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ED-B FIBRONECTIN IN NON–SMALL CELL LUNG CARCINOMA

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□ *Fibronectin (FN), a matrix glycoprotein, has been shown to undergo alternative splicing exclusively during organogenesis and tumorigenesis. One such splice variant, extradomain-B (ED-B) FN, is normally absent in normal adult tissues and is proposed to be a marker of tumoral angiogenesis. The present study was aimed at elucidating whether ED-B FN is expressed in non–small cell lung carcinomas and whether such aberrant expression correlates with tumoral angiogenesis. Frozen tissues from 28 non–small cell lung carcinomas (consisting of both squamous cell carcinomas and adenocarcinomas) along with paired normal tissue samples were collected from the tissue bank collection of the Department of Pathology, London Health Sciences Center, Canada. Frozen tissue specimens were subjected to RNA extraction and real time reverse transcriptase–polymerase chain reaction (RT-PCR) with respect to total and ED-B FN isoform expression. In addition, paraffin-embedded tissue sections from the same cases were collected for histological analysis using ED-B FN antibody. Tumor tissues were further stained with CD34 antibody and analyzed semiquantitatively for tumor microvessel density. The results demonstrate up-regulation of ED-B FN mRNA levels in lung tumor tissues as compared to paired normal tissues. Furthermore, ED-B FN expression was localized specifically to tumor cells and was found to correlate with tumor microvessel density. These findings provide evidence of possible involvement of ED-B FN in pulmonary tumoral angiogenesis. Furthermore, ED-B FN may potentially be used as a diagnostic marker and a target for antiangiogenic therapy.*

Keywords angiogenesis, cancer, ED-B, extracellular matrix, lung

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Non–small cell lung carcinoma (NSCLC) is the leading cause of cancer mortality in North America [1, 2]. Chemotherapy, radiation, and surgery can lead to modest increases in the survival of patients with lung cancer [2, 3]. However, new treatment options are necessary for advanced NSCLC and for patients in which surgery is not amenable. A critical process, which represents the next generation of therapeutic target, is angiogenesis [2–4]. Tremendous enthusiasm in antiangiogenic therapy has paved the path to the identification of over 40 novel targets [3]. Although shown to be efficacious in animal models, antiangiogenic therapy has yet to be established in clinical trials.

The challenges in antiangiogenic therapy arise from our incomplete understanding of the characteristics that distinguish tumor tissue from normal host tissue. Recently, a matrix protein, extradomain-B–containing fibronectin (ED-B FN), has been identified as being exclusively expressed during embryogenesis and tumorigenesis [5, 6]. ED-B has also been shown to be induced in a number of human cancers and in proliferative diabetic retinopathy [5–7]. We have shown re-expression of ED-B FN in vascular endothelial cells in response to mitogenic growth factors [8]. These findings suggest an important role of ED-B FN in both physiological and pathological angiogenesis. Furthermore, expression of ED-B FN in tumoral angiogenesis may provide an efficient means of targeting tumor cells.

Although ED-B FN exhibits restrictive expression in an oncodevelopmental manner, the biological significance of ED-B FN is not fully understood. Using small interfering RNA–based gene silencing, we have shown that ED-B is involved in endothelial cell–derived vascular endothelial growth factor (VEGF) expression and cellular proliferation [8]. Such a biological property of ED-B could promote angiogenesis and tumor metastasis. Evidence also suggests that ED-B FN may have a role in cell adhesion and spreading [9]. Given these findings, and its preferential localization to neoplastic lesions, ED-B FN may have major implications for immunologic imaging in vivo, and in prognosis and therapy [10, 11].

In the present study, we used a sensitive and specific real time reverse transcriptase–polymerase chain reaction (RT-PCR) assay to identify any changes in ED-B FN expression level between tumor and nontumor tissue. We also used immunologic staining to determine the level of ED-B FN protein expression and localization. In addition, we have studied whether a correlation between ED-B FN expression and microvascular density in NSCLC exists.

MATERIALS AND METHODS

Patient Samples

NSCLC tumor samples were obtained with normal tissue counterparts from twenty eight patients with the approval of the Tissue and Archive

Committee through the London Health Sciences Centre tissue bank. The samples comprised of 13 squamous cell carcinoma and 15 adenocarcinoma cases. Frozen and paraffin-embedded tissue sections were used for real time RT-PCR and immunohistochemical analysis, respectively.

RNA Isolation and cDNA Synthesis

RNA was obtained from frozen samples using TRIzol reagent (Invitrogen, Burlington, ON, Canada) followed by the addition of chloroform to extract the aqueous RNA solution [7]. RNA was precipitated using isopropyl alcohol, which was subsequently dissolved in diethylpyrocarbonate-treated water. First-strand cDNA was generated through reverse transcription of RNA (2.5 μg) using the Superscript II system (Invitrogen) and oligo dT primers.

Real Time RT-PCR

RT-PCR analysis of total FN and ED-B FN was performed using the Lightcycler (Roche Diagnostic Canada, Laval, QC, Canada) with SYBR Green I system as described previously [7]. For each specimen, 2 μL of cDNA and 18 μL of reaction mixture comprising of 10 μL SYBR Green Taq ReadyMix (Sigma-Aldrich, St. Louis, MO), 1.6 μL 25 mM $MgCl_2$, 1 μL of both forward and reverse primers (Table 1), and 4.4 μL of H_2O was placed in a glass capillary. mRNA levels were measured by standard curve method and β-actin mRNA expression was quantified to normalize for differences in reverse transcription efficiencies and amount of template within each RT-PCR sample.

TABLE 1 Primer Sequences and PCR Temperature Profile

Gene	Sequence (5′ → 3)	PCR parameters*	
EDB^+ FN	CCGCCATTAATGAGAGTGAT	Denaturation	95°C, 0 s
	AGTTAGTTGCGGCAGGAGAAG	Annealing	55°C, 5 s
		Extension	72°C, 8 s
		Signal	81°C, 1 s
Total FN	GATAAATCAACAGTGGGAGC	Denaturation	95°C, 0 s
	CCCAGATCATGGAGTCTTTA	Annealing	50°C, 6 s
		Extension	72°C, 10 s
		Signal	81°C, 1 s
β-Actin	CCTCTATGCCAACACAGTGC	Denaturation	95°C, 0 s
	CATCGTACTCCTGCTTGCTG	Annealing	58°C, 5 s
		Extension	72°C, 8 s
		Signal	83°C, 1 s

*Initial denaturation was carried out at 95°C, 1 minute. Ramp rate for all PCR phases was 20°C/s.

Singleplex RT-PCR assays were performed to quantify the expression level of total and ED-B FN. In order to accurately compare the expression level between genes, we optimized our total FN, ED-B FN, and β-actin PCR assays to a mean standard curve slope of -3.20 ± 0.32. Equal slope/efficiency allows comparison between singleplex reactions.

After the extension phase of the real-time PCR, product melting temperature (T_m) was obtained in order to distinguish PCR amplicon from nonspecific products such as primer dimers. T_m was determined via melting curve analysis (MCA), allowing the amplicon to anneal at 60°C, followed by a progressive increase in temperature by 0.10°C per second. Signals were then obtained 2°C to 3°C below the determined T_m of the expected amplified product.

ED-B FN Antibody Production

ED-B FN antibody was prepared by immunization of BALB/c mice (The Jackson Laboratory, Bar Harbor, ME). The synthetic human EDB peptide (ITDSSIGLRWTPLNSSTIIGY; [8, 12]) conjugated to keyhole limpet hemocyanin (KLH) (Multiple Peptide Systems, San Diego, CA) was injected (100 μg/animal) in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The antibody has previously been characterized and shown to specifically detect ED-B FN [8].

Immunohistochemistry

For each tumor case, 5 micron thick sections were analyzed for ED-B FN expression and microvessel density using CD34 staining. The samples were deparaffinized in xylene and rehydrated in graded alcohols. For ED-B FN staining, antigen retrieval was carried out by heating the slides in 10 mM citrate buffer (pH 6.0). Slides were blocked with 10% hydrogen peroxidase for 5 minutes. The sections were subsequently incubated for 1 hour at room temperature with mouse anti-human CD34 antibody (1:200; Clone QBEnd 10; Novocastra Laboratories, Newcastle, UK) and ED-B FN mouse antiserum (1:200). After washing, the sections were treated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000; Vector Laboratories, Burlingame, CA). The slides were then scored arbitrarily by 3 investigators. For each tumor sample, 5 fields under the 10× objective were quantified based on the proportion of tumor cells and intensity of the stain. The greatest intensity was assigned a score of three, and a score of 0 was assigned to baseline staining. Microvessels that stained for anti-CD34 were manually counted in tumor samples using light microscopy. Microvessel density was then expressed as CD34-positive cells/400× field.

Statistical Analysis

Data are presented as mean ±SEM. All statistical analyses were performed by ANOVA followed by student's *t* test. A *P* value of less than .05 was considered significant.

RESULTS

ED-B FN mRNA Expression in NSCLC

Frozen normal and tumor tissue were obtained from patients with either lung squamous cell carcinoma or adenocarcinoma. mRNA was obtained and expression of total FN and ED-B FN levels were determined using real time RT-PCR. MCA of post-PCR products showed amplification of single cDNA sequence as assessed by T_m (Figure 1*A*). Compared to normal counterparts, tumor tissue demonstrated a significant upregulation of total FN (Figure 1*B*; $P = .0014$) and relative ED-B FN (ED-B FN:Total FN) mRNA (Figure 1*C*; $P = .0276$). Furthermore, 85% and 82% of all tumor samples showed upregulation of Total FN and ED-B FN mRNA, respectively.

Analysis of tumor tissue revealed no significant difference in total FN mRNA between adenocarcinoma and squamous cell carcinoma (Figure 2). However, relative ED-B FN expression was found to be higher, although not statistically significant, in squamous cell carcinoma compared to adenocarcinoma samples (Figure 2).

ED-B FN Distribution in NSCLC

We next determined the level and pattern of ED-B protein expression by immunohistochemical staining of the tumor samples. Staining revealed a consistently positive stain of ED-B FN in the tumor tissues compared to no staining in normal tissues (Figure 3). Furthermore, anti–ED-B FN antibody showed ED-B FN localization to the tumor compartments and vascular endothelium (Figure 3). Interestingly, squamouse cell carcinoma samples showed modestly higher intensity of ED-B FN staining as compared to adenocarcinoma (data not shown).

Microvessel Density

In order to determine whether ED-B FN expression correlates with tumoral angiogenesis, the tissue sections were subjected to CD34 staining. Linear regression analysis showed a modest, although significant, positive correlation between ED-B FN expression and tumor microvessel density

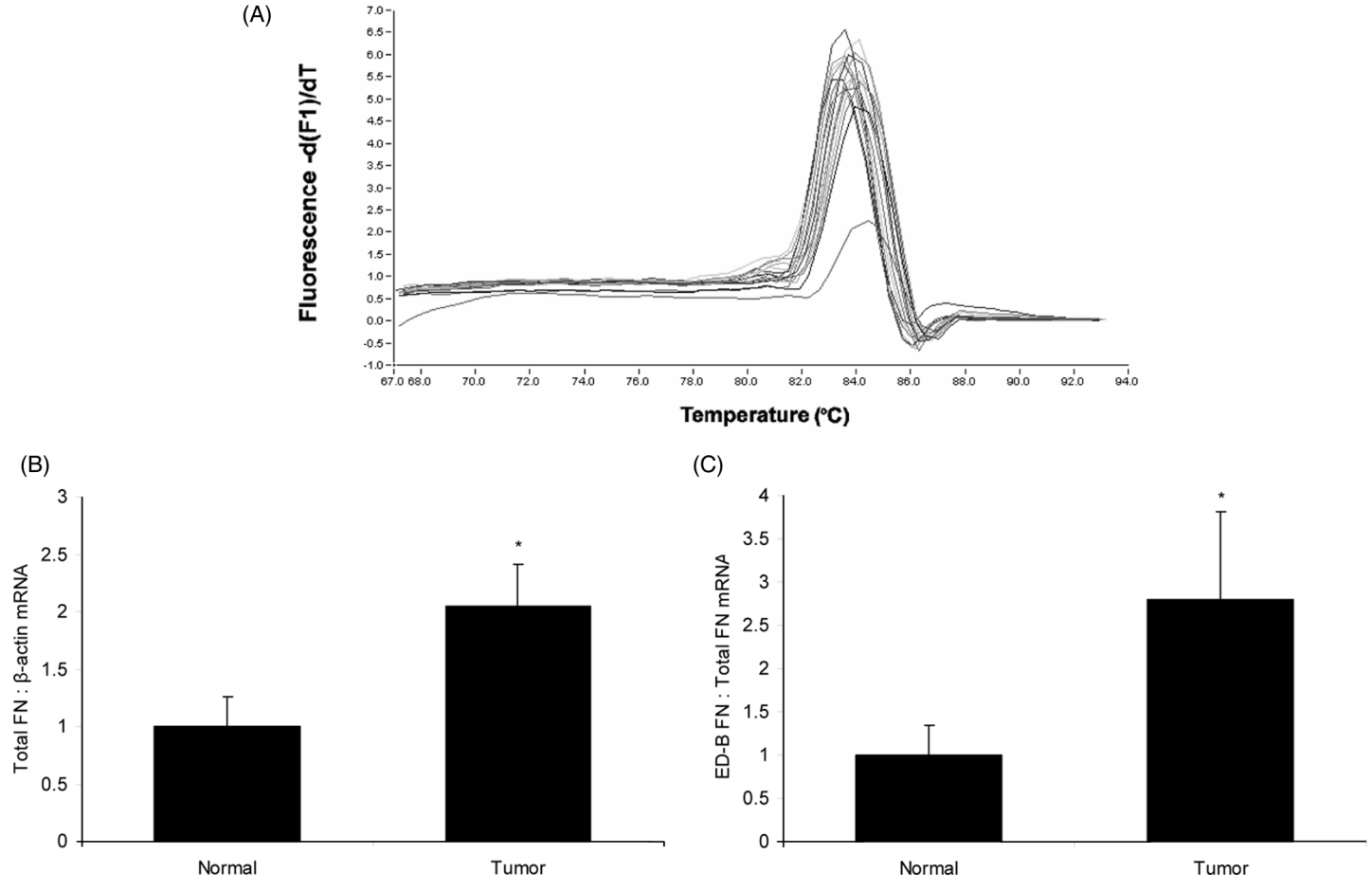

FIGURE 1 ED-B FN mRNA expression. mRNA expression of total FN and ED-B FN as assessed by real time RT-PCR, showing (*A*) MCA of ED-B FN post-PCR products, (*B*) total FN mRNA levels, and (*C*) ED-B FN mRNA levels. MCA was used to determine specificity of PCR. A single peak represents one specific amplicon. (Total FN mRNA is expressed as ratio of FN to β-actin mRNA; ED-B FN mRNA is expressed as ratio of ED-B to total FN mRNA; $^{*}P < .05$ compared to normal counterparts; n = 28 for total FN and 23 for relative ED-B FN).

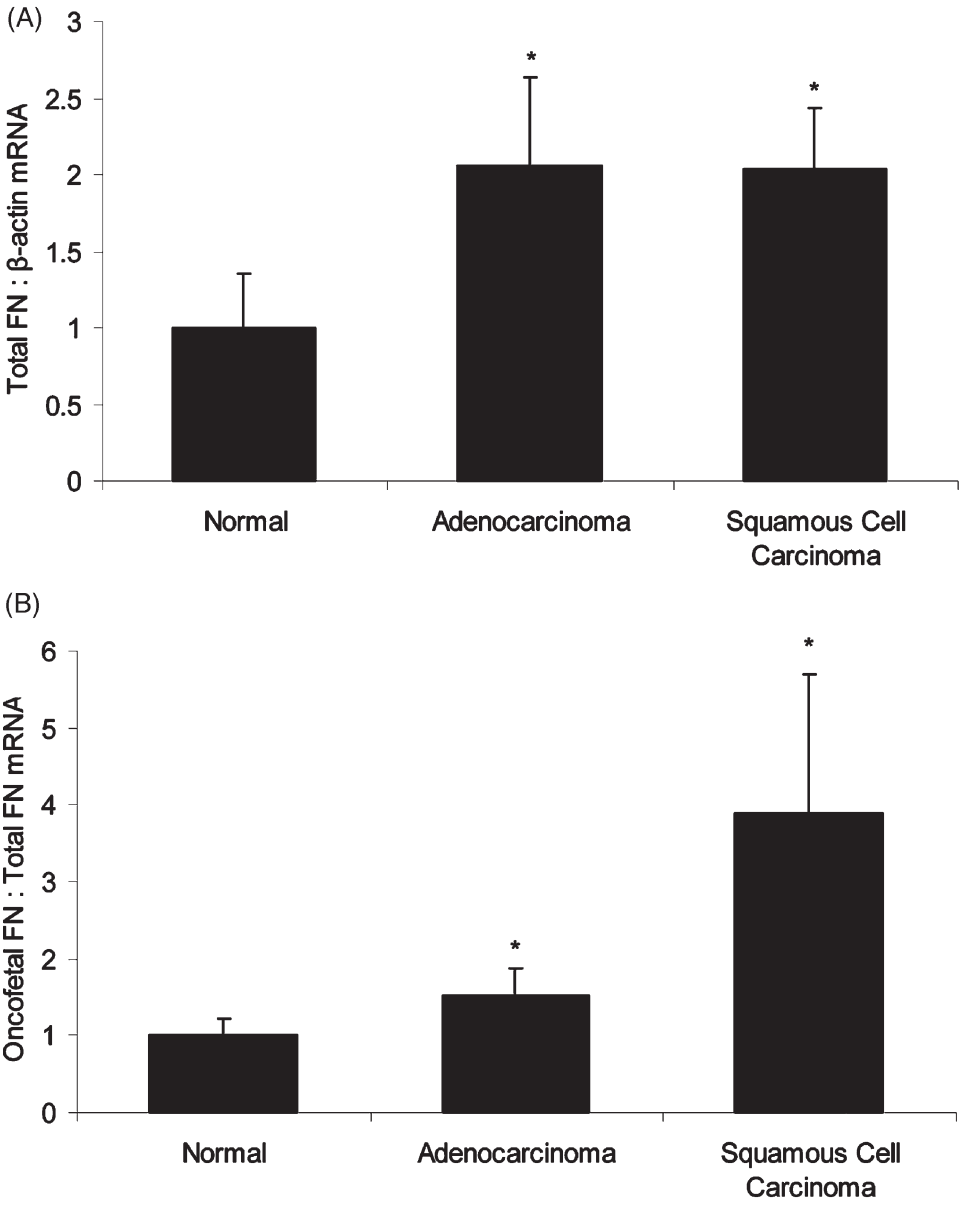

FIGURE 2 ED-B FN mRNA expression in adenocarcinoma and squamous cell carcinoma. mRNA expression of total FN and ED-B FN, showing (*A*) total FN mRNA levels and (*B*) ED-B FN mRNA levels. (Total FN mRNA is expressed as ratio of FN to β-actin mRNA; ED-B FN mRNA is expressed as ratio of ED-B to total FN mRNA; $^{*}P < .05$ compared to normal counterparts; n = 15 for adenocarcinoma and 13 for squamous cell carcinoma).

(Figure 4; $P < .05$). These data suggest a possible role of ED-B FN expression in lung tumor development and progression.

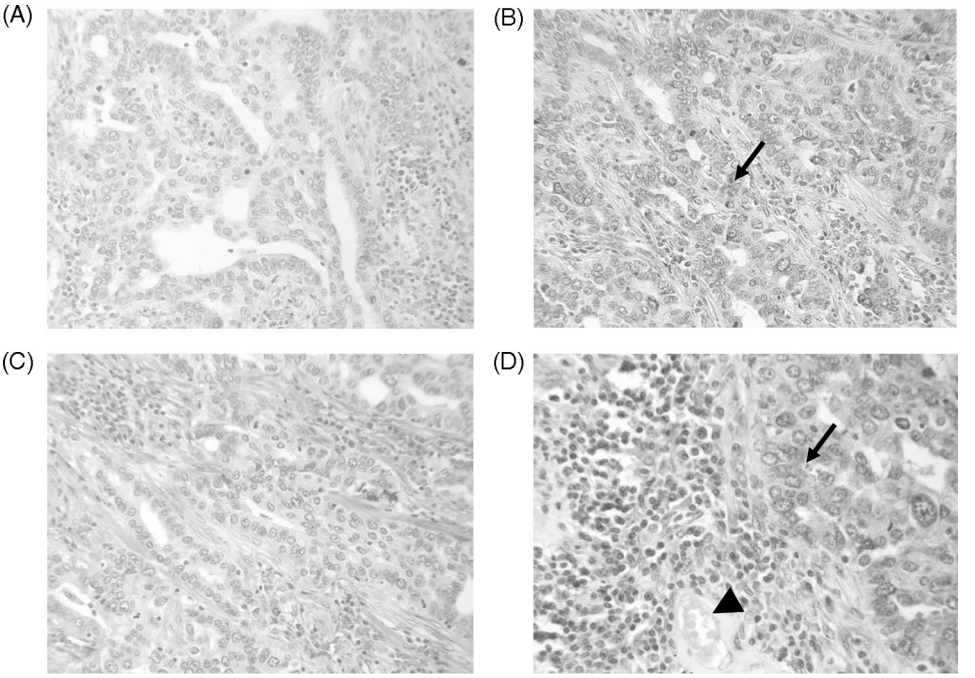

FIGURE 3 ED-B FN protein expression and localization. Immunohistochemical analysis of ED-B FN protein, showing (*A*) negative control, (*B*) positive staining (*arrow*) in the adenocarcinoma cells, (*C*) corresponding hematoxylin and eosin–stained section, and (*D*) higher magnification showing positive staining in the tumor cells (*arrow*) and in the endothelium (*arrowhead*) of the blood vessels (original magnification ×100 in *A* to *C* and ×400 in *D*).

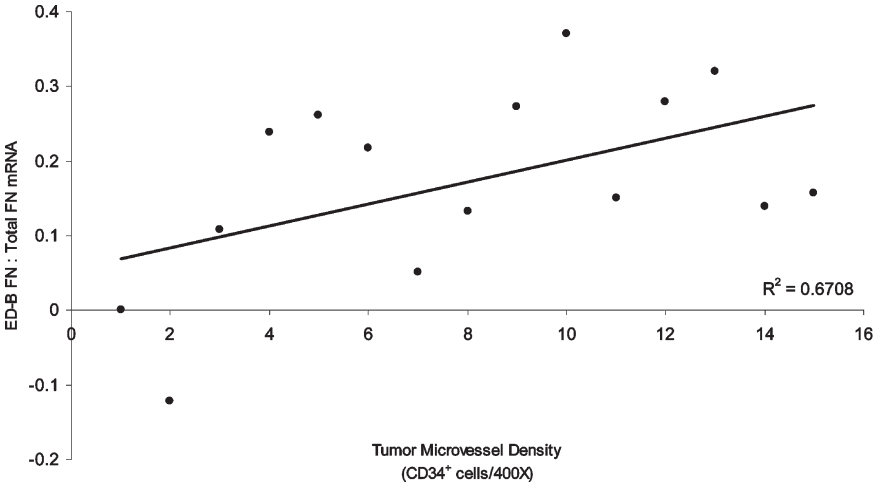

FIGURE 4 Tumor microvessel density. Measurement of tumor microvessel density as assessed by CD34 staining, showing correlation between ED-B FN mRNA and microvessel density. Microvessel density is expressed as number of positive cells per ×400 magnification ($r^2 = .67$; $P < .05$).

DISCUSSION

Although ED-B FN has been shown to be induced in a number of human cancers, little is known about its role in NSCLC. In the present study, we have demonstrated that ED-B FN expression is up-regulated in NSCLC. We have also demonstrated a parallel increase in ED-B FN protein expression specifically in the tumor compartment. Furthermore, ED-B FN expression correlated with tumor microvessel density. These results suggest an important role of ED-B FN in NSCLC that could also serve as a therapeutic target.

The expression ED-B FN has been investigated extensively in a number of human cancers. Preferential expression of ED-B FN in neoplastic blood vessels suggests a potential role of this extracellular matrix protein in angiogenesis. We have previously demonstrated that ED-B FN is also up-regulated in diabetic retinopathy [7], a condition that entails microvascular endothelial cell proliferation and unregulated angiogenesis. Although a possible mechanism by which ED-B FN may mediate vascular cell proliferation and angiogenesis has yet to be completely elucidated, several studies have been directed towards its potential significance in the clinical management of cancer [10]. We have previously shown that ED-B FN is involved in vascular endothelial cell–derived VEGF expression and cell proliferation [8]. Whether ED-B FN causes up-regulation of VEGF in NSCLC requires further investigation. Our studies, however, do show a significant correlation between ED-B FN expression and tumor microvessel density.

To our knowledge, there are very limited findings that address the role of ED-B FN in NSCLC [13]. A recent study has shown increased ED-B levels in various lung cancer cell lines including adenocarcinoma cells [13]. Furthermore, ED-B–expressing cells were shown to be more adhesive as compared to ED-B^{-} cells. Our studies provide further evidence that ED-B FN is induced in samples from patients with NSCLC. However, one interesting contrast between our study and that by Nanki and colleagues [13] is the expression of ED-B in squamous cells carcinoma. Nanki and colleagues concluded that lung adenocarcinoma cell lines produced significantly higher levels of ED-B FN as compared to the squamous cell carcinoma cell lines in vitro. Our study with human NSCLC, however, demonstrates that ED-B FN mRNA and protein expression is modestly higher in squamous carcinoma compared to adenocarcinoma tissue. It is plausible that bystander cells may contribute to ED-B FN expression. However, our immunhistochemical analyses revealed only tumor cell–specific expression. These findings, therefore, could be attributed to possible aberrant behaviour of lung cancer cell lines in culture. Studies of squamous cell carcinoma of the head and neck have revealed prominent ED-B expression in the stroma with a modest increase in the tumor cells [14]. Our studies,

however, suggest a more pronounced role of the tumor cells in ED-B expression as compared to the stroma. Although the contribution of the stroma cannot be ruled out, it is plausible that depending upon the nature of the tumor cell and the microenvironment, the source of ED-B FN may be different. It should also be noted that regardless of the source of ED-B FN, the assembly of this embryonic FN isoform by the vascular endothelial cells may very well represent a more important event in the process of angiogenesis. A recent study of cultured endothelial cells supports such a notion [15]. It has been shown that microvascular endothelial cells express and assemble ED-B FN into fibrils [15]. Furthermore, endothelial cells possess the ability of assembling exogenously administered nonendothelial ED-B FN [15]. These findings may provide important cues for targeting tumor endothelial cells.

The exclusivity of ED-B FN expression during angiogenesis may prove to be invaluable in identifying patients with poor prognosis and survival. A recent study aimed at determining the possible association between ED-B FN expression and clinicopathological parameters and patient survival has shown some promise [14]. In squamous cell carcinoma of the head and neck, ED-B FN expression was shown to be associated with tumor grade and shorter time to tumor progression. Furthermore, the overall survival rate of patients with ED-B–positive tumor was lower [14]. These findings suggest that ED-B FN may be re-expressed during tumoral angiogenesis and may provide a novel target for therapy. Our studies do support such a notion. We have shown that tumor cells in NSCLC express ED-B FN, which was also positive in the endothelium. It is interesting to note that other FN isoforms, such as extradomain-A FN (ED-A FN), may play a role in the pathogenesis of NSCLC.

Although, ED-A FN does not exhibit the same restrictive pattern of expression as ED-B FN, it is plausible that ED-A may play a role in NSCLC distinct from that of ED-B. However, such a notion requires further information.

In summary, our results have shown increased expression of both ED-B FN mRNA and protein in lung tumor tissue. Correlative increase of ED-B FN mRNA expression in tumor cells with microvascularity is additional support for a possible involvement of this embryonic protein in the development of NSCLC and angiogenesis. Our results provide novel avenues for the development of adjuvant therapeutic modalities for NSCLC.

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