RESEARCH ARTICLE

Cell Free EGFR mRNA Expression and Implications for Survival and Metastasis in Non-Small Cell Lung Cancer Cases

Mirza Masroor[1], Rashid Mir[2], Jamsheed Javid[2], Y Prasant[1], A Imtiyaz[1], Z Mariyam[1], Anant Mohan[3], PC Ray[1], Alpana Saxena[1]*

Abstract

Background: NSCLC is a disease involving uncontrolled cell growth, which could result in metastases into nearby tissues beyond the lungs. Materials and Methods: The aim of the present study was to analyze the influence of epidermal growth factor receptor (EGFR) gene expression on metastasis and survival in NSCLC patients. The present case-control study included 100 cases of NSCLC patients and 100 age and sex matched controls. EGFR gene expression was analyzed by quantitative real time PCR using serum RNA. Association with NSCLC patient survival was analyzed by the Kaplan-Meier method. Results: We analyzed EGFR gene expression and observed mean increased gene expression of 13.5 fold in NSCLC patients. Values reflected overall survival of patients with a median of 15.8 months in the cases of <13 fold increased gene expression *vs* 6.7 months with >13 fold increased EGFR gene expression (p=0.005). Distant metastatic patients with <13 fold increased EGFR gene expression had 7.9 months of median survival time while>13 fold increased EGFR gene expression had only 5 months of median survival time (p=0.03). Non metastatic patients with <13 fold increased EGFR gene expression had 18 months of median survival time as compared to only 7.1 months with >13 fold increased expression. Conclusions: Higher cell free EGFR mRNA expression may play an important role in causing distant metastases and reducing overall survival of NSCLC patients in the Indian population.

Keywords: EGFR gene expression - metastases - survival - NSCLC patients

Asian Pac J Cancer Prev, **16 (15)**, 6445-6449

Introduction

Lung cancer is the major cause of cancer-related deaths and has the highest incidence, mortality rates amongst all malignancies worldwide (Siegel et al., 2013). Lung cancer has an incidence of over 1.6 million cases every year accounting for 13% of all new cancer diagnoses and 1.4 million deaths every year recorded for 18% of all cancer-related deaths (WHO, 2012; Lewis et al., 2013). Quantitative real-time RT-PCR (qPCR) was recently shown to be useful for early NSCLC diagnosis, prognosis prediction and gene expression analysis (Hayes et al., 2006).

The molecular mechanisms involved in NSCLC are still relatively unknown and are being investigated extensively. Over the last decade, extensive research has been performed on tyrosine kinases (Engelman et al., 2005). All members have an extracellular ligand-binding region, a single membrane-spanning region and a cytoplasmic tyrosine-kinase-containing domain (Yarden et al., 2001; Riese et al., 2006).

Ligand binding to EGFR receptors induces the formation of receptor homo and heterodimers and activation of the intrinsic kinase domain, resulting in phosphorylation on specific tyrosine residues within the cytoplasmic tail. These phosphorylated residues serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signaling pathways (Schlessinger et al., 2004). EGFR is a critical component for signal transduction, blocking to EGFR can inhibit tumor growth and targeting EGFR tyrosine kinase activity could important therapeutic target in cancer therapy (Utsugi et al., 2013). Qing Li revealed that EGFR gene expression, and variations in gene copy number can be used in the differential diagnosis of high-grade and low-grade CIN but also in the early diagnosis of cervical cancer (Qing et al., 2014). The EGFR receptors are implicated in the development of many types of cancer, and EGFR was the first tyrosine-kinase receptor to be linked directly in human tumors (Gschwind et al., 2004). The EGFR tyrosine kinase works through the auto-activation of the receptor via its homo/heterodimerization and autophosphorylation on tyrosine-rich cytosolic domains after the binding of the ligand (Hynes et al., 2005). There is evidence that

*[1]Department of Biochemistry, Maulana Azad Medical College and Associated Hospitals, [3]Department of Pulmonary Medicine and Sleep Disorders, All India Institute of Medical Sciences, New Delhi, India, [2]Faculty of Applied Medical Sciences, University of Tabuk, Tabuk, Saudi Arabia *For correspondence: biochempublications@gmail.com*

the activated EGFR can also mediate signals through the STAT (Signal transducer and activator of transcription) transcription factors (Quesnelle et al., 2007; Hirsch et al., 2009). The EGFR is expressed in all cell types with the exception of hematopoietic cells. More importantly, the EGFR has been found to be over expressed in 50-70% of human primary colon, lung, and breast carcinomas as well as in other tumor types (Salomon et al., 1995). Over-expression of EGFR-TK results in increased cell proliferation, survival, invasion and metastases. This has been implicated in the pathogenesis and progression of many malignancies as well as in the poor prognosis of patients (Wang et al., 2007, 2008).This study aimed to investigate the putative role of cell free EGFR gene expression with survival and metastases of NSCLC patients.

Materials and Methods

Study population and sample collection

Present study was approved by Institutional ethics committee of Maulana Azad Medical College & associated hospitals and All India Institute of Medical Sciences New Delhi. This study included 100 newly diagnosed NSCLC patients and 100 age and sex matched healthy controls. Patients' 3 ml blood was collected from each subject and serum was separated within 30 minute of the sample collection and stored at -80^{o}C until analyzed. Patients included in study were followed from the 2013 to 2014 for survival analysis.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from serum using Trizol reagent according to the manufacturer's protocol (Invitrogen) and stored at -80^{o}C until further processing. cDNA was synthesised by using 100ng total RNA following manufacturers protocol (Verso, Thermo scientific,USA).

Quantitative Real time PCR

EGFR gene expression was studied by QRT-PCR (SYBR Green I technology) with β-actin gene as internal control. The primer sequences for EGFR gene expression were forward primer 5'-GGA GAA CTG CCA GAA ACT GAC C-3', reverse primer 5'-GCC TGC AGC ACACTGGTTG-3' (Ivan Bieche et al., 2003), for β-actin were forward primer 5'-CGACAACGGCTCCGGCATGTGC-3, reverse primer 5- GTCACCGGAGTCCATCACGATGC-3'. The expression of EGFR and β-actin was performed by PCR programme for 40 cycles, denaturation at 95°C for 40 s, annealing at 60°C for 40 s, extension at 72°C for 40s and reaction volume was 20 µl. A final extension step at 72°C for 5 min to complete the reaction and melting curve analysis was performed between the range of 35°C to 90°C to ensure the specific amplification. A control without cDNA was included in each experiment as non template control and all reaction were performed in duplicate. The relative quantification method (ΔΔCT) was used to analyse the EGFR gene expression level by using β-actin as internal control and final results were expressed as mean fold change in EGFR gene expression in NSCLC patients as compared to control.

Statistical analysis

Statistical analysis of data was performed using the SPSS 16 and Graph Pad version 6.0. Mann Whitney and Kruskal Wallis test were used to analyze the association with different variables. The Kaplan-Meier method was used to calculate the overall survival of NSCLC patients. A p value <0.05 was considered indicative of a statistically significant difference.

Results

Demographics

All demographic features of the subjects are depicted (Table 1). In brief, total of 100 Non-small cell lung cancer patients (adenocarcinoma) were analyzed. This study included both males (71%) and females (29%) and mean age of 54.37 years. 44% patients were in stage IV and 15%, 15%, 26% patients in stage I, II and III respectively while 44% patients had distant metastases . Patients with different pathological grade, grade 1 (well differentiated) includes 24%, grade 2 (moderately differentiated) includes 41% and grade 3 (Poorly differentiated) includes 35% cases. We included smoker 45% as well as non smoker 55% with different smoking type as cigarette, bidi, and hukka, 18% cases smoked cigarette, 16% cases smoked bidi and 11% cases smoked hukka.

EGFR gene expression and NSCLC patients:

We analyzed EGFR gene expression with several variables of Non-small Cell Lung Cancer patients in this study and observed mean value of increased gene expression was 13.54 fold in Non-small Cell Lung Cancer patients. Patients in stage I showed 4.07 fold increased EGFR gene expression, stage II showed 7.08 fold increased gene expression while in stage III showed 14.81 fold increased gene expression and stage IV showed 18.35 fold increased gene expression which is significantly associated (p<0.0001). Patients with metastases had 18.35 fold increased gene expression while patients without metastases had 9.86 fold increased gene expression also showed significant differences (p<0.0001) in gene expression. Patients who had pleural effusion showed 17.66 fold increased gene expression while patients without pleural effusion showed 12.88 fold increased gene expression. Patients with different smoking type is significantly associated (p=0.002) with EGFR gene expression. Patients who smoked bidi had 18.55 fold increased gene expression in comparison with cigarette (15.51fold) and hukka (7.22 fold) smokers. Patients with different smoking level had also significantly associated (p=0.03) with EGFR gene expression, data showed in Table 2.

EGFR gene expression and patients' survival

We assessed overall survival between two groups of NSCLC patients expressing above 13 fold and below 13 fold gene expression of EGFR in NSCLC patients. Patients with <13 fold increased gene expression had 15.8

Table 1. Demographic Characteristics of NSCLC Patients

Variables	NSCLC Patients (%)
Total no. of cases	100
Gender	
Males	71
Females	29
Age (years)	
<55	56
>55	44
Mean ± SD age (years)	54.37+10.77 (range 32-75years)
TNM Stage	
Stage I	15
Stage II	15
Stage III	26
Stage IV	44
Distant Metastases	
Positive	44
Negative	56
Histopathological Grade	
Grade 1	24
Grade 2	41
Grade 3	35
Pleural effusion	
Yes	15
No	85
Smoking Status	
Non Smoker	55
Smoker	45
Smoking Status	
Non Smoker	55
Current Smoker	24
Ex. Smoker	21
Smoking Type	
Cigarette	18
Bidi	16
Hukka	11
Smoking Level (Pack Year)	
Mild (<10)	23
Moderate (< 40)	18
Heavy (>40)	4

Table 2. EGFR Gene Expression of Patients with Different Variables

Variables	Mean + SD	Range	p value
NSCLC patients	13.54+7.87	2.07- 37.53	
Gender			
Males	13.45+7.66	2.07- 31.78	0.84
Females	13.96+8.49	2.41- 37.53	
Age (years)			
<55	13.90+8.46	2.13- 37.53	0.98
>55	13.21+7.12	2.07- 29.65	
TNM Stage			
Stage I	4.07+2.71	2.13-12.55	<0.0001
Stage II	7.08+4.06	2.07-13.18	
Stage III	14.81+4.86	8.88-29.86	
Stage IV	18.35+7.11	8.51-37.53	
Distant Metastases			
Positive	18.35+7.11	8.51-37.53	<0.0001
Negative	9.86+6.29	2.07-29.86	
Histopathological Grade			
Grade 1	13.19+8.75	2.41-31.78	0.72
Grade 2	13.47+7.55	2.07-30.70	
Grade 3	14.43+7.28	3.56-37.53	
Pleural Effusion			
Yes	17.66+5.86	9.06-30.27	0.009
No	12.88+7.98	2.07-37.53	
Smoking Status			
Non Smoker	12.81+7.03	2.07-30.70	0.57
Smoker	14.57+8.77	2.22-37.53	
Smoking Status			
Current Smoker	15.24+7.11	2.97-29.04	0.44
Ex. Smoker	13.79+10.48	2.22-37.53	
Smoking Type			
Cigarette	15.51+8.57	3.01-31.78	0.002
Bidi	18.55+8.52	2.43-37.53	
Hukka	7.22+4.29	2.22-13.18	
Smoking Level (Pack Year)			
Mild (<10)	15.60+9.41	2.43-37.53	0.03
Moderate (< 40)	15.47+7.54	2.22-29.65	
Heavy(> 40)	4.57+3.22	2.60-9.38	

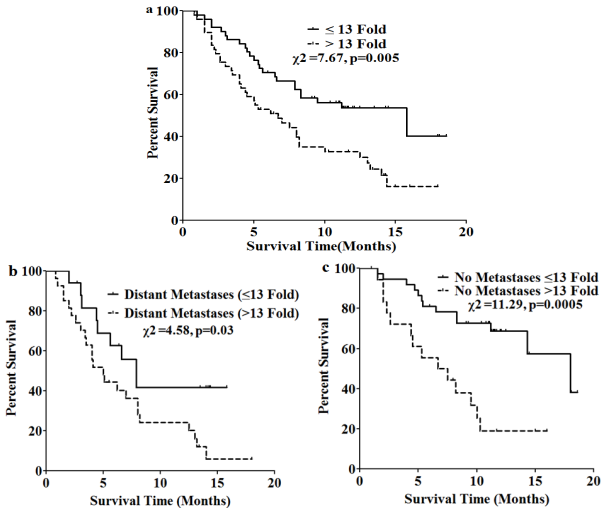

Figure 1. Kaplan-Meier survival Curves with Respect to EGFR Gene Expression: (a) Overall Survival (b, c) Distant Metastases and no Metastases

month median survival time while >13 fold increased gene expression had only 6.7 month median survival time (Figure 1a). We further assessed the role of EGFR gene expression in survival of NSCLC patients with respect to their metastases status. Distant metastatic patients with <13fold increased gene expression had 7.9 months of median survival time while>13 fold increase gene expression had 5.0 months of median survival time (p=0.03) (Figure 1b). Non metastatic patients with<13 fold increased gene expression had 18 months of median survival time while patients with >13 fold increased gene expression had 7.1 months of median survival time (Figure 1c).

Discussion

In our present study we made an attempt to identify cell free EGFR mRNA expression in NSCLC patients. Amongst the lung cancers, non-small cell lung cancer (NSCLC) comprises 80% - 85% of all cases and more than 70% patients are diagnosed in advanced stage (Siegel et al., 2013; Xu et al., 2014). Several experimental evidence suggests that the EGFR is involved in tumour

formation and progression. Overexpression of EGFR has been correlated with prognosis and metastatic spreading in several carcinoma types, such as breast, head and neck, and lung malignancies (Salomon et al., 1995). Overexpression of EGFR has been reported and implicated in the pathogenesis of many human malignancies, including NSCLC (Inamura et al., 2010). Scagliotti et a. (2004) also have analysed that EGFR expression in NSCLC is associated with reduced survival (Scagliotti et al., 2004). In present study we also observed that higher EGFR gene expression has been found to be associated with poor survival of NSCLC patients. We observed a significant difference in gene expression level in different stages. Patients in stage IV, stage III and stage II had 4.5, 3.63 and 1.73 fold higher gene expression, in comparison to patients in stage I respectively. When patients overall survival time compared with <13 fold and >13 fold increased gene expression and found patients with >13 fold increased gene expression had 2.35 times reduced overall survival in comparison to patients with <13 fold increased gene expression. Distant metastatic patients with >13 fold increase gene expression had 1.58 times reduced overall survival in comparison to patients with <13 fold increased gene expression. In addition, Non metastatic patients with >13 fold increased gene expression had 1.28 times reduced overall survival while distant metastatic patients with >13 fold increased gene expression had 2.53 times reduced overall survival. EGFR overexpression in pancreatic cancer to range from 30% to 95% in various studies (Bloomston et al., 2006) and EGFR expression has been correlated with local advanced and metastatic stage of disease (Tobita et al., 2003). In NSCLCs particularly adenocarcinomas, EGFR is overexpressed in 70% of the patients (Sekido et al., 2003). EGFR is overexpressed in the advanced NSCLC patients, and is associated with the poor survival and EGFR expression is clearly involved in the lung cancer pathogenesis (Dowell et al., 2005). Travis et al in his study suggested that EGFR expression level may be used as prognostic and predictive marker in NSCLC (ADC) (Travis et al., 2013). EGFR was found to be a region for metastatic competency, and is thought to promote cancer cell migration and invasion (Masuda et al., 2012). Franklin WA and Hirsch FR et al. also observed that in lung carcinomas, EGFR is more commonly over expressed (Franklin et al., 2002; Hirsch et al., 2002). Its expression has been detected in a wide variety of human malignancies, including up to 50-78% of breast cancers (Ge et al., 2002). It has been revealed that high EGFR expression was found in many solid tumors such as, gastric cancer and breast cancer (Atmaca et al., 2012; Zhang et al., 2013). Lee et al. (2014) also suggested, EGFR overexpression may be the independent poor prognostic factor in primary breast carcinoma (Lee et al., 2014).

Here in this study we conclude cell free EGFR mRNA expression may be a predictive factor for patients' poor overall survival and metastatic behavior of NSCLC patients. Due to the small sample size in the present study our findings need to be validated by further independent and prospective studies on larger population.

Acknowledgements

The authors thank to all the study subjects and All India Institute of Medical Sciences, New Delhi, for assistance in recruiting the subjects.

References

Atmaca A, Werner D, Pauligk C, et al (2012). The prognostic impact of epidermal growth factor receptor in patients with metastatic gastric cancer. *BMC Cancer*, **12**, 524.

Bloomston M, Bhardwaj A, Ellison EC, et al (2006). Epidermal growth factor receptor expression in pancreatic carcinoma using tissue microarray technique. *Dig Surg*, **23**, 74-9.

Dowell JE, Minna JD (2005). Chasing mutations in the epidermal growth factor in lung cancer. *N Engl J Med*, **352**, 830-2.

Engelman JA, Janne PA, Mermel C, et al (2005). ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinibsensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci U S A,* **102**, 3788-93.

Franklin WA, Veve R, Hirsch FR, et al (2002). Epidermal growth factor receptor family in lung cancer and premalignancy. *Semin Oncol*, **29**, 3-14.

Ge H, Gong X, Tang CK (2002). Evidence of high incidence of EGFRvIII expression and co-expression with EGFR in human invasive breast cancer by laser capture microdissection and immunohistochemical analysis. *Int J Cancer*, **98**, 357-61.

Gschwind A, Fischer OM, Ullrich A (2004). The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nature Rev Cancer,* **4**, 361-70.

Hayes DC, Secrist H, Bangur CS, et al (2006). Multigene real-time PCR detection of circulating tumor cells in peripheral blood of lung cancer patients. *Anticancer Res*, **26**, 1567-75.

Hirsch FR, Varella-Garcia M, Capuzzo (2009). Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. *Oncogene*, **28**, 32-7.

Hirsch FR, Varella-Garcia M, Franklin WA, et al (2002). Evaluation of HER2/ neu in non-small cell lung cancer by immunohistochemistry and fluorescence in-situ hybridization (FISH) techniques. *Br J Cancer*, **86**, 1449-56.

H J Lee, A N Seo, E J Kim et al (2014). Prognostic and predictive values of EGFR overexpression and EGFR copy number alteration in HER2-positive breast cancer. *British J Cancer*, **112**, 103-11.

Hynes NE, Lane HA (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer*, **5**, 341-54.

Inamura K, Ninomiya H, Ishikawa Y, et al (2010). Is the epidermal growth factor receptor status in lung cancers reflected in clinicopathologic features? *Arch Pathol Lab Med,* **134**, 66-72.

Ivan Bieche, Peter Onody, Sengul Tozlu, et al (2003). Prognostic value of ERBB family mRNA expression in breast carcinomas. *Int J Cancer*, **106**, 758-65.

Lewis SZ, Diekemper R, Addrizzo Harris DJ (2013). Methodology for development of guidelines for lung cancer: diagnosis and management of lung cancer, 3rd ed: american college of chest physicians evidence-based clinical practice guidelines. *Chest*, **143**, 41-50.

Masuda H, Zhang D, Bartholomeusz C, et al (2012) Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Res Treat*, **136**, 331-45.

Qing Li, Yongfeng Tang, Xue Cheng, et al (2014). EGFR protein expression and gene amplification in squamous intraepithelial lesions and squamous cell carcinomas of the cervix. *Int J Clin Exp Pathol,* **7**, 733-41.

Quesnelle KM, Boehm AL, Grandis JR (2007). STAT-mediated EGFR signaling in cancer. *J Cell Biochem*, **102**, 311-9.

Riese D J, Stern DF, (1998). Specificity within the EGF family/ ErbB receptor family signaling network. *Bioessays*, **20**, 41-8.

Salomon DS, Brandt R, Ciardiello F, et al (1995). Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol*, **19**, 183-232.

Scagliotti GV, Selvaggi G, Novello S, et al (2004). The biology of epidermal growth factor receptor in lung cancer. *Clin Cancer Res*, **10**, 4227-32.

Schlessinger J (2004). Common and distinct elements in cellular signaling via EGF and FGF receptors. *Science*, **306**, 1506- 07.

Sekido Y, Fong KM, Minna JD, (2003) Molecular genetics of lung cancer. *Annu Rev Med*, **54**, 73-87.

Siegel R, Naishadham D, Jemal A (2013). Cancer statistics, *CA Cancer J Clin*, **63**, 11-30.

Tobita K, Kijima H, Dowaki S, et al (2003). Epidermal growth factor receptor expression in human pancreatic cancer: Significance for liver metastases. *Int J Mol Med*, **11**, 305-9.

Travis WD, Brambilla E, Noguchi M, et al (2013). Diagnosis of lung cancer in small biopsies and cytology: implications of the 2011 international association for the study of lung cancer/american thoracic society/european respiratory society classification. *Arch Pathol Lab Med*, **137**, 668-84.

Utsugi T (2013). New challenges and inspired answers for anticancer drug discovery and development. *Jpn J Clin Oncol,* **43**, 945-53.

Wang X, Zhang S, MacLennan GT, et al (2007). Epidermal growth factor receptor protein expression and gene amplification, in small cell carcinoma of the urinary bladder. *Clin Cancer Res*, **13**, 953-7.

Wang X, Zhang S, MacLennan GT, et al (2008). Epidermal growth factor receptor protein expression and gene amplification in the chemorefractory metastatic embryonal carcinoma. *Mod Pathol*, **22**, 7-12.

World Health Organization (2012). Cancer fact sheet. http:// www.who.int/mediacentre/factsheets/ fs297/en/.

Xu W, Yang G, Xu Y, et al (2014). The possibility of traditional chinese medicine as maintenance therapy for advanced nonsmall cell lung cancer. *Evid Based Complement Alternat Med,*. **2014**, 278917.

Yarden Y, Sliwkowski MX, (2001). Untangling the ErbB signalling network. *Nature Rev Mol Cell Biol*, **2**, 127-37.

Zhang YJ, Gallis B, Taya M, et al (2013). pH-Responsive artemisinin derivatives and lipid nanoparticle formulations inhibit growth of breast cancer cells *in vitro* and in duce down-regulation of her family members. *PLoS One*, **8**, 59086.