Elevated Vascular Endothelial Growth Factor mRNA is a consequence of Epidermal Growth Factor Receptor alterations in lung cancer patients

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Abstract: Several kinases are involved in transduction pathways via sequential signalling activation. These kinases include trans- membrane receptor kinases like epidermal growth factor receptor (EGFR). Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis involving tumor growth and metastasis. In cancer cells, signalling pathways are often altered and result in uncontrolled growth and increased capability to invade surrounding tissue. Screening of EGFR in 60 tumours with non-small cell lung cancer (NSCLC), 51 Squamous cell carcinoma (SCC), 8 adenocarcinomas (AD), 44 small cell lung cancer (SCLC) and 9 large cell carcinomas (LCC).Alterations in EGFR were identifiedby direct sequencing, VEGF expression in tumours and matching non-malignant tissues from 48 NSCLC patients by quantitative polymerase chain reaction (qPCR) system. EGFRmutations were present in 11.6%; all the mutations have been reported first time except L858R mutation of exon 21. EGFR mutations didn't show statistical significance with the clinical parameters of NSCLC patients. Increased expression of VEGFA mRNA was noted in tumor compared to non-tumorous tissue ($P < 0.0001$).Overexpression of the gene was considered at $\Delta C_T > 6.0$. Within the group of patients with conventional tumor, those with histology other than SCC had a higher level of VEGFA mRNA ($P=0.04$) and the late stages show significantly higher expression than early stages of the disease ($P= 0.001$).Elevated mRNA expression of VEGFA may be due to activation of EGFR caused by molecular alterations in the gene; therefore the study presumes the link between EGFR activation and VEGFA expression in regulating the lung cancer growth and proliferation.

Key words: EGFR; VEGFA; Mutation; Expression; Lung cancer; Kashmir

Introduction

Lung cancer is the leading cause of death for men and women around the world. Non-Small Cell Lung Cancer (NSCLC) accounts for about 85% of lung cancer and its advanced form are refractory to most chemotherapies (Dowell and Minna 2005; Minna et al. 2004). The histological heterogeneity of non- small cell lung cancer encompasses different types, the major of them being-squamous cell lung cancer, adenocarcinoma and large call lung cancer (Ginsberg et al. 2001). Phosphorylated tyrosine serves as a binding site for several signal transducers that initiate multiple signalling pathways, resulting in cell proliferation, migration, metastasis, resistance to apoptosis, and angiogenesis (Arteaga 2002).EGFR (also known as erbB-1) is member of the erbBgene family and encode for trans membrane receptor-type tyrosine-protein kinases (Hynes and Stern 1994; Parsonsand Parsons1993). The ligands of EGFR include epidermal growth factor and transforming growth factor α, which upon the binding of EGFR, transmit growth-stimulatory signals (Gill et al. 1987). The EGFR TK domain has been reported to be mutated in lung cancer (Fukuoka et al. 2003;Kris et al. 2003;Perez-Soler et al. 2004). The mutations cluster in the first 4 exons 18-21 of the tyrosine kinase domain of the gene (Shigematsu et al. 2005; Shigematsu et al. 2006). In spite of many proposed hypotheses, there is still controversy in

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how the mutations affect EGFR function and which role they play in the mutagenesis process (Dowell 2006, Johnson and Janne2005, Pao and Miller 2005).The EGFR mutational spectrum has been reported to show geographical and ethnic differences, as incidence of EGFR mutations is more in East Asian compared to Caucasians patients and those with adenocarcinoma type of lung cancer (Paez et al. 2004; Eberhard et al. 2005). Because the prevalence of genetic alterations often varies depending on the patient's ethnicity, we searched for EGFR mutations in surgically resected NSCLC samples to determine the prevalence of these mutations in Kashmiri lung cancer patients by sequencing exons 18-21 amplified from genomic DNA samples. In the present study 60 cases of lung cancer patients have been screened for alterations in the aforementioned exons and the sequencing data revealed mutation profile quite different from the published data.

Angiogenesis is a complex process and represents a critical step for tumour formation and progression (Ferrara et al. 2003; Folkman 2003). It is now evident that angiogenesis is not only essential for tumour growth, but also for initial progression from a pre-malignant lesion to a fully invasive cancer, and in the growth of dormant micro-metastases into clinically detectable metastatic lesions (Ferrara etal.2003; Folkman 2003). Therefore, the targeting of angiogenesis has become a major therapeutic strategy for cancer treatment, and a wide variety of drugs interfering with this process are under development. To grow beyond a critical size or metastasise to another organ, a tumour must recruit a network of new blood vessels. This switch to an angiogenic phenotype is regulated by a balance between pro- and anti-angiogenetic molecules (Ferrara etal.2003; Folkman 2003). Although this is a complex and coordinated process, requiring the sequential activation of a series of receptors by numerous ligands. There are five recognized isoforms of VEGF, named from A to D, of which VEGF-A is considered the most important for angiogenesis, while others like VEGF-C and D seem to play a role for lymphangiogenesis. The link between EGFR alteration, leading to its activation and VEGF mRNA expression that is likely to be important in tumor progression lead the way for evaluating EGFR mutation profile w.r.t VEGF expression kinetics in lung cancer patients.

Materials and Methods

Patient samples

Tumor and corresponding normal lung tissue specimens were obtained from 60 lung cancer patients who underwent curative resection obtained from SKIMS Srinagar (India) from May 2009 to Aug 2011. There were no restrictions on age, sex, histology or stage, but patients with a prior history of cancer other than lung cancer and the patients who received chemotherapy were excluded from the study. Written informed consent was obtained from each patient before the surgery. This study was approved by the Institute Ethical committee. Clinical characteristics of the lung cancer patients are shown in Table 1. Tumor and normal lung tissue samples were obtained at the time of surgery, and these were rapidly frozen and stored at - 80^0C. The normal lung tissue specimens were obtained from either the opposite end of resected surgical samples or as distant as possible from the site of tumor.

EGFR Gene Analysis

Genomic DNA was extracted from tumors and normal lung tissues according to standard procedures. DNA content was quantified by spectrophotometric absorption (Nanodrop Spectrophotometer, BioLab, Scoresby, VIC, Australia). Purity of DNA was obtained by checking the optical density (OD) 260/280 ratios. Genetic analysis of the EGFRgene was performed by PCR amplification of exons 18, 19, 20 and 21 with flanking intronic sequences and direct sequencing of the PCR products. Mutations of the first four exons (exon 18-21) of the tyrosine kinase (TK) domain of the EGFR gene were detected using polymerase chain reaction based

Table 1: Clinical characteristics of the subjects

VARIABLE	PATIENTS	
	No.	%
Gender		
Male	93	83
Female	19	17
Age(Yrs.)	58±11.56	-
Smoking status		
Smoker	84	75
Non-smoker	28	25
Histology		
NSCLC	68	60.7
SCLC	44	39.3
Grade		
G1(well differentiated)	57	50.9
G2(moderately differentiated)	30	26.8
G3(poorly differentiated)	25	22.3
Stage		
I	39	34.8
II	33	29.4
III	28	25.0
IV	12	10.7

NSCLC: Non-small cell lung cancer; SCLC: small cell lung cancer

direct sequencing. The PCR reactions were performed in a total volume of 40 uL containing 100 ng genomic DNA, 0.2 mmol/L of each primer, and 0.2 mmol/L dNTPs, 1 unit of Taq polymerase (Takara, Shuzo Company, Otus, Shiga, Japan), and 1X reaction buffer (10 mmol/L Tris-HCl, pH 8.3; 50 mmol/L KCl; and 1.5 mmol/L MgCl2).

Table 2: Association of *EGFR* gene mutations with clinical variables in lung cancer patients

Variable	N	EGFR Mutation		(Odds Ratio) * P Value
		wild	mutant	
Age(years)				
>50	37	32	05	**(0.60)**
≤50	23	21	02	0.57
Gender				
Male	46	42	04	**(2.42)**
Female	16	13	03	0.28
Smoking Status				
Non-Smoker	19	14	05	**(0.14)**
Smoker	41	39	02	0.02
Family History				
Yes	03	02	01	**(0.23)**
No	57	51	06	0.26
Grading				
G2+G3	39	36	03	**(2.8)**
G1	21	17	04	0.2
Histology				
NSCLC	41	36	05	**(0.84)**
SCLC	19	17	02	0.85
Stage				
I & II	38	33	05	**(0.66)**
III & IV	22	20	02	0.63

NSCLC: Non-small cell lung cancer; SCLC: small cell lung cancer

TK exons were amplified in a 96-well format PCR setup. The PCR cycle conditions consisted of an initial denaturation step at 95^0C for 7 minutes followed by 35 cycles of 30 seconds at 95^0C; 30 seconds at 58^0C/62^0C; 30 seconds at 72^0C; and a final elongation at 72^0C for 10 minutes. The primers and conditions for PCR amplification are shown in Table 3. All samples were subjected to 2% agarose gel electrophoresis for ensuring the correct product amplification. Purification and Sequencing was done by MacrogenInc.1001 World Meridian Venture Center.

Table 3: Primer sequences and annealing temperatures for direct sequencing

Gene	Exon	Primer Sequence	T_m (^{0}C)	Product size (bp)
EGFR	18	F: 5′-tccaaatgagctggcaagtg-3′ R:5′-tcccaaacactcagtgaaacaaa-3′	58	397
	19	F: 5′-cccagtgtccctcaccttc-3′ R: 5′-gcagggtctagagcagagca-3′	62	306
	20	F: 5′-cattcatgcgtcttcacctg-3′ R: 5′-catatccccatggcaaactc-3′	58	377
	21	F: 5′-gctcagagcctggcatgaa-3′ R: 5′-catcctcccctgcatgtgt-3′	62	348

F= forward primer; R= reverse primer; T_m = annealing temperature, bp=base pairs

Q RT-PCR to assess VEGF mRNA expression

An RNA extraction kit (RNeasy Mini Kit; Qiagen, Valencia, CA)/Trizol were used to extract total RNA from resected lung tissue and adjacent normal tissue. The primers used to amplify and quantify the 4 isoforms of VEGF mRNA were designed from sequences within exons 3 to 4, which are common to all isoforms of VEGF mRNA. Integrity of RNA was assessed by observing the RNA on 1% agarose gel. Purity of RNA was obtained by checking the optical density (OD) 260/280 ratios. RNA was converted to

cDNA using RevertAid™ Premium First Strand cDNA Synthesis Reverse Transcription kit according to the manufacturer's instructions (Fermentas).Each cDNA sample was diluted for providing uniformly concentrated samples. Primers were designed for the analysis of expression of VEGF mRNA with Primer Express 3 (Applied biosystems Inc.), and GAPDH mRNA (GenBank accession number NM_002046). GAPDH was used as the endogenous control gene. For VEGF-A, the primers were: forward 5′-CGAGGGCCTGGAGTGTGT -3′; reverse-5′-GATCCGCATAATCTGCATGGT -3′) whereas for GAPDH, the primers were: forward-5′-GATCCGCATAATCTGCATGGT-3′; Reverse-5′ GATCCGCATAATCTGCATGGT-3′. Real-time quantitative PCR was performed for detection of VEGF-A gene expression by recruiting Appliedbiosystems Inc.Step One Software v2.0). PCR was performed containing Maxima®SYBR Green qPCR Master Mix (2X) (Fermentas) and all the samples (unknown and standards) were run in triplicate and accompanied by a non-template control. Thermal cycling conditions included 40 cycles of 30 s at 55^0C. The melting curves of all final real-time PCR products were analysed for determination of genuine products and contamination by non-specific products and primer dimers. All samples were also subject for separation on 2% agarose gel electrophoresis for ensuring the correct product amplification.

Statistical analysis

Statistical analysis was performed using independent t-test and paired t-test for continuous variables and Pearson's χ^2 test or Fisher's exact test for categorical variables as appropriate. All reported P values were based on two-sided tests. Significance level was taken at $p<0.05$. Statistical tests were performed using the software SPSS 8.0 (SPSS Inc., Chicago, Illinois).

Results

Association of EGFR mutations with clinical parameters

We looked for alterations (mutations) of EGFR exons 18-21. EGFR TK domain mutations were found in 07 cases (11.6%) of the 60 lung cancer patients, 03 mutations in exon 18, 01 in exon 19, 01 in exon 20, and 02 in exon 21. The EGFR mutations were not associated with any of the clinical parameters as shown in Table 2. Of these 7 mutations, all (100%) were substitutions in exon 18, 19, 20 and 21. The detailed analysis of EGFR mutations is shown in table 4.

VEGF mRNA Expression in Lung Cancer and Healthy Lung Tissue Assessed by RT-QPCR

In the present study, total VEGF mRNA was detected and quantified in 48 lung-cancer tissues. The amount of starting total RNA used for RT- QPCR is much less than that used for conventional RT-PCR. VEGF mRNA expression was detected in all 48 (100%) paired lung cancer and non-tumorous lung tissues by RT- QPCR. The VEGF mRNA expression ratio (amount of VEGF mRNA/amount of GAPDH mRNA) is expressed as $\Delta C_T = (C_T\text{VEGF} - C_T\text{GAPDH})$. The ΔC_T values for VEGF in 48 samples of lung cancer tissue ranged from 1.4 to 26.8, with a mean ± standard deviation (SD) of 15.68± 6.27, while the corresponding values in the matched non-tumorous lung tissue ranged from 1.0 to 17.53, with a mean ± SD of 9.42±6.14. VEGF mRNA expression in lung cancer tissue was significantly higher

Table 4: Genetic Alterations in *EGFR* Gene in lung cancer patients

GENE	Sex	Age (yrs)	Smoking Status	Grade	Exon	Nucleotide no. Nucleotide Change	Amino Acid Change
EGFR	M	38	SM	G1	18	c.2071C>A	p.Pro691Thr
	M	56	SM	G3	18	c.2123G>A	p.Lys708Lys
	M	66	SM	G1	18	c.2082C>A	p.Pro694Pro
	M	58	SM	G1	19	c.2190C>T	p.Leu730Leu
	M	70	SM	G2	20	c.2356C>A	p.Thr785Thr
	F	47	NONSM	G2	21	c.2573T>G	p.Leu858Arg
	M	58	SM	G1	21	c.2585T>A	p.Leu862Gln

G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated; M: Male; F: Female; SM: Smoker; NONSM: Non smoker

than in the matched non-tumorous lung tissue ($p < 0.0001$) shown in (Table 5).

Relationship between VEGF mRNA Expression and Clinicopathologic Variables

Table 5 shows the relationship between VEGF mRNA expression and the clinicopathologic characteristics. VEGF expression was higher in advanced stages of the disease and in adenocarcinoma, large cell carcinoma than in squamous cell carcinoma (SCC) (P= 0.001, P= 0.04). The association of VEGF mRNA expression with age, dwelling, sex, smoking status, and pathologic grade was not statistically significant. In the present study, VEGF mRNA expression was evaluated by Reverse Transcriptase-quantitative polymerase chain reaction (RT-qPCR) to better define the role of this angiogenic factor in the pathogenesis of lung cancer.

Table 5: The relationship between clinico-pathological variables and VEGFA expression in lung cancer

Clinical parameter	VEGF mRNA[#] expression (mean ± SD)	No.	P value
Tissue			
Tumor	15.68±6.27	48	**< 0.0001**[*]
Non-tumorous	9.42±6.14	48	
Age			
≤50 years	11.38±3.48	15	0.93
>50 years	11.29±3.28	33	
Sex			
Male	14.34±4.58	36	0.56
Female	15.23±4.69	12	
Smoking Status			
Smoker	12.45±2.98	32	0.34
Non smoker	13.34±3.11	16	
Histology			
NSCLC	13.37±4.58	30	**0.04**
SCLC	16.23±4.69	18	
Pathologic grade			
G2+G3	9.34±2.31	36	0.55
G1	8.89±2.12	12	
Pathologic Stage			
I & II	09.80±3.11	29	0.001
III & IV	13.34±3.87	19	

[#] VEGF mRNA expression calculated from real-time quantitative RT-PCR: $-\Delta CT = -[CT_{(VEGF)} - CT_{(GAPDH)}]$

[α] adenocarcinoma, large cell carcinoma

SCC: Squamous cell carcinoma, G1: well differentiated; G2: moderately differentiated; G3: poorly differentiated

Discussion

EGFR remains the best-studied Receptor Tyrosine Kinase (RTK) that is frequently mutated in NSCLC.In the present study, we looked for EGFR mutations in patients who underwent curative surgical resection to find out the effect of EGFR alterations on VEGF mRNA expression in lung cancer patients of Kashmir. The present study shows mutations in the TK domain of EGFR gene in SCC (Squamous cell carcinoma) and Adenocarcinoma. EGFR mutations in SCC (Squamous cell carcinoma) were more frequent in men than in women and in smokers than in non-smokers. Overall, EGFR mutation frequencies largely depend on smoking status (~50% in non-smokers, 5-15% in smokers) and histologic sub-classification (Marchetti et al. 2005;Paoetal.2004;Sugio et al. 2006;Toyooka et al. 2007). In the current study EGFR mutations were reported in 11.6% (Squamous cell carcinoma 13.6% and Adenocarcinoma

12.5%), comparison to data already published which shows EGFR mutations in nearly 40% of adenocarcinomas (AD), 30% of mixed adenosquamous carcinomas, and <5% of squamous (SQ) or large-cell (LC) carcinomas (Sanders and Albitar2010). In previous studies, EGFR mutations were reported in about 22% to 67%, 10% to 24%, and 3% to 25% of Asian population, Southern European, and American populations with lung adenocarcinomas (Chou et al. 2005;Cortes-Funes et al. 2005). Mutation frequencies also vary by region in the EGFR gene. In all histologic subtypes, >70% of the EGFR mutations occur in exons 19 and 21. Only a small fraction of EGFR mutations have been found outside exons 18-21, which encode part of the TK domain that is frequently activated by such mutations. A large proportion of EGFR mutations (44%) in NSCLC are in-frame deletions, especially those in exon 19. Point mutations are seen mainly in exon 21.In this study all mutations are of missense type and found in all exons of EGFR gene. The alterations (mutations) shown in the present study have been reported first time from the Valley and it appears that there is no literature available as for as the mutations are concerned except the point mutation (L858R) in exon 21 of EGFR gene. Although EGFR mutations in lung cancers have been extensively studied, most previous studies have investigated mutations in patients with advanced stage of lung cancer. In this study 14.2% mutations were found in advanced stages while as 10.8% mutations were in early stages of Non-small cell lung cancer (NSCLC). In May 2004, two independent groups of investigators reported the discovery of somatic mutations in the TK domain (exons 18–23) of EGFR (Chen et al. 2006;Eberhard et al. 2005). Practically all mutations that have been reported are on exons 18 through 21. A meta-analysis (Shigematsu and Gazdar 2006) of nine published studies showed that EGFR mutations were limited to the first four exons (exons 18-21) of the TK (Tyrosine Kinase) domain, which encode the N-lobe and the 5′ portion of the αC-lobe of EGFR. The mutations consisted of three different types (deletions, insertions, and missense point mutations) and they all targeted key structures around the adenosine triphosphate binding cleft, including the glycine-rich GXGXXG motif of the phosphate binding loop (P-loop), the αC-helix, and the aspartic acid - phenylalanine - glycine sequence (DFG motif) in the activation loop (A-loop). In-frame deletions in the region spanning codons 746 to 750 in exon 19 accounted for 44% of all mutations, and inframe duplications -insertions in exon 20 accounted for 5% of all mutations (Shigematsu and Gazdar 2006). These two types of mutation (in-frame deletions and in-frame duplications-insertions) occur on either side of the αC-helix. The third type of mutation, missense point mutation occurs in all four exons, particularly L858R located near the DFG motif in exon 21 (accounting for 41% of all mutations) and G719X (where the X indicates A, C, or S) in the GXGXXG motif in exon 18 (accounting for 4% of all mutations). Consistent with previous studies (Tokumo et al. 2005), we found that 90% of the mutations were either in-frame deletions in exon 19 or L858R in exon 21, and all in-frame deletions in exon 19 targeted the four-codon region (codons 747-750).

Total VEGF mRNA was expressed at significantly higher levels in cancer tissue than in the adjacent normal lung tissue. Adenocarcinomas and large cell carcinomas had significantly higher total VEGF mRNA expression than squamous cell carcinomas. High VEGF mRNA expression ($\Delta C_T \geq 10$) in tumors was associated with advanced tumor stage and histology. The finding that adenocarcinoma has significantly higher VEGF expression than squamous cell carcinoma has not been reported by other investigators (Fontanini et al. 1999;Ohta et al. 1996).Although there was a trend for adenocarcinoma to have higher VEGF expression in the study of (Fontanini et al. 1997), it did not reach statistical significance. The different results may be due to the use of different methods for quantitation of VEGF mRNA (Ohta et al. 1996;Ohta et al. 1997), different primer designs resulting in different PCR efficiencies (Ohta et al. 1996;Ohta et al.

1997), relative small sample size (Ohta et al. 1997). VEGF expression in adenocarcinomas was significantly higher than that in squamous cell carcinoma (Joseph et al. 1997). Although VEGF is constitutively expressed by many tumour cells and transformed cell lines, its expression is subject to several mechanisms of control. A key pathway is the regulation by oxygen concentration. Decreased intratumour oxygen concentration potently upregulates VEGF expression (Ferrara et al. 2003;Forsythe et al. 1996). In addition, alterations in oncogenes and tumour suppressor gene function promote tumour growth by modulating the angiogenic response induced by VEGF (Ferrara et al. 2003). The VEGFs mediate angiogenic signals to the vascular endothelium via high-affinity TK receptors. Following its binding to cognate receptors, VEGF initiates a cascade of signalling events that begins with dimerization and trans-autophosphorylation of TK residues in the VEGFRs which in turn, activates phospholipase C-γ (PLC-γ), PI3-K, GAP, MAPK, and others. Overexpression of VEGF in cancer cells may be an indicator of poor prognosis in many types of human tumours, including carcinomas of the breast, kidney, colon and prostate (Tortora et al. 2004). VEGF levels may have a potential value for predicting the effectiveness of conventional treatments, including radiotherapy, chemotherapy, and hormonal therapy in different diseases (Kerbel and Folkman 2002;Tortora et al. 2004). Recent experimental evidence has demonstrated that VEGF overexpression and secretion represent a major escape pathway when tumour cells develop resistance to selective EGFR inhibitors (Ciardiello et al. 2004;Viloria-Petit et al. 2001).

Conclusion

The present study reveals role of EGFR alterations and corresponding increase in VEGF mRNA in non-small cell lung cancer patients, Hence the study presumes the link between EGFR downstream signalling proteins which probably act on promoter of the VEGF gene, thus resulting its activation.

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