RESEARCH ARTICLE

Clinical Implication of EGF A61G Polymorphism in the Risk of Non Small Cell Lung Adenocarcinoma Patients: A Case Control Study

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Abstract

Background: The epidermal growth factor (EGF) plays important roles in non-small cell lung cancer (NSCLC) susceptibility and functional polymorphism in the EGF (+61A/G) gene has been linked to increased risk of NSCLC. This study aimed to evaluate the role of the EGF +61A/G polymorphism in risk of NSCLC adenocarcinoma (ADC) occurrence and survival in an Indian population. Materials and Methods: This case-control study included 100 histopathologically confirmed NSCLC (ADC) patients and 100 healthy controls. EGF (A61G) was genotyped by AS–PCR to elucidate putative associations with clinical outcomes. The association of the polymorphism with the survival of NSCLC patients was estimated by Kaplan–Meier curves. Results: It was found that EGF 61AG heterozygous and GG homozygous genotype is significantly associated with increased risk of NSCLC (ADC) occurrence compared to AA genotype, [OR 2.61 (1.31-5.18) and 3.25 (1.31-8.06), RR 1.51(1.15-2.0) and 1.72 (1.08-2.73) and RD 23.2 (6.90-39.5) and 28.53(7.0-50.1) for heterozygous AG (p=0.005) and homozygous GG (p=0.009)]. Patients homozygous for the G allele exhibited a significantly poor overall survival. The median survival time for patients with EGF 61 AA, AG, and GG genotypes was 10.5, 7.4, and 7.1 months (p=0.02), respectively. NSCLC (ADC) patients with GG + AG exhibited 7.3 months median survival compared to the AA genotype (p=0.009). Conclusions: The present study revealed that the EGF A61G genotype may be a novel independent prognostic marker to identify patients at higher risk of occurrence and an unfavourable clinical outcome.

Keywords: EGF gene (+61A/G) polymorphism - AS-PCR - NSCLC (ADC) patients

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Introduction

Non-small cell lung cancer (NSCLC) is the major cancer killer disease worldwide in both males and females accounting for more than1.2 million deaths each year (Alberg et al., 2005). NSCLC accounts for 75%-85% of all histotypes of lung cancer and the overall prognosis of NSCLC patients remains poor with a 5-year survival rate only 14% while 5-year survival rate less than 70% in stage I (Naruke, 1997; Spira et al., 2004). Cancer cells produce high level of their own peptide growth factors and this turn on the cellular proto-oncogenes (Goustin et al., 1986; Aaronson et al., 1991). The epidermal growth factor (EGF) and its receptor (EGFR) play a central role in lung carcinogenesis. The EGF gene is located in chromosome 4q25-27 and its protein may activate DNA synthesis and promotes cellular proliferation by stimulating mitosis (Laurence and Carpenter, et al 1990). EGF mRNA is 4.8-kb long and their gene is 110-kb long containing 24 exons (Salomon et al., 1995). The interaction between EGF and EGFR may be a risk factor for susceptibility and prognosis in various tumours, such as melanoma, glioblastoma and gastric cancer (Moulder et al., 2001). EGFR signal promote cell proliferation, invasion, metastasis, angiogenesis, and inhibition of apoptosis (Tabernero et al., 2005). The EGF A61G polymorphism is located in the 5'-untranslated region at position 61 and reported to functional influence on increase EGF production. Mononuclear cells from individuals with the AA genotype have been reported to decrease levels of EGF production than cells with GG genotype (Shahbazi et al., 2002). Epidermal growth factor (EGF) is ligand which binds to EGFR receptors and transmits signal further, and it was observed in cancer cells EGFR signalling pathway is often deregulated and which increases proliferation, resistance to apoptosis, metastases and angio-genesis (Ciardiello et al., 2001; 2008; Hynes

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et al., 2005; Gan et al., 2012). EGF ligand binding with its receptor (EGFR) pathway has been demonstrated to play a significant role in transducing growth signals to mitogen-activated protein kinase pathway, as well as the phosphatidylinositol 3-kinase causing NSCLC (Harris et al., 2003; Goto et al., 2005; Araujo et al., 2007). EGF can also disrupt different pathways and contribute to metastasis due to intercalation of integrin α6β4 with EGFR, which can be important factor for cell migration (Araujo et al., 2007; Teixeira et al., 2008). Shahbazi et al first time found that individuals homozygous for the 61AA genotype produced significantly low EGF level than the homozygous 61GG (p=0.0004) or heterozygous 61AG genotype (p=0.001) and suggested that high EGF synthesis is important to melanoma development (Shahbazi et al., 2002; Lim et al., 2005). Many studies have evaluated the role of EGF A61G single nucleotide polymorphism. However, conflicting findings have been reported of EGF A61G polymorphism in lung cancer risk (Kang et al., 2007). In the present study, we hypothesized that EGF (A61G) polymorphism may also be associated with cancer susceptibility, unfavourable clinical behaviour and risk of NSCLC in Indian population.

Materials and Methods

Cases and controls

Present study included histo-pathologically confirmed 100 newly diagnosed NSCLC (ADC) patients and 100 healthy controls. 3 ml of peripheral blood sample collected in EDTA vials from each subjects included in the study. This study was approved by the Institutional Ethics Committee of MAMC, New Delhi and written informed consent was obtained from all subjects. Patient follow-up was obtained through the hospital records and follow-up done from May 2013 to May 2015.

DNA extraction and genotype

Genomic DNA extraction was done by phenol chloroform method from blood samples collected in EDTA vials from NSCLC ADC cases as well as healthy controls. The +61 A/G polymorphism was analysed by allele specific PCR method with EGF +61A Forward 5' GCCCCAATCCAAGGGTTGTA3',EGF+61G Forward 5' GCCCCAATCCAAGGGTTGTG 3'and and reverse primer for both alleles is 5'GCCAAGGGAAGCCACAGGAAAG 3' (Kenneth K et al., 2008). PCR was performed in 25 µl reaction volume containing 3 µl of 100 ng template DNA, 0.25 µl, 25 pmol each primer 2.5 µl, 10 mM dNTPs, 1.5 µl of 20mM $MgCl_2$, 0.3 µl of 5 U/ µl Taq polymerase with 2.5 µl of 10X Taq Buffer (Fermantas) and 14.7 µl of nuclease-free ddH_2O. The PCR was performed with initial denaturation at 94°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 40 seconds, annealing at 58°C for 40 seconds, extension at 72°C for 40 seconds and the final extension was at 72°C for 10 minutes. Amplified 206bp PCR products were separated by electrophoresis on a 2 % agarose gel containing ethidium bromide (Figure 1).

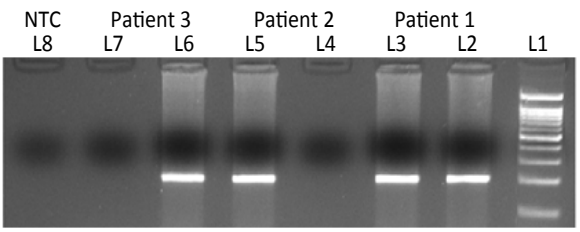

Figure 1. Agarose Gel Picture of EGF+61A/G Amplification. L1:100bp ladder. Patient1; L2 – L3: Both normal (A) and mutant allele (G) amplified: Patients 1 positive for Heterozygous A and G allele. Patient2; L4 – L5: Mutant allele (T) amplified: Patients2 Positive for Homozygous G allele. Patient3; L6 – L7: Normal allele (A) amplified: Patients 1 positive for Homozygous A allele. NTC; L8: Non template control

Statistical analysis

Genotype frequencies between the cases and controls were evaluated using the Chi square test, Hardy-Weinberg equilibrium test used to check the allele frequency and values below 5 were analyzed by Fisher exact test. The associations between A61G genotypes and risk of NSCL cancer (ADC) were estimated by computing the odds ratios (ORs) and risk ratios (RRs) with 95 % confidence intervals (CIs). Kaplan-Meier methods were used to evaluate the relationship between A61G genotype and

Table 1. Distribution of Selected Characteristics among NSCLC Patients and Healthy Controls

Variables	NSCLC patients (%)	Healthy controls (%)
Total no.	100	100
Gender		
Males	65	71
Females	35	29
Age at diagnosis (In years)		
< 55	43	56
> 55	57	44
Mean + SD age (years)	55.4+12.29 (range32-89 years)	54.25+10.82 (range 30-70 years)
Smoking status		
Non smokers	56	55
Smokers	44	45
Current smokers	25	24
Ex- smokers	19	21
Smoking type		
Cigarette	15	18
Bidi	23	16
Cigarette + Bidi	6	11
Smoking level (pack year)		
Mild (< 10)	33	23
Moderate (< 40)	11	18
Heavy (> 40)	0	4
TNM Stage		
Stage III	25	
Stage IV	75	
Distant Metastases		
Positive	75	
Negative	25	
Histopathological Grade		
Grade 1	41	
Grade 2	23	
Grade 3	36	
Pleural effusion		
Yes	28	
No	72	

Table 2. Genotype frequencies of EGF (A61G) among NSCLC cases and controls

Variables	AA	AG	GG	p value	A allele frequency	G allele frequency
Patients(n=100)	17 (17%)	63 (63%)	20 (20%)	0.008	0.45	0.55
Controls(n=100)	36 (36%)	51 (51%)	13 (13%)		0.61	0.39

Table 3. EGF Genotype Frequencies in Cases & Controls and Associations with NSCLC Risk

EGF (A61G) Genotype	Control (n=100)	Cases (n=100)	OR(95% CI)	RR(95% CI)	RD(95% CI)	P value
AA	36(36%)	17(17%)	Ref (1)	Ref (1)		
AG	51(51%)	63(63%)	2.61(1.31-5.18)	1.51(1.15-2.0)	23.19(6.90-39.47)	0.005
GG	13(13%)	20(20%)	3.25(1.31-8.06)	1.72(1.08-2.73)	28.53(7.0-50.05)	0.009
AG+GG	64(64%)	83(83%)	2.74(1.41-5.32)	1.56(1.20-2.02)	24.39(8.68-40.09)	0.002

OR odd ratio, RR risk ratio, RD risk differences

Table 4. Association between the EGF (A61G) Genotype and Clinico-pathological Characteristics in Cases

Variables		Group I	Group II	OR	RR
Gender		Male	Female		
	AA	9	8	Ref (1)	Ref (1)
	AG	41	22	0.60(0.20-1.78)	0.81(0.50-1.31)
	GG	15	5	0.37(0.09-1.50)	0.70(0.42-1.18)
Age (in years)		< 55	> 55		
	AA	8	9	Ref (1)	Ref (1)
	AG	28	35	1.11(0.37-3.25)	1.05(0.59-1.88)
	GG	7	13	1.65(0.43-6.62)	1.34(0.61-2.93)
Smoking behaviour		Non-smokers	Smokers		
	AA	14	3	Ref (1)	Ref (1)
	AG	31	32	4.81(1.25-18.43)	1.67(1.19-2.33)
	GG	11	9	3.81(0.82-17.58)	1.49(0.95-2.35)
Smoking status		Current Smokers	Ex-smokers		
	AA	3	0	Ref (1)	Ref (1)
	AG	17	15	6.20(0.29-129.8)	1.88(1.35-2.60)
	GG	5	4	5.72(0.22-142.7)	1.80(1.00-3.22)
Smoking type		Cigarette	Bidi		
	AA	0	1	Ref (1)	Ref (1)
	AG	12	16	0.44(0.01-11.75)	-
	GG	3	6	0.61(0.01-19.60)	-
Smoking type		Cigarette	Cigarette + Bidi		
	AA	0	2	Ref (1)	Ref (1)
	AG	12	4	0.07(0.002-1.80)	-
	GG	3	0	0.02(0.0004-1.99)	-
Smoking type		Bidi	Cigarette + Bidi		
	AA	1	2	Ref (1)	Ref (1)
	AG	16	4	0.12(0.008-1.75)	0.41(0.08-2.09)
	GG	6	0	0.04(0.001-1.55)	0.33(0.06-1.65)
Smoking level (pack year)		Mild(<10)	Moderate(<40)		
	AA	2	1	Ref (1)	Ref (1)
	AG	24	8	0.66(0.05-8.37)	0.88(0.38-2.02)
	GG	7	2	0.57(0.03-10.08)	0.85(0.35-2.08)
TNM Stage, Stage III, Stage IV					
	AA	3	14	Ref (1)	Ref (1)
	AG	18	45	0.53(0.13-2.09)	0.61(0.20-1.85)
	GG	4	16	0.85(0.16-4.50)	0.88(0.22-3.40)
Distant Metastases		Positive	Negative		
	AA	14	3	Ref (1)	Ref (1)
	AG	45	18	1.86(0.47-7.28)	1.15(0.88-1.51)
	GG	16	4	1.16(0.22-6.13)	1.02(0.75-1.40)
Pleural Effusion		No	Yes		
	AA	10	7	Ref (1)	Ref (1)
	AG	47	16	0.48(0.15-1.49)	0.78(0.51-1.20)
	GG	15	5	0.47(0.11-1.93)	0.78(0.48-1.25)

overall survival of NSCLC patients. All statistical analyses were performed using Graph Pad Prism 6.0 and SPSS 16.0.

Results

Study population

All demographic features of the subjects are depicted in table-1. In brief, total of 100 Non-small cell lung ADC patients and same number of healthy control were analyzed. Both NSCLC (ADC) cases and controls include 65% males and 35% females of age < 55 group (43 %) and >55group (57%) with mean ± SD in cases of 55.4+12.29 (range32-89 years) and controls of 54.25+10.82 (range 30-70 years). 75% patients were in stage IV, and 25% patients in stage III while 75% patients had distant metastases. Patients with different pathological grade, grade 1 (well differentiated) includes 41%, grade 2 (moderately differentiated) includes 23% and grade 3 (Poorly differentiated) includes 36% cases. We included smoker 44% as well as non smoker 56% with different smoking type as cigarette, bidi, and both, 15% cases smoked cigarette, 23% cases smoked bidi and 6% cases smoked both cigarette and bidi.

Genotype (A61G) distribution among Cases and Controls

The genotype and allele distributions of A61G in cases and controls are summarized in Table 2 and 5. We found statistically significant difference in genotype distribution of A61G in cases and healthy controls (p=0.0008). The frequency of G allele (fG) was to be higher among NSCLC (ADC) patients (0.55) compared to healthy controls (0.39) and the frequency of A allele (fA) in healthy controls was to be higher (0.61) compared to NSCLC cases (0.45).

EGF (A61G) genotype and NSCLC risk

Odds ratio and risk ratio with 95 % confidence intervals was calculated for each group to estimate the degree of association between the EGF (A61G) genotypes and risk of NSCLC in patients. Compared to AA genotype, OR 2.61(1.31-5.18) and 3.25(1.31-8.06), RR 1.51(1.15-2.0) and 1.72(1.08-2.73) and RD 23.19(6.90-39.47) and 28.53(7.0-50.05) for heterozygous AG (p=0.005) and homozygous GG (p=0.009) genotype were estimated. It was found and suggesting that possible dominant effect of EGFA61G polymorphism on NSCLC (ADC) risk in Indian population. It was also observed that smoking behaviour and smoking status with heterozygous AG and homozygous GG had increased risk of NSCLC disease Table 3, 4.

EGF (A61G) genotype and NSCLC survival analysis

Survival analysis of 100 NSCLC (ADC) patients, based on genotype distribution was done and it was found that the mean follow-up time of the patients was 8.64 months (median 10.60; range 1- 24.3 months) for the overall survival. NSCLC-related deaths events 86 (86% %) with mean follow-up time of 7.1months (median 7.35; range 1-15.8 months) and for the patients

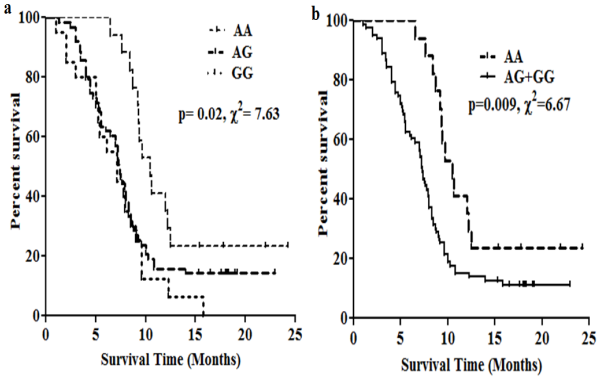

Figure 2. Kaplan–Meier Survival Curves of NSCLC Patients with Respect to EGF (A61G) Polymorphism

Table 5. Association and Stratification Analysis of EGF (A61G) Polymorphism and NSCLC

Variables		Total	AA Genotype n (%)	AG Genotype n (%)	GG Genotype n (%)	A allele frequency	G allele frequency
Gender	Male	65	9(13.84%)	41(63.08%)	15(23.08%)	0.45	0.55
	Female	35	8(22.85%)	22(62.86%)	5(14.29%)	0.54	0.46
Age (in years)	< 55	43	8(18.60%)	28(65.12%)	7(16.28%)	0.51	0.49
	> 55	57	9(15.79%)	35(61.40%)	13(22.81%)	0.41	0.59
Smoking status	Nonsmokers	56	14(25%)	31(55.35%)	11(19.65%)	0.52	0.48
	Smokers	44	3(6.81%)	32(72.73%)	9(20.46%)	0.43	0.57
	Current Smokers	25	3(12%)	17(68%)	5(20%)	0.46	0.54
	Ex-smokers	19	0(0%)	15(78.94%)	4(21.06%)	0.39	0.61
Smoking type	Cigarette	15	0(0%)	12(80%)	3(20%)	0.4	0.6
	Bidi	23	1(4.35%)	16(69.56%)	6(26.09%)	0.39	0.61
	Cigarette+ Bidi	6	2(33.4%)	4(66.7%)	0(0%)	0.66	0.34
Smoking level (pack year)	Mild(<10)	33	2(6.06%)	24(72.72%)	7(21.22%)	0.42	0.57
	Moderate(<40)	11	1(9.09%)	8(72.73%)	2(18.18%)	0.45	0.55
TNM Stage	25	3(12%)	18(72%)	4(16%)	0.48	0.52	
IV	75	14(18.67%)	45(60%)	16(21.33)	0.49	0.51	
Distant Metastases	Positive	75	14(18.67%)	45(60%)	16(21.33%)	0.49	0.51
	Negative	25	3(12%)	18(72%)	4(16%)	0.48	0.52
Histopathological Grade	Grade I	41	2(4.87%)	35(85.37%)	4(9.76%)	0.47	0.53
	Grade II	23	2(8.69%)	10(43.48%)	11(47.83%)	0.3	0.7
	Grade III	36	13(36.12%)	18(50%)	5(13.88%)	0.61	0.39
Pleural effusion	No	72	10(13.88%)	47(65.28%)	15(20.84%)	0.46	0.54
	Yes	28	7(25%)	16(57.14%)	5(17.86%)	0.53	0.47

who survived, the follow-up time was approximately 18.12 months (median 18.1; range 9.1-24.3 months). Patients homozygous for G allele exhibited a significant poor overall survival (p=0.02). Median survival time for patients with EGF 61 AA, AG, GG + AG and GG genotype was 10.5, 7.4, 7.3 and 7.1 months, respectively. Significant poor overall survival was observed in NSCLC (ADC) patients presented with EGF 61 GG genotype (Figure2a, b).

Discussion

Presence of the EGF +61G allele is a key point in the steps towards carcinogenesis by increasing serum EGF and stimulating proliferation, angiogenesis and metastasis (Zhang et al., 2010). Interaction between serum EGF and its receptor (EGFR) is very important in NSCLC framework. EGF interaction with its receptor (EGFR) has been demonstrated to play a critical role in lung cancer carcinogenesis and tumour aggressiveness, mainly in NSCLC patients. The EGF/EGFR pathway transduces growth signals to mitogen-activated protein kinase pathway, phosphatidylinositol 3-kinase and other downstream pathways (Harris et al., 2003; Goto et al., 2005; Araujo et al., 2007; Zhang et al., 2010; Hu-Lieskovan et al., 2011). Costa et al in 2007 showed that the +61 G allele was associated with a high EGF expression level *in vitro* (Costa et al., 2007). Wu et al in 2009 found a statistically significant association between EGF +61 G/G genotype and the +61 G allele with risk for colorectal cancer and pancreatic cancer risk development (Wu et al., 2009). Lim and colleagues conducted a study on schizophrenic patients and lung cancer patients in Korean population to analyse the EGF A61G polymorphism and observed an association of the EGF +61 A/G and EGF +61 G/G genotypes with lung cancer risk (OR 2.3, 95%CI 1.6082-3.3687) (Lim et al., 2005). Meta-analysis studies also showed that EGF A61G polymorphism is associated with overall cancer risk (Zhang et al., 2010; Li et al., 2012). The present data explored the association of EGF (A61G) polymorphism with risk and unfavourable clinical outcome of non-small cell lung cancer. We also observed a positive association between EGF +61 A/G polymorphism with NSCLC (ADC) patients. A significant difference was observed in distribution of EGF (A61G) genotype in NSCLC cases and controls. Our study, the first report from Indian population, suggested that homozygous EGF (+61)GG genotype is strongly associated with the risk of developing NSCLC ADC with approximately more than 3 fold increase than homozygous EGF (+61)AA genotype. EGF (+61) GG genotype was also been found to be an independent factor for unfavourable clinical outcome, However patients with GG genotype at higher risk for death than AA genotype of NSCLC patients. Increased risk of NSCLC (ADC) is also associated smoking behaviour and smoking status. EGF A61G polymorphism is also associated with other cancers, Ying Piao et al suggested that G allele and GG genotype of EGF A+61G (rs4444903) polymorphism has correlations with esophageal and colorectal cancer (Ying et al., 2013). In a study, Vauleon et al (Vauleon et al., 2007) showed that the +61 A/G is functional polymorphism and the promoter with G allele had 40% more active than the A variant ($p < 0.001$). Costa et al. (Costa et al., 2007) also found that the G allele conferred higher risk for gliomas, glioblastomas, and oligodendrogliomas and it was significantly associated with increased risk for gliomas. The prevalence of the G/G genotype was significantly higher in melanoma patients, the G allele was present in nearly 66% of patients with malignant melanoma (odds ratio 4.9 [95% CI 2.3-10.2]; $p < 0.0001$) (Shahbazi et al., 2002). Ichiro O et al in 2007 found a significant association between the high-expression homozygous G/G genotype of the EGF gene and shorter disease-free period and Malignant Melanoma specific survival compared to A/G and A/A carriers (Ichiro O et al., 2007).

In conclusion, the present study we showed first time that EGF +61AG heterozygous and GG homozygous genotype are associated with reduced overall survival and risk of developing NSCLC (ADC) in the Indian population. EGF represents a novel prognostic marker to identify patients at higher risk for unfavourable clinical outcome. In addition, EGF (A61G) genotyping can be useful to decide specific EGF targeted therapy as well as in the management of NSCLC (ADC) patients.

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