

RESEARCH ARTICLE

Overexpression of EGFR Protein in Bruneian Lung Cancer Patients

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Abstract

Background: Lung cancer is the leading cause of cancer death in Brunei Darussalam, accounting for almost 20% of the total. The epidermal growth factor receptor (EGFR) is a member of the erbB family of tyrosine kinase receptor proteins, which includes c-erbB2 (HER2/neu), erb-B3, and erb-B4. EGFR overexpression is found in a third of all epithelial cancers, often associated with a poor prognosis. **Materials and Methods:** Protein expression of EGFR in 27 cases of lung cancer tissue samples and 9 cases of normal lung tissue samples was evaluated using an immunohistochemical approach. **Results:** The results demonstrated significant increase and overexpression of EGFR in Bruneian lung cancer tissue samples in comparison to normal lung tissue. However, there was no significant relationship between clinicopathologic variables (age and sex) of patients and EGFR protein expression. **Conclusions:** EGFR is overexpressed in Bruneian lung cancer patient tissue samples in comparison to normal lung tissue samples. This may indicate that EGFR protein over expression plays an important role in the genesis of this type of cancer in Brunei Darussalam.

Keywords: Lung cancer - EGFR - immunohistochemistry - overexpression

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Introduction

Lung cancer has been the most common cancer in the world for decades. It is estimated to have 1.8 million new cases in 2012 (World Health Organisation, 2013). Lung cancer also is the most common cause of cancer mortality worldwide, estimated to be the cause of nearly one in five (1.59 million deaths, 19.4% of the total) [World Health Organisation, 2013]. Each year, more people die of lung cancer than of colon, breast, and prostate cancers combined (American Cancer Society, 2014).

The chances of a man developing lung cancer in his lifetime are 1 in 13, while it is at 1 in 16 for women. These numbers include smokers and non-smokers, with smokers having a higher incidence rate of lung cancer (Sanchez et al., 2004).

The 5-year survival rate for non-small cell lung cancer (NSCLC) varies from 60-80% for early Stage I to below 10% for advanced Stage IV disease (Srisam et al., 2005). Less than 10% of lung cancer cases are discovered at an early stage and can undergo surgical resection with potentially long-term survival (Srisam et al., 2005). The lack of capability to detect lung cancer in early stages is due to the lack of valid methods for early detection (Mutti, 2008). The death from lung cancer is the result of late detection, frequent recurrence after surgery and

poor responsiveness to chemotherapy (Wang et al., 2010).

Epidermal Growth Factor Receptor (erb-B1) is a member of the erbB family of tyrosine kinase receptor proteins, which includes c-erbB2 (HER2/neu), erb-B3, and erb-B4 (Oda et al., 2005). EGFR consists of a single polypeptide chain of 1186 amino acids (Scagliotti et al., 2004). As a transmembrane glycoprotein, the EGFR molecule consists of an extracellular domain that binds with ligands; an intracellular region with tyrosine kinase activity and a transmembrane region with a single hydrophobic anchor sequence (Scagliotti et al., 2004; Oda et al., 2005).

Previous studies have shown that EGFR is expressed or highly expressed in various human tumor cells (Abdel Salam et al., 2009). Studies have reported a correlation between levels of EGFR protein expression and mutation with poor prognosis and reduced survival rates of lung cancer (Phsaki et al., 2000). These receptors play a key role for tumor cell survival and proliferation (Phsaki et al., 2000).

EGFR is commonly overexpressed in non-small cell lung cancer and head and neck cancers (Hirsch et al., 2003; Grandis et al., 2003). The prognostic association of EGFR overexpression in lung cancer is a controversial issue. Many reports indicated that EGFR overexpression is associated with poor prognosis, whereas other reports

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indicated that overexpression of EGFR was not associated with poor prognosis (Hirsch et al., 2003). The differences in reported EGFR protein expressions from one study to another most likely reflect differences in the assessment techniques, definition of the level of overexpression, and differences in the study populations.

Recent trials have suggested that for advanced NSCLC patients with EGFR mutant tumors, initial therapy with a TKI instead of chemotherapy may be the best choice of treatment (Ciardiello and Tortora, 2001). However, an optimal method of determining EGFR levels in tumors has yet to be found (Ettinger, 2006). The correlation between levels of EGFR and treatment effectiveness is debated. Reports of EGFR as a prognostic factor showed disparate results, though for some tumors, EGFR is a good indicator of reduced survival rates in more aggressive disease (Dacic et al., 2006).

Most studies on EGFR protein expression use semiquantitative immunohistochemistry (IHC) analysis. Due to lack of technique standardization, staining intensity interpretation, and result evaluation, there is a certain difficulty in comparing results from different laboratories and authors (Grandis et al., 1996).

In the present study, we have extended the previously published reports by looking at the expression of EGFR in Bruneian lung cancer tissue samples versus normal lung tissues. In addition, the expression of EGFR in lung cancer tissue samples was correlated with the clinicopathologic variables of these patients.

Materials and Methods

Tissue samples

This study was conducted after obtaining the approval of the Medical and Health Research Ethics Committee and Universiti Brunei Darussalam Research Ethics Committee. Twenty seven Formalin Fixed Paraffin Embedded (FFPE) biopsies of cancer lungs (13 Adenocarcinoma, 8 Squamous carcinoma, 2 Large cell carcinoma, 1 Bronchoalveolar carcinoma, 1 Malignant lymphoma, 1 Small-cell carcinoma, 1 Carcinoid tumor), were obtained from the tissue archive at the Department of Pathology of RIPAS Hospital, Brunei Darussalam from year 2011 to 2012. Nine samples of macroscopically/ microscopically normal lung tissue obtained from surgery were also examined for EGFR protein expression.

Immunohistochemistry

DAKO's protocol (DAKO Corporation, Glostrup, Denmark) for EGFR pharmDx was followed for immunohistochemistry assay of lung tissue biopsies. Briefly, tissue sections were deparaffinised with xylene and then dehydrated with 95% and then with absolute alcohol. A negative control was included in every experiment conducted, in which the primary antibody was omitted. In addition, each experiment included a positive control consist of lung tissue sample known to be positive for EGFR protein expression.

The tissue sections were digested at 37°C for 30 minutes with proteinase K (DAKO Corporation, Denmark). Peroxidase blocking was carried out for 5

minutes by using Peroxidase-Blocking Solution, (Dako Corporation, Denmark). The primary antibody consisted of DAKO Mouse Anti-Human EGFR (F4) antibody (Dako Corporation, Denmark). The primary antibody, was added to each tissue section (except negative control tissue section) and tissue sections samples were incubated at room temperature for one hour. After washing with Phosphate-buffered saline (PBS), the secondary antibody DAKO Anti-mouse (Dako Corporation, Denmark), was added for 30mins. Between all of the above mentioned step sections were washed with PBS for 4 minutes.

Visualization of positive signals for EGFR was carried out for 12mins by using substrate-chromogen solution (3,3'-Diaminobenzidine, DAB).

After washing in running water for at 10 minute sections were counterstained with Haematoxylin and then mounted. Slides were examined microscopically and positive and negative signals for the expression of EGFR were evaluated and data were recorded.

Evaluation of the immunohistochemical staining of EGFR in Lung cancer tissue samples

Evaluation of immunohistochemistry results for EGFR expression in Lung cancer was carried out following the guidelines and interpretation manuals as provided by DAKO Corporation, Ltd. Samples were classified into four categories (0, 1+, 2+, 3+). Tumor with a complete absence of staining was scored 0. Weak, incomplete membranous staining was scored +1. Tumors with either strong, incomplete basolateral staining or weak, completely membranous staining in more than 10% of the tumor cells were scored 2+. Those with strong, complete membranous staining in more than 10% of the tumor cells was scored as 3+.16 According to the HercepTest EGFR guidelines for scoring, tumors scored as 0 and 1+ were considered 'negative', those at 2+ would be 'equivocal' and tumors classified under 3+ were considered to be 'positive'. Tumors with a score of 2+ and higher were considered as showing overexpression.

Statistical analysis

Cases were evaluated for demographic and histological variables. Distribution between these variables were compared using the χ^2 test or Fisher's exact test.

Statistical analysis was performed using SPSS 16 for Windows.

Dichotomous variables are reported as numbers (%). Continuous variables are reported as medians and ranges.

Results

Twenty-seven tissue samples obtained from patients with lung carcinoma were examined for levels of expression of EGFR. Of the lung cancer specimens, thirteen (48.1%) were adenocarcinoma, two (7.4%) were large cell carcinoma, and eight (29.6%) were squamous carcinoma. Other histological findings include one (3.7%) broncho-alveolar carcinoma, one (3.7%) malignant lymphoma, one (3.7%) small-cell carcinoma, and one (3.7%) carcinoid tumor, totaling up to four (14.8%). The racial distribution of lung cancer, 18 (66.7%) Malay, 8

Chinese (29.6%) and 1 others (3.7%) reflects the racial distribution in the population of Brunei Darussalam.

The cancer histology was equally distributed across the sexes except for squamous cell carcinoma, which was three times more for men than women.

Twenty-two of the lung cancer tissue samples (81.5%) were positive for the expression of EGFR (2+/3+)[Figure.1], with twelve (44.4%) being strongly positive (3+). In the normal lung samples, four (44.4%) showed expression of EGFR (2+/3+), with none being strongly positive (3+). The greatest number of EGFR overexpression was seen in adenocarcinomas (69.2%), followed by squamous carcinoma (50%). Patients with adenocarcinoma had a greater range of EGFR protein expression from 0 to 3+. Patients with squamous carcinoma, large cell carcinoma, malignant lymphoma and carcinoid tumour tend to show a greater amount of EGFR protein expression. (Table 1). Of the nine normal lung tissue samples, one (11.1%) showed expression of EGFR (Table1).

The rate of EGFR overexpression is about equal between the two sexes, with male patients at ten (76.9%) and female patients at eleven (78.6%). Seven (53.8%) males showed a 3+ score in EGFR compared to five (35.7%) females (Table 1). One (11.1%) normal lung tissue showed overexpression of EGFR protein with a score of 2+. The remaining eight (88.9%) presented with a score of 1+ for EGFR protein expression. The lung cancer tissues showed significantly higher rate

intensity of staining for EGFR as compared to normal lung tissue($p=0.01$; $p<0.05$). The positive rate of EGFR protein in lung cancer tumor cells were 81.5%, which was significantly higher than its expression in normal lung ($p=0.01$ $p<0.05$)

The pattern of the expression of EGFR are summarized in Table 2. Twelve (44.4%) samples of lung cancer showed both membranous and cytoplasmic staining. Two (7.4%) showed membranous staining only(Figure.1), and seven

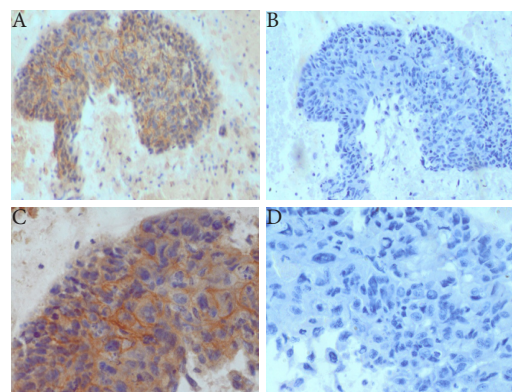


Figure 1. Examples of Expression of EGFR Protein in Lung Cancer Tissue Samples. Positive signals for EGFR expression appears as brown color stain. A) Adenocarcinomas; 10x magnification; B) Serial section to Figure (A), in which the primary antibody was omitted (negative control); C) 40x magnifications showing EGFR membranous staining. D) Negative control to Figure 1 (C).

Table 1. Distribution of EGFR Protein Expression Scores According to Lung Cancer Histology and Sex in 27 Lung Carcinomas and Lung Cancer Histology in 9 Normal Lung Tissues

Histology	0	EGFR expression scores			Total	Positive (2+/3+)	P value
		1+	2+	3+			
Lung Carcinoma							
Adenocarcinoma	1	3	4	5	13	9	0.01
Squamous carcinoma	0	0	4	4	8	8	
Large cell carcinoma	0	0	1	1	2	2	
Bronchoalveolar carcinoma	0	0	1	0	1	1	
Malignant lymphoma	0	0	0	1	1	1	
Small cell carcinoma	1	0	0	0	1	0	
Carcinoid tumour	0	0	0	1	1	1	
Total					27	22	
Normal	0	8	1	0	9	1	
Sex							
Male	1	2	3	7	13	10	
Female	1	2	6	5	14	11	
Total					27	21	

* $p<0.05$

Table 2. Distribution of Staining Location in Lung Tissue According to Lung Cancer Histology

	Staining Location		
	Membranous only	Cytoplasmic only	Membranous & cytoplasmic
Adenocarcinoma	3	1	8
Squamous carcinoma	1	1	6
Large cell carcinoma	1	0	0
Bronchoalveolar carcinoma	1	0	0
Malignant lymphoma	0	0	1
Small cell carcinoma	0	0	0
Carcinoid tumour	1	0	0

(25.9%) showed cytoplasmic staining only. Two (7.4%) were unstained. (Table 2).

Discussion

Lung cancer is the major cause of death in Brunei Darussalam (Ministry of Health, Brunei Darussalam, 2013). Lung cancer claims the highest mortality rate with over 24.9% of all cancer morbidity cases in Brunei Darussalam (Ministry of Health, Brunei Darussalam, 2013).

Previous studies have shown that EGFR is expressed or highly expressed in various human tumour cells (Salomon et al., 1995). Previous studies have reported a correlation between levels of EGFR protein expression and gene mutation with poor prognosis and reduced survival rates of lung cancer (Sanja et al., 2006; Ekrem et al., 2010). In non-small cell lung cancer, overexpression of EGFR or mutations in intracellular EGFR have been observed in 43-89% of cases (Gupta et al., 2009). These mutations occur within EGFR exons 18-21 (Soh et al., 2009; Baek et al., 2014). These mutations increase the kinase activity of EGFR, leading to activation of signaling pathways associated with cell over growth and survival (Soh et al. 2009). Due to the vital role played by EGFR in lung cancer development, its consequently, became a target for anti-cancer drug therapy (Giaccone, 2005; Zhao et al., 2014). Treatment with EGFR inhibitors have shown a promising results in NSCLC (Ciardiello and Tortora, 2001). Gefitinib and Erlotinib are two drugs used for the treatment of advanced NSCLC. The mode of action of these two drugs is based on their capability to inhibit the tyrosine kinase activity of EGFR by competing with ATP for the ATP-binding site (Barghi et al., 2014; Han et al., 2015; Hsieh et al 2015). Recently published study developed a nanosized polymeric delivery system for the drug "Erlotinib" (Barghi et al., 2014). This new approach may become the future tool for the treatment of lung cancer.

In the present study, we have evaluated the expression of EGFR in lung cancer and in normal lung tissue samples, using immunohistochemical approach.

The present study, demonstrated overexpression of EGFR in Bruneian lung cancer tissue samples versus normal lung tissue samples. To our knowledge, this is a unique study as it compared between the expressions of EGFR in normal lung tissue samples with the expression of EGFR in lung cancer tissue samples obtained from Bruneian patients.

In the present study, we have used immunohistochemical approach to evaluate the expression of EGFR in lung tissue samples. The reason for using Immunohistochemistry (IHC), being IHC is one of the most commonly used methods of evaluating EGFR levels (Cooper et al., 2013). Other techniques such as immunoassay, enzyme-linked immunosorbent assay (ELISA) and fluorescence in-situ hybridization (FISH) are also used (Pauletti et al., 2000; Salam et al., 2009) However, due to IHC being relatively simple to perform, and easily replicable in almost any laboratory, it has potential to be a screening test for identifying molecular biomarkers for lung cancer (Grands

et al., 1996).

In the current study, 81.5% of the lung cancer tissue samples obtained from Bruneian patients showed overexpression of EGFR protein. This is consistent with the findings in other studies (Ekrem et al., 2010). Even so, the range of overexpression of said studies can widely vary, from 43-89% (Hirsch et al., 2003). This a greater variance in expression levels of EGFR in lung cancer, thus casting doubt on its reliability as a prognostic marker, it indicates that there may be certain factors that are causing this spread in results (Hoang et al., 2000; Tubbs et al., 2001). Differences in findings can occur as a result of variations in the IHC techniques used by different authors. Standardized interpretation criteria are also lacking as the length and method of fixation, not to mention the degree of antigen retrieval can significantly affect the resultant slides produced by IHC for analysis (Hoang et al., 2000; Tubbs et al., 2001). Moreover, specificity and sensitivity of the antibodies used clearly affects the results, as mentioned previously, whereby mutant-specific antibodies react better to their specific target 0.36 Interobserver variance cannot be discounted as interpretation of the IHC results is highly subjective. This is especially true in studies which focus on membranous staining. Studies involving cytoplasmic staining with a positive staining cut-off is more standardized. Studies on membranous staining, however, have their attention on the intensity of the staining only. It appears that the techniques for IHC assay and analysis should be standardized for its utility in clinical use (Tubbs et al., 2001).

In the present study and in order to overcome these issues, we have included a proper negative, positive controls and normal lung tissues in each experiment we have conducted and also, the evaluation process of our data was carried out by two evaluators.

In the present study, 22 of the lung cancer tissue samples (81.5%) were positive for the expression of EGFR (2+/3+), with twelve (44.4%) being strongly positive (3+). In the normal lung samples, four (44.4%) showed expression of EGFR (2+/3+), with none being strongly positive (3+). The greatest number of EGFR overexpression was seen in adenocarcinomas (69.2%), followed by squamous carcinoma (50%). Patients with adenocarcinoma had a greater range of EGFR protein expression from 0 to 3+. Patients with squamous carcinoma, large cell carcinoma, malignant lymphoma and carcinoid tumour tend to show a greater amount of EGFR protein expression. In principal, our finding is consistent with previously published work (Cooper et al., 2013)

In addition, our data showed that the EGFR protein expression does not correlate with the clinicopathologic variables such as sex ($p=0.004$), age ($p=0.02$) and race ($p=0.008$). These findings are in agreement with previous findings (Li et al., 2011).

In conclusion, the findings of the present study demonstrate a significant increase and overexpression of EGFR in Bruneian lung cancer tissue samples in comparison to normal lung tissue samples. This may indicate that EGFR protein over expression plays an important role in the genesis of this type of cancer in Brunei Darussalam.

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