly indicated a correlation between microvascularity in NSCLC and VEGF protein expression as evaluated by immunohistochemistry, and prominent VEGF immunoreactivity in the tumor stroma is also correlated with high tumor angiogenesis.

We then compared VEGF expression among 99 evaluable ADC cases with or without activated Ki-ras oncogene. Fig. 3B

Fig. 1  Vascularity or microvessel density in NSCLC. There is a good correlation among the three methods of assessing vascularity [between subjective vascular grading versus microvessel density as assessed by the SAMBA system (A) and between microvessel density and direct microvessel counts at ×200 magnification (B)]. C, the scattergram shows that the vascularity of ADC is higher than that of SQCC. The vascularity of LCUC is not significantly different from that of ADC and SQCC. D, there is also no significant difference in the microvessel counts of ADCs with (K+) or without (K−) Ki-ras mutation. The two-tailed Ps were obtained using the Mann-Whitney nonparametric test.
demands that when only the levels of cytoplasmic staining were considered, the distribution of VEGF scores was similar between these two groups of ADCs, indicating no correlation between Ki-ras oncogene activation and VEGF expression.

**VEGF mRNA Expression in Vitro.** The mRNA expression of VEGF in 14 NSCLC cell lines and an immortalized normal bronchial epithelial cell line (HBE135) was studied (Fig. 4). The HBE135 cells expressed only the approximately 4.3-kb VEGF mRNA, whereas NSCLC cell lines variably expressed the other alternatively spliced VEGF mRNAs of approximately 3.4-, 2.0-, and 1.8-kb sizes.

The levels and pattern of expression of the various VEGF mRNA transcripts were not correlated with the histological type of the tumor cells. Three cell lines (H520, MGH-13, and A549) expressed VEGF mRNA at levels significantly lower than that of HBE135 cells, whereas eight cell lines (MGH-7, H157, MGH-8, MGH-24, MGH-30, H125, H1264, and RVH-6849) overexpressed the 4.3-kb VEGF mRNA as compared to the HBE135 cells. Three other lines (H226, H358, and H661) showed similar expression levels when compared to the HBE135 cells.

The levels of VEGF mRNA expression were not correlated with activation of ras family genes among these NSCLC cell lines. The highest-expressing cell lines (MGH-24 and H125) had wild-type ras genes, whereas the A549 cell line with mutated ras (Ki-ras codon 12, AGT) demonstrated a low 4.3-kb VEGF transcript but a prominent 3.4-kb VEGF transcript. All of the other ras-mutated cell lines (H157, Ki-ras codon 12, CGT; MGH-8, Ki-ras codon 12, GAT; MGH-30, Ki-ras codon 12, TGT; H358, Ki-ras codon 12, TGT; and H1264, Ki-ras codon 12, CGT) demonstrated VEGF mRNA levels comparable to or slightly higher than that of the immortalized HBE135 cells, which have the wild-type ras gene.

**DISCUSSION**

The present investigation yields several conclusions: (a) our data confirm that the oncogenic activation of ras occurs almost exclusively in ADC and, by an overwhelming majority (>90%), occurs on codon 12 of the Ki-ras gene; (b) there is no correlation between the activation of ras and tumor vascularity or VEGF mRNA/protein expression; and (c) there is a correlation between vascularity of tumor stroma and VEGF protein expression, as assessed by immunohistochemistry.

Rodenhuis and coworkers (20, 21) were the first to report
a high incidence of activation of the Ki-ras oncogene in resected primary ADC of the lung. Their report of the adverse clinical prognostic implication of Ki-ras mutation, independent of stage and tumor differentiation, has been confirmed by other investigators (4, 5). The prevalence of ras mutations reported by various investigators ranged from 10-32% (2-5, 22-25). Except for the results reported by Rosell et al. (23, 26) and Vachenthal et al. (27), all other investigators have found mutations mainly in ADCs. Occasional cases of SQCC tissue and cell lines that harbor activated ras have been reported, but they are distinctly rare (28). Studies that have assayed potential mutations in all three ras family genes have also found that the mutation most commonly observed (90% of cases) occurs on Ki-ras and predominantly occurs on codon 12.

Several techniques have been developed to detect point mutations on the ras oncogene, all based on PCR technique coupled to different methods of mutant detection. The most commonly used technique of detection is the ASOH method, which is the one we used. We obtained a mutation prevalence rate of 35% in ADCs. In an analysis of 280 human lung cancer specimens using this technique, Rodenhuis et al. (3) reported a prevalence of 25.4% (44 of 181) in ADCs, but among smokers, this rate was 30%. No mutation was detected in 48 cases of SQCC analyzed. Using the same technique, but on formalin-fixed and paraffin-embedded tissues, Westra et al. (29) reported a prevalence of 42% in 18 ADCs analyzed. Applying the single-strand conformational polymorphism technique on archival formalin-fixed paraffin blocks, Sarkar et al. (30) detected mutations on K-ras codons 12 and 13 in 30% of ADCs screened but in none of 25 SQCCs studied. Keohavong et al. (31) used denaturing gradient gel electrophoresis to detect mutations and found positive results in 32% of 127 ADCs analyzed; 1 of the 37 cases of SQCC they studied also contained mutation. However, Mills et al. (32), who used the technique of PCR primer-introduced restriction with enrichment for mutant alleles, reported that the prevalence of ras mutation in ADCs may be as high as 56%. The type and location of the mutations that we detected were also similar to all other reports, being most common on codon 12 of the Ki-ras gene, with predominantly a G to T transversion. All together, these data indicate that ras mutations in human lung cancers occur almost exclusively and approximately in one-third of ADCs and very rarely in SQCCs. This suggests three potential hypotheses: (a) the molecular pathogenesis of ADC is different from that of SQCC; (b) the activation of ras genes is less compatible with the growth of transformed lung epithelial cells with squamous differentiation; and (c) the activation of ras dominantly influences the differentiation commitment of transformed lung epithelial cells. These hypotheses may lead to a reevaluation of our concepts on the etiology and pathogenesis of ADC and SQCC of the lung and may provide clues to the mechanisms that determine the differentiation phenotype of lung cancers.

Since the pioneering work of Srivastava et al. (33) and Weidner et al. (19), many studies have confirmed the adverse clinical implication of high neovascularization in cancers of many organs, including lung cancer (6-8). The degree of tumor angiogenesis, as quantitated by counting the microvessel density in the most vascularized area of a tumor after immunostaining of endothelial cells with antibodies to von Willebrand’s factor VIII, CD34, or CD31, has been correlated with the presence and absence of distant metastases in various tumor types (19, 34, 35). The correlation has been confirmed by using an alternative quantitation method using the image analysis systems (36, 37). Our pilot comparative studies have also confirmed that the various techniques to estimate vascularity, including a semiautomated image analysis counting system, correlate well with each other.

The results of our study indicate that the microvessel density or vascularity of NSCLC is not correlated with the activation of ras oncogene but is correlated with VEGF protein expression. Our in vitro data also fail to demonstrate any correlation between the constitutive VEGF mRNA expression levels and the presence or absence of Ki-ras mutation in NSCLC cell lines. This is in contrast to recent reports indicating that activated ras oncogenes may up-regulate the expression of VEGF. Rak et al. (9) reported that when a rat normal intestinal epithelial cell line (IEC-18) expressed a transfected Ha-ras oncogene, the VEGF mRNA expression and VEGF protein
secretion were up-regulated. Furthermore, the disruption of the activated (mutant) Ki-ras allele of two human colon carcinoma cell lines led to a down-regulation of the VEGF mRNA expression and protein synthesis. Grugel et al. (10) also reported that the stable transfection and expression of v-Ha-ras and v-raf in NIH 3T3 cells led to constitutive up-regulation of VEGF mRNA expression. Recent reports indicated that VEGF expression is also induced by insulin-like growth factor I (38) and that tumor vascularity in NSCLC is correlated with the expression levels of platelet-derived endothelial cell growth factor (39). These suggest that regulation of VEGF expression is multifactorial and that ras mutation may not be the dominant regulating factor for VEGF expression in vivo, which would explain the lack of direct correlation between ras oncogene activation and levels of VEGF expression. Nevertheless, the results also suggest that ras mutation and tumor microvessel density may be used as independent prognostic factors in NSCLC, and additional studies should explore the potential synergism between these two parameters in the prognostication of these tumors.

The correlation between vascularity of tumor stroma and VEGF protein levels as assessed by immunohistochemistry is consistent with the evidence that VEGF is the major angiogenic factor in lung cancers (11, 12). There are several alternatively spliced mRNA transcripts for VEGF. These alternatively spliced transcripts produce VEGF protein isoforms of 121, 165, 189, and 206 amino acids. VEGF121 and VEGF165 are the secreted forms, but only VEGF165 is capable of binding to heparin. VEGF189 and VEGF206 are mainly cell-associated, and they display higher heparin affinity than VEGF165. In contrast to VEGF165, VEGF189 seems to be sequestered on cell surface heparan-sulfates and binds to the extracellular matrix of cells. Interestingly, in most of the tumors that we studied, VEGF immunostaining was confined to the cytoplasm of the tumor cells. In occasional cases, however, we observed strong staining of basal lamina lining the islands or glandular structures formed by the cancer cells or intense staining of the tumor stroma but not of the tumor cell cytoplasm. We speculate that the pattern of VEGF immunostaining may reflect the VEGF isoform synthesized by the cells. Whereas most cases of NSCLC produce smaller forms of VEGFs (isoforms 121 and 165) that do not bind extracellular matrix, hence demonstrating only cytoplasmic localization, tumors that demonstrate strong positive VEGF staining of the stromal matrix or basal lamina may also or preferentially secrete the larger forms of VEGF (isoforms 189 and 206). Furthermore, the latter tumors consistently demonstrate very high vascularity, which suggests that the extracellular matrix-binding VEGFs are highly angiogenic. Variability in the expression of the different VEGF mRNA transcripts is also evident among the NSCLC cell lines we studied and seemed not to correlate with the differentiation phenotypes of these tumor cells.

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M S Tsao, N Liu, T Nicklee, et al.


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