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RESEARCH ARTICLE

VEGFR-2 IN LATE STAGE/HIGH GRADE SQUAMOUS CELL CARCINOMA OF THE LUNG: A COHORT STUDY

[1]Qazi Danish, [1]Taseem A. Mokhdomi, [1]Shoiab Bukhari, [2]Azra Shah, [*1]Raies A. Qadri

[1]Department of Biotechnology, University of Kashmir, Srinagar, J and K, 190006, India
[2]Department of Pathology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar, J and K, 190011 India

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ABSTRACT

Background: VEGFR-2 is a critical molecule for tumor angiogenesis and a target for assessment of anti-angiogenic therapy. Although, its expression and functionality has been correlated in various cancers, not many studies are available in the context of lung cancer.
Patients and Methods: In this study, the expression of VEGFR-2 was analyzed in clinically relevant tissue samples of Non-Small Cell Lung Cancer (NSCLC) patients (n=108, surgically resected archival tumor samples) with histopathologically confirmed Squamous Cell Carcinoma (SCC) sub-type and compared with appropriate controls. Expression of endogenous level of VEGFR-2 was assessed by Western blotting and densitometry.
Results: Clinicopathological data suggested that the incidence of SCC in local population with respect to age, sex, tumor stage and histology was typical and consistent with reported global incidence of NSCLC. We observed differential expression of VEGFR-2 based on the staging of tumors. Poorly differentiated SCC samples show higher expression of VEGFR-2, expression levels of VEGFR-2 in moderately differentiated SCC were comparable while expression was lower in well differentiated SCC samples when compared to their available respective controls. Further corroborating this observation, external control samples (non-tumor patient samples) expressed VEGFR-2 levels comparable to patient tissue controls in turn pointing to a possible stage-based prognostic significance for SCC.
Conclusion: Our results highlight a strong correlation of VEGFR-2 expression, with late stage SCC compared to early stage SCC. Thus, VEGFR-2 may be actively involved in late progression of SCC, which constitutes a major chunk of NSCLC and may also have prognostic and therapeutic relevance.

INTRODUCTION

Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) or fetal liver kinase-1 (*flk*-1) or Kinase –domain region (*KDR*), is a major receptor for signaling in endothelial cells and mediates the angiogenic and permeability enhancing effects of VEGF (Ferrara *et al*., 2003). Within the cell, VEGFR-2 protein is produced as a 150 kDa protein which is rapidly processed to a mature 230 kDa form, and expressed on the cell surface (Takahashi and Shibuya, 1997). Vascular endothelial growth factor (VEGF) produced from tumor cells induce activation of VEGF/VEGFR signaling axis in endothelial cells and tumor cells instituting neovascularization and cancer development. Thus, targeting angiogenesis has become a key therapeutic strategy for cancer treatment (Kerbel, 2008; Weis and Cheresh, 2011) with the advent of monoclonal antibody against VEGFR-2 which were first reported to show suppressive effect on solid tumor growth in mice (Lyden *et al.,* 2001). Furthermore, a report suggests that oral administration of a bacterial-type vector (non-toxic *Salmonella typhimurium*) containing VEGFR-2-expressing vector DNA induces an immune response to VEGFR-2, leading to an efficient inhibition of solid tumor growth in the immunized animals (Niethammer *et al*., 2002). Seto *et al.* was first to examine the prognostic value of the expression of VEGF and of the VEGF-Rs, Fms-like tyrosine kinase receptor-1 (flt-1) and kinase insert domain-containing receptor (KDR) in NSCLC and their data suggested that expression of VEGF and VEGF-Rs are associated with a poor prognosis via autocrine and paracrine growth stimulation of cancer cells. Moreover, tumors expressing higher flt-1 and KDR had greater malignant potential associated with poor prognosis of the disease (Seto *et al*., 2006). Furthermore, the synergism of ligands for VEGFR-1 and VEGFR-2 are indicative of a molecular ''cross-talk'' allowing modulation of a variety of VEGFR dependent signals (Carmeliet *et al*., 2001). The family of VEGF receptors is thus, critical for tumor angiogenesis. Among the family of VEGF receptors, VEGFR-2 is the most important and functionally active receptor. Very few studies have been done to study the expression and significance of VEGFR-2 in NSCLC (Pajares *et al*., 2012).

Corresponding author: Raies A. Qadri
Department of Biotechnology, University of Kashmir, Srinagar, J and K, 190006, India.

So far, no such study has been done in the local population of Kashmir valley. To identify and establish the probable importance of VEGFR-2 in lung tumorigenesis, we study the expression of VEGFR-2 in clinical samples of Squamous cell carcinoma subtype of lung cancer by Western blot analysis, a technique profoundly used in such expression studies (Bashir *et al*., 2010).

MATERIALS AND METHODS

Patient and control tissue samples

Surgically resected tissue samples of clinically diagnosed lung cancer patients preserved after formalin fixation and embedding were collected from Department of Pathology, Sher-i-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, J&K, India after proper permission from the Institutional Ethics Committee (SKIMS) and pre-informed consent from patients. The surgeries were performed from March 2007 to October 2009. The tumor samples (n=108) included tissue embedded blocks containing histopathologically confirmed tumor tissue. Adjacent normal tissue (parenchyma at least 3cm away from tumor area) was also resected in some cases (n=60) from the same patients diagnosed with tumor, which corresponded for respective control samples. All the samples in this study were from those patients who had not received any radio- or chemotherapy prior to resections. Additionally, FFPE sections of patients with other lung diseases (non-specific) were collected (n=24) to be analyzed as external controls.

Antibodies

VEGF receptor 2 (55B11) rabbit monoclonal antibody (Cat. No. 2479) Cell Signaling Technology, Inc.; Actin Mouse monoclonal antibody (Cat. No. CP-01) Calbiochem, Inc.; Horse-radish peroxidase (HRP) conjugated anti-Rabbit IgG (Cat. No. AB-21) Pierce (Fisher), Inc.; HRP conjugated anti-Mouse IgG (Cat. No.115-035-003) Jackson Laboratory; VEGF Receptor-2 Control Proteins (Cat. No. #2904) Cell signaling technology, Inc.

Protein Extraction

Protein extraction from FFPE section was done by Antigen retrieval method. ~10 mg sections were cut from FFPE specimens avoiding paraffin, using a fresh scalpel for each sample. Sections were incubated in three washes of Xylene for 5 minutes each. Then sections were given three washes of 100% ethanol, followed by three washes each of 95% ethanol, 90% ethanol, 70% ethanol and 50% ethanol, in that order for with each wash for 5 minutes. Finally sections were washed twice in dH_20 for 5 minutes each. After rehydration, sections were treated with 500µl Protein Extraction buffer (SDS lysis buffer-pH 7.5) and boiled for 30 minutes. After cooling, the samples were centrifuged at 10000 rpm for 10 minutes. The supernatant collected was quantified for protein content and used as protein extract.

Protein estimation

Protein concentrations were determined using the Micro BCA^{TM} (bicinchoninic acid) protein assay kit (Pierce, USA). The assay was performed according to the instructions provided by the manufacturer. The dilutions of the sample were made in PBS and mixed with equal volume of reagent mix (B. C. A.: 24: 1: 25). The plate was incubated at 37°C for 1 hr and absorbance was measured at 540 nm. BSA of known concentration provided with the kit was used as a standard.

Western Blot

Western blot was carried out by the method described by Towbin *et al.* (1979). The sample to be analyzed separated in a 8% or 10% SDS-PAG and transferred to a nitrocellulose (NC) membrane (MDI, India) at a constant current of 300mA for 2 h using a Bio-Rad transfer apparatus (BioRad, USA). The transfer of proteins was ascertained by staining the NC membrane with Ponceau-S (1X). The membrane was blocked for 1 h at room temperature with 1% non-fat milk protein prepared in TBS or PBS and subsequently probed with the appropriate primary antibody, followed by HRP-labeled secondary antibody and developed using Enhanced Chemiluminescence reagents (Amersham Biosciences, USA).

Densitometry

The blots were scanned and the bands were quantitated using Quantity One software (Ver. 4.5.0 BioRad, Hercules, CA). The level of VEGFR-2 expression was quantified by calculating the densitometric reading of the bands for VEGFR-2 after normalization of β-actin across the blots. The data were expressed as percentage change in expression of VEGFR 2 in tumor tissues compared to their respective control tissues. The β-actin values did not usually differ more than 1.5-fold. The quantification was independently confirmed by Odyssey (Li-Cor) quantification software.

Statistical Analysis

Statistical analysis was done using Graph Pad Prism (Ver. 5.0) software. For case controls studies the data were analyzed using t-test (one-tailed). For comparative studies, data were analyzed by One Way ANOVA with multiple comparisons using Dunnetts test. Data were represented as Mean (SEM) and values were considered as statistical significant for $P < 0.05$ from at least three independent experiments.

RESULTS AND DISCUSSION

Clinico-pathological Analysis

Table 1 gives the histopathological and clinical details of the resected lung carcinoma samples. The clinical details of resected samples from non-tumor diseases (external controls) are provided in Table 2. The number of samples and age of patients for each sub-type based on tumor grade is comparable in the cohort. An overwhelming majority of samples are males, which could be due to poor reporting rather than actual incidence. The peculiar feature of these patients was that all were smokers or ex-smokers, which meant a strong correlation of smokers with Lung SCC incidence. The non-tumor controls resections were from patients suffering with Aspergilloma, Bronchiastiasis etc.

Table 1. Interpretation of Clinical Data of Tumor samples with their available controls

Clinical details of patients diagnosed for scc of the lung (n=108)						
Patients with SCC of the lung	PD		MD		WD	
	n	%	n	%	n	%
	30	27.7	42	38.88	36	33.33
Average Age*	57(5.70)		58.85(7.86)		52.83(9.36)	
Sex						
Males	30	100	36	85.72	36	100
Females	-		6	14.28	-	
Smoking status						
Current smokers	24	80	30	71.43	30	83.33
Ex smokers	6	20	12	28.57	6	16.67
Controls available	18	60	30	71.42	12	33.33

*Mean Age (Standard deviation); PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated

VEGFR-2 expression in Poorly Differentiated Squamous Cell Carcinoma (SCC) of Lung

In the study presented here, we analyzed the expression levels of VEGFR-2 in the tumor samples and their controls. Paraffinization and formalin fixation of tissues, routinely followed in pathological evaluations, are known to deglycosylate and crosslink the proteins. Heat antigen retrieval of protein extraction overcomes the cross linking phenomenon but deglycosylation cannot be undone. Upon western blotting of tissue extracts, a single band corresponding to ~150 kDa was detected by the antibody and we know that VEGFR2 is endogenously expressed as a 150 kDa protein before glycosylation and other posttranslational modifications. Western blots showed varied expression as per the staging and type of the tumor.

Table 2. Interpretation of Clinical Data of non-specific controls

Clinical details of control patients diagnosed for non-tumor diseases (nonspecific controls), n = 36		
Patients with Non-Tumor Diseases	n	%
	36	100
Average Age*	42.16(8.9)	
Sex		
Males	18	50
females	18	50
Smoking status		
Current smokers	36	100
Ex smokers	-	-

*Mean Age (Standard deviation)

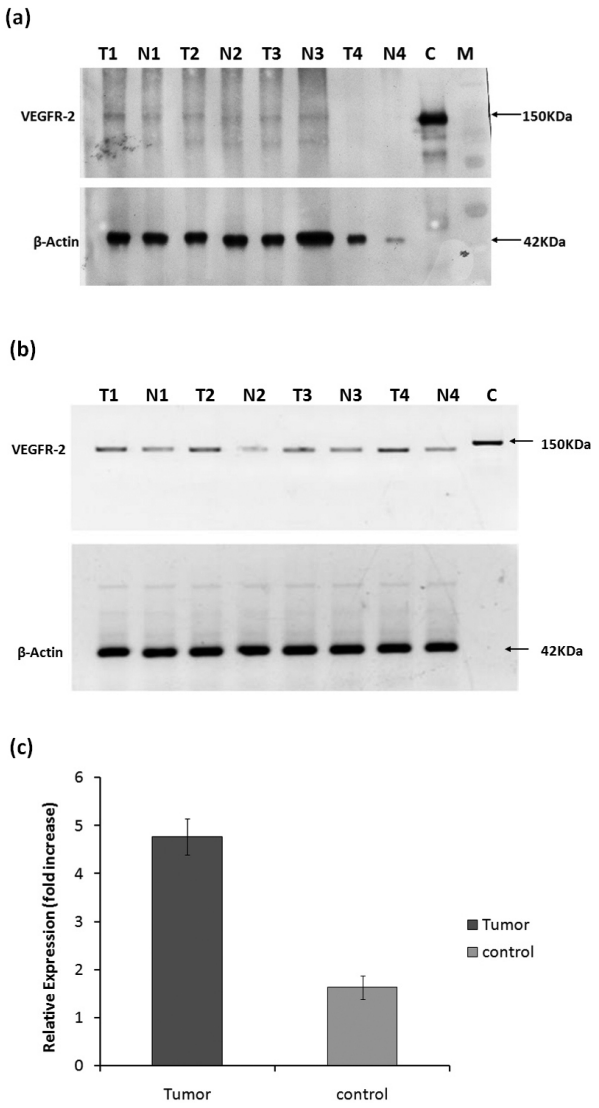

Figure 1. VEGFR-2 Expression in Poorly differentiated lung SCC. (a) Representative Western blots; (b) Western blots after normalised β-actin levels Lanes T1, T2, T3, T4 represent Poorly differentiated Lung SCC randomly selected samples while N1, N2, N3, N4 represents normal control lung tissue from the same patients, Lane C represents VEGFR-2 control proteins, Lane M is the marker lane; (c) Histograms, showing the expression of VEGFR-2 in Poorly Differentiated SCC samples and respective controls. Relative Expression (Fold increase) represents band intensity of VEGFR-2 in comparison to normalized actin.

A high grade tumor is histologically represented by tumor cells which are poorly differentiated and have least resemblance to normal tissue (Towbin *et al*., 1979). This type of tumor is highly aggressive and therapeutically resistant, hence has very poor prognosis (AJCC, 2002). The actual expression of β-actin varied across samples invariably owing to the heterogeneity of tissue in the resections, when loading equal protein concentrations Figure 1(a).

We did try other known loading controls like Tubulin, total ERK, Histone-H3 (data not shown) but all showed varied expression, hence we stuck to β-actin as normalizing control. Using Densitometry to assess the band intensities for VEGFR-2 and normalizing to actin levels, we evaluated the expression of this receptor Figure 1(b). Figure 1 (b) represents sample

protein concentrations loaded such that β -actin levels are equal i.e. normalization of β-actin levels. Statistical analysis implied that Poorly differentiated SCC samples showed higher expression of VEGFR-2 compared to their respective controls Figure 1(b), (c). Histogram in Figure 1(c) shows relative expression of VEGFR-2 as fold increase in Poorly differentiated tumors in comparison to their controls.

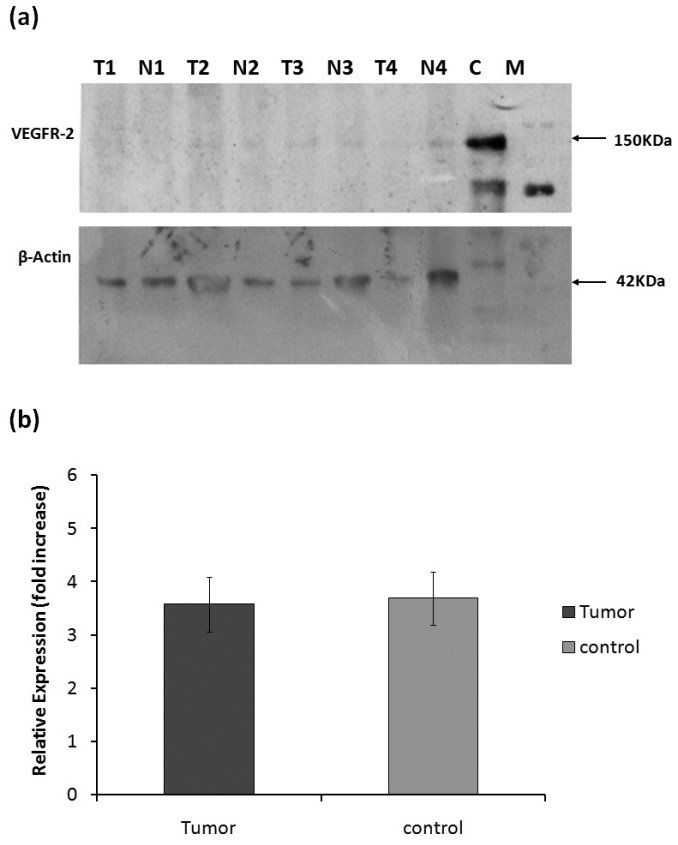

Figure 2. VEGFR-2 Expression in Moderately differentiated lung SCC. (a) Representative Western blots Lanes T1, T2, T3, T4 represent Moderately differentiated Lung SCC randomly selected samples while N1, N2, N3, N4 represents normal control lung tissue from the same patients, Lane C represents VEGFR-2 control proteins, Lane M is the marker lane; (b) Histograms, showing the expression of VEGFR-2 in Moderately Differentiated SCC samples and respective controls. . Relative Expression (Fold increase) represents band intensity of VEGFR-2 in comparison to normalized actin

VEGFR-2 expression in Moderately Differentiated and Well Differentiated Squamous Cell Carcinoma (SCC) of Lung

Well differentiated and moderately differentiated carcinomas are less aggressive than those of poorly differentiated ones and as such have better prognosis (AJCC, 2002). To compare VEGFR-2 expression in these subtypes, we blotted for VEGFR-2. In Figure 2(a), actual VEGFR-2 expression is compared to β-actin expression in selected moderately differentiated SCC and moderately well differentiated SCC representative samples by Western blotting (equal protein concentrations are loaded). Though feeble levels of VEGFR-2 are visible in the blots, the levels are evaluated by Densitometry after normalization and relative expression calculated.

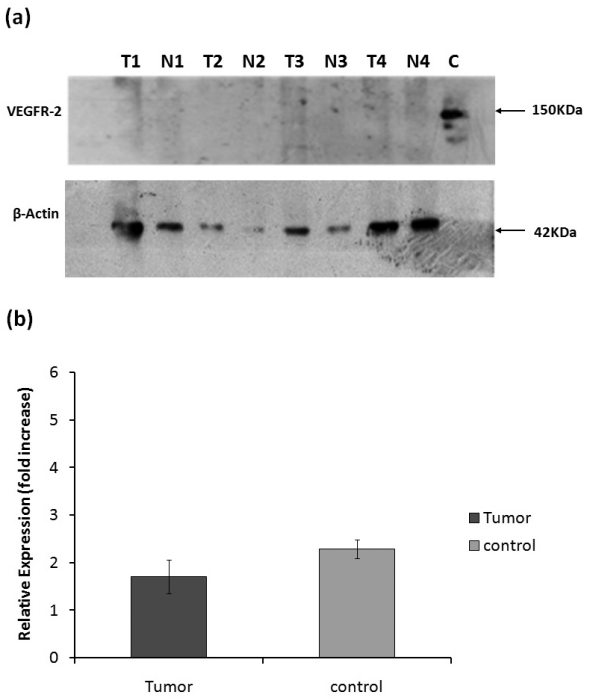

Figure 3. VEGFR-2 Expression in Well differentiated lung SCC. (a) Representative Western blots Lanes T1, T2, T3, T4 represent Moderately differentiated Lung SCC randomly selected samples while N1, N2, N3, N4 represents normal control lung tissue from the same patients, Lane C represents VEGFR-2 control proteins, Lane M is the marker lane; (b) Histograms, showing the expression of VEGFR-2 in Well Differentiated SCC samples and respective controls. Relative Expression (Fold increase) represents band intensity of VEGFR-2 in comparison to normalized actin

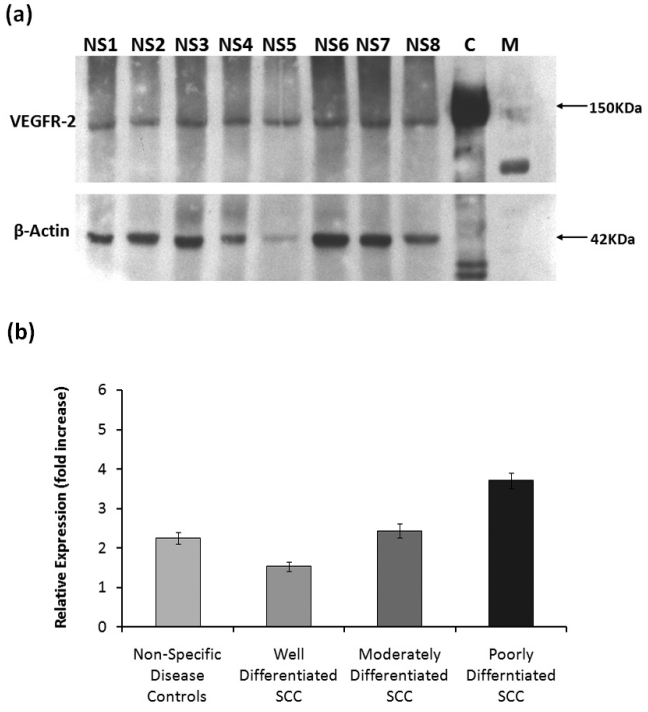

Figure 4. Comparison between stage wise expression of VEGFR-2 in SCC samples and Non-specific disease controls (external controls). (a) Representative Western blots of VEGFR-2 in Non-tumor patient controls Lanes NS1, NS2, NS3, NS4, NS5, NS6, NS7, NS8 represents normal control lung tissue from the same patients, Lane C represents VEGFR-2 control proteins, Lane M is the marker lane; (b) Histograms, showing comparative expression of VEGFR-2 in various stages of SCC and Non-tumor patient controls. Relative Expression (Fold increase) represents band intensity of VEGFR-2 in comparison to normalized actin

Densitometry followed by Statistical analysis (t-test comparison) implies that the expression of VEGFR-2 in tumor tissue is comparable to VEGFR-2 expression in autologous control tissue, represented in the histogram Figure 2(b). Figure 3(a) depicts western blot for VEGFR-2 and βactin levels in selected well differentiated SCC samples. Very low or negligible levels of VEGFR-2 are detected in well differentiated SCC tissue and their autologous controls. When compared, after normalization to β-actin levels, the tissue from well differentiated SCC showed lower expression than the available controls, shown as relative fold change in the histogram of Figure 3(b). Evaluation of the blots show varied expression as per the staging and type of the tumor. Poorly differentiated SCC samples show higher expression of VEGFR-2 compared to their respective autologous controls. Moderately differentiated SCC showed comparable expression of VEGFR-2 in tumor samples (most cases) compared to their available respective controls. Moderately well differentiated SCC, often grouped together with moderate SCC, seemed to show similar profile of VEGFR-2 in tumor samples against available control samples. Well differentiated SCC samples or their controls expressed VEGFR-2 in very low or negligible levels. Thus, the higher expression of VEGFR-2 in poorly differentiated SCC than moderately differentiated SCC than well differentiated SCC, in that order, points out to need for excess VEGFR-2 in tumor progression and possibly metastasis.

Comparative expression of VEGFR-2 in SCC tumors and non-specific disease controls

To verify the observed expression of VEGFR-2 in tumor tissue when compared to their autologous controls, we decided to likewise compare this expression with lung resected tissue from patients having non-tumor diseases, all of whom were smokers. Figure 4(a) shows western blot which represents VEGFR-2 and β-actin expression in other (non-tumor) lung disorders (external controls) compared to a tumor sample (Moderately differentiated SCC) and its control. VEGFR-2 expression on its own is comparable in all the samples. Calculating the intensities of VEGFR-2 bands by densitometry and normalizing to β-actin levels of Non-specific control samples shows that the levels of VEGFR-2 in them are comparable to moderately differentiated sub-type of Squamous cell carcinoma. Statistical Analysis proves that the expression in non-autologous controls and well differentiated SCC are comparable Figure 4(b). Indeed, the VEGFR-2 levels expressed in non-tumor lung tissue were comparable with control (normal) lung tissue from tumor patients. It would be fair to compare VEGFR-2 levels in non-tumor lung with tumor samples. The comparative representation of the calculated VEGFR-2 levels in SCC samples by Densitometry and Statistical Analysis (Dunnet's test; one way ANOVA) of the blots, Figure 4(b), shows a peculiar and interesting stage based expression of VEGFR-2, pointing to a possible role of VEGFR-2 in tumor progression and suggesting a diagnostic application of VEGFR-2 expression in Non Small Cell Lung cancer. Although, a diagnostic implication of this study is limited as most of the tumor resections were in stage II of cancer development.

Conclusion

This study points to a role of VEGFR-2 in possible prognosis of Squamous Cell carcinoma of lung. It also emphasizes the rethinking of implications of anti-angiogenic therapy based on VEGFR-2 expression at various stages or grades of tumor development. Currently, there are no validated molecular predictive biomarkers for patients undergoing anti-angiogenic therapy (Gerger *et al*., 2011). Because only a minority of patients achieve benefit with increased risk of toxicity, the establishment of predictors for response is critically important. Validation of Western blot analysis technique promises to be an easy and accessible tool for evaluation of NSCLC. In this regard, further studies become imperative to look into the aspects of specific localization of VEGFR-2 in tissues by immunostaining etc. and testing the functional significance of this varied expression of the receptor in clinical samples. A comparison with other sub-types of NSCLC would also be helpful in deducing expression and significance of VEGFR-2. Comprehensive studies on expression and significance of VEGFR-2 and its signaling in lung cancer, especially NSCLC, may provide new insights in tumor development and possible specific therapeutic targeting for lung cancer, the highest prevalent cancer worldwide (Jemal *et al*., 2011).

Competing Interests

The author's declare that they have no competing interests.

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Abbreviations

NSCLC: non-small cell lung cancer, SCC: Squamous Cell carcinoma, VEGF: Vascular Endothelial Growth Factor, VEGFR: Vascular Endothelial Growth Factor Receptor.

REFERENCES

American Joint Committee on Cancer. AJCC Cancer Staging Manual. 6th ed. New York, NY: Springer, 2002

Bashir, M., Kirmani, D., Bhat, H.F., Baba, R.A., Hamza, R., Naqash, S., *et al*. 2010. P66shc and its downstream Eps8 and Rac1 proteins are upregulated in esophageal cancers. *Cell Commun Signal*., 8:13.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V. and De Mol, M., *et al*. 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med*., 7: 575–583.

Ferrara, N. 2010. Vascular endothelial growth factor and age-related macular degeneration. From basic science to therapy. *Nat Med*., 16: 1107-1111.

Ferrara, N., Gerber, H.P. and LeCouter, J. 2003. The biology of VEGF and its receptors. *Nature Medicine,* 9: 669–676.

Gerger, A., LaBonte, M., Lenz, H.J. 2011. Molecular predictors of response to antiangiogenesis therapies. *Cancer J.,* 17:134-141.

Herbst, R.S., Onn, A. and Sandler, A. 2005. Angiogenesis and lung cancer: Prognostic and therapeutic implications. *J Clin Oncol*., 23: 3243-3256.

Jemal. A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D. 2011. Global Cancer Statistics. *CA Cancer J Clin.,* 61:69–90.

Kerbel, R.S. Tumor angiogenesis 2008. *N. Engl. J. Med*., 358: 2039-2049.

Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P. and Butros, L., *et al* 2001. Impaired recruitment of bone marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med*., 7: 1194–1201.

Niethammer, A.G., Xiang, R., Becker, J.C., Wodrich, H., Pertl, U. and Karsten, G., *et al*. 2002. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat Med.,* 8: 1369–1375.

Pajares, M.J., Agorreta, J., Larrayoz, M., Vesin, A., Ezponda, T., Zudaire, I., *et al*. 2012. Expression of Tumor-Derived Vascular Endothelial Growth Factor and Its Receptors Is Associated With Outcome in Early Squamous Cell Carcinoma of the Lung. *J Clin Oncol*., 30: 1129-1136.

Seto, T., Higashiyama, M., Funai, H., Imamura, F., Uematsu, K. and Seki, N., *et al*. 2006. Prognostic value of expression of vascular endothelial growth factor and its flt-1 and KDR receptors in stage I nonsmall-cell lung cancer. *Lung Cancer,* 53: 91-96.

Takahashi, T. and Shibuya, M. 1997. The 230 kDa mature form of KDR/Flk-1 (VEGF receptor¬2) activates the PLCγ pathway and partially induces mitotic signals in NIH3T3 fibroblasts. *Oncogene,* 14: 2079–2089.

Takahashi, T., Yamaguchi, S., Chida, K. and Shibuya, M. 2001. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC and DNA synthesis in vascular endothelial cells. *EMBO J*., 20: 2768–2778.

Towbin, H., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci., U S A*. 76: 4350–4354.

Wedge, S.R., Ogilvie, D.J., Dukes, M., Kendrew, J., Curwen, J.O.and Hennequin, L.F., *et al*. 2000. ZD4190-an orally active inhibitor of vascular endothelial growth factor signaling with broad-spectrum antitumor efficacy. *Cancer Res*., 60: 970–975.

Weis, S.M., Cheresh, D.A. 2011. Tumor angiogenesis: Molecular pathways and therapeutic targets. *Nat Med*., 17: 1359-1370.

Wood, J.M., Bold, G., Buchdunger, E., Cozens, R., Ferrari, S., Frei, J. and Hofmann, F., *et al*. 2000. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res*.,60: 2178–2189.

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