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Original Paper

A Comparison of Epidermal Growth Factor Receptor Levels and Other Prognostic Parameters in Non-small Cell Lung Cancer

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Epidermal growth factor receptor (EGFR) was measured using a competitive radioligand binding assay in membrane preparations from 74 primary human non-small cell lung cancer (NSCLC) tissues and 20 pathologically normal peripheral lung tissues. The mean EGFR level in tumours was 30.38 fmol/mg (± 41.95 S.D.) of membrane protein (mg·p), significantly higher ($P = 0.00016$) than in normal tissues (mean, 10.26 ± 10.02 fmol/mg·p). The mean EGFR concentration was also significantly higher in pathological stage IV tissue than in stages I ($P = 0.049$) and II ($P = 0.040$), and the mean EGFR concentration was significantly higher in cases with mediastinal involvement than in cases without it ($P = 0.029$). The mean EGFR level was higher in DNA aneuploid and multiploid cases than in DNA diploid cases, but there was no significant difference. No significant relationships were found to exist between receptor concentrations and pathological tumour size or histological type, or patient gender or age. From the above findings, a possible prognostic role for EGFR in primary NSCLC should be investigated.

Key words: EGFR, NSCLC, DNA ploidy, competitive radioligand binding assay, prognostic parameter

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INTRODUCTION

EPIDERMAL GROWTH factor receptor (EGFR), first purified from the A431 cell line [1], is a 170 kDa transmembrane glycoprotein with an intracellular domain that exhibits tyrosine kinase activity and presumably has binding sites for ATP (adenosine 5′-triphosphate) [2]. The DNA sequence coding for EGFR is related to the *ERBB2* oncogene product, a membrane protein with receptor function, and to the *V-ERBB* product, which represents the intracellular and transmembrane domain of EGFR [1]. The enzymatic activity is stimulated by epidermal growth factor (EGF) and transforming growth factor alpha (TGFα), and autophosphorylation of the receptor occurs [3, 4], which regulates the growth and differentiation of ectodermal-derived cells.

These growth factors and EGFR play an important role in cellular proliferation and differentiation [5, 6]. Some evidence suggests that EGFR could be related to malignant transformation. High concentrations of EGFR have been found in many ectodermal-derived malignancies [7–10]. Some reports have suggested that examination of this receptor is useful for predicting the prognosis of tumours. High levels of EGFR in breast cancer have been related to poorer prognoses [11, 12] and advanced gastric carcinomas have shown a higher level of expression of EGFR than early gastric carcinomas [13].

In this study, EGFR levels were investigated in non-small cell lung cancer (NSCLC) tissue using a competitive radioligand binding assay, and the relationships between EGFR expression and other prognostic parameters were analysed.

PATIENTS AND METHODS

Patients

74 patients (54 male, 20 female) with primary NSCLC were evaluated (mean age 64.3 years, range 39–85 years). 42 patients had adenocarcinomas, 26 squamous cell carcinomas, 3 large cell carcinomas and 3 adenosquamous cell carcinomas. All patients were diagnosed and treated in the Second Department of Surgery at Shiga University of Medical Science between 1992 and 1993. Patients were

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staged according to the tumour, node and metastasis (TNM) classifications of the International Union Against Cancer (UICC) [14]. 33 patients had stage I lung cancer, 7 stage II, 15 stage IIIA, 9 stage IIIB and 10 stage IV (7 of the stage IV cases were diagnosed as stage IV by pulmonary metastasis after surgery and distant metastases were found within 3 months in the 3 other patients). As a control, 20 pathologically normal, peripheral lung tissue samples were examined. Of the control group patients (mean age 55.6 years), 6 had surgical treatment for lung cancer, 6 for metastatic lung tumour, 4 for pneumothorax, and 4 for benign lung disease. Samples from patients with malignant diseases were collected at least 10 cm away from the malignancies.

Competitive radioligand binding assay for EGFR

All tissue samples were collected during surgery and minced, quick frozen, and stored in liquid nitrogen until processed.

The competitive radioligand binding assay for EGFR was carried out as described by Fitzpatrick and associates [15]. Briefly, unlabelled recombinant human-EGF (Otsuka Assay, Tokushima, Japan) was iodinated with ^{125}Na using chloramine-T with specific activity ranging from 200 to 400 μCi/μg. Supercooled samples were pulverised and homogenised in an ice-cold TEMG buffer (10 mM Tris–HCl, 1.5 mM EDTA (ethylenediaminetetraacetic acid), 10 mM monothioglycerol, 10% glycerol, pH 7.5) and the homogenate thus obtained was centrifuged at 40000 rpm for 30 min at 4°C. The membrane pellet was resuspended in phosphate buffer saline (PBS), pH 7.0, then centrifuged at 3200 rpm for 10 min at 4°C, after which the supernatant was adjusted to a protein concentration of 200–300 μg/100 μl. The protein concentration was measured by the Bradford protein-dye binding method [16]. Competitive radioligand binding assay using ^{125}I-EFG and unlabelled EGF was performed consecutively. Five concentrations of ^{125}I-EGF (0, 0.26, 0.52, 1.04, 2.08 nM) and 100-fold concentrations of cold EGF for non-specific binding were used; ^{125}I-EGF was incubated for 2 h at 20°C. The binding reaction was stopped by adding ice-cold PBS containing 5 mg/ml bovine serum albumin (BSA), and it was then filtrated through glass microfibre filters (Whatman GF/B, International Ltd Maidstone, U.K.). The filters were washed twice with ice-cold PBS containing BSA and counted in an Aloka auto gamma counter for 1 min. The high-affinity contents of EGFR were calculated from the binding data by Scatchard analysis. Results were expressed as fmols of EGFR per mg of membrane protein (fmol/mg·p).

Analysis of DNA contents by flow cytometry

From the same supercooled tissue samples as above, DNA contents were analysed by flow cytometry, and the DNA index (DNAI) was calculated [17]. The DNA patterns were divided into diploid ($0.9 \leqslant$ DNAI $\leqslant 1.1$), aneuploid (0.9 > DNAI or 1.1 < DNAI; aneuploidy with a single aneuploid peak), and DNA multiploid patterns (aneuploidy with multiple aneuploid peaks). When the DNAI was approximately 2.00, peaks with a proportion of over 10% of total cell count were judged to be aneuploid peaks.

Statistical analysis

Statistical analysis was performed using unpaired *t*-tests; when the *F* test was significant, Welch's *t*-test was used, and when the *F* test was not significant, the Student's *t*-test was used. In all statistical analyses, the difference was considered to be significant when the *P* value was less than 0.05.

RESULTS

The mean EGFR concentration (± standard deviation [S.D.]) in 20 samples of pathologically normal lung tissue was 10.26 ± 10.02 fmol/mg·p (range, 0–38.0). The calculated upper limit of the normal range (mean + 2 S.D.) was taken as 30.3 fmol/mg·p. Cancer tissue had a mean EGFR concentration of 30.38 ± 41.95 fmol/mg·p (range 0–210.3), which was significantly higher than that in normal lung tissue ($P = 0.00016$). The median value was 7.90 fmol/mg·p in normal lung tissue and 15.15 fmol/mg·p in cancer tissue.

Histological type and EGFR concentrations

The mean EGFR concentration was 26.34 ± 35.32 fmol/mg·p (range 0–160.1) in the 42 adenocarcinomas and 40.96 ± 53.37 fmol/mg·p (range 0–210.3) in the 26 squamous cell carcinomas. A trend towards higher EGFR levels in squamous cell carcinomas than in adenocarcinomas was observed, but no significant differences were found between any histological types. The median values were 14.80 fmol/mg·p in the adenocarcinomas and 21.35 fmol/mg·p in the squamous cell carcinomas.

Pathological stage and EGFR concentrations

The mean EGFR concentrations were 22.17 ± 25.84 fmol/mg·p (range 0–117.6) in the 33 stage I cancers; 17.00 ± 15.45 fmol/mg·p (range 0.8–39.8) in the 7 stage II cancers; 32.98 ± 51.70 fmol/mg·p (range 0–210.3) in the 15 stage IIIA cancers; 36.20 ± 50.77 fmol/mg·p (range 0–145.3) in the 9 stage IIIB cancers; and 57.67 ± 63.50 fmol/mg·p (range 3.4–160.1) in the 10 stage IV cancers. Significant differences were observed between stages I and IV ($P = 0.049$) and between stages II and IV ($P = 0.040$) (Figure 1). The early stage cancers (stages I and II) had a significantly lower mean value (21.27 ± 24.26 fmol/mg·p) than the advanced stage cancers (stages III and IV) (41.09 ± 54.57 fmol/mg·p) ($P = 0.028$). The median fmol/mg·p values were 19.30 in the stage I cancers, 13.90 in the stage II cancers, 26.50 in the stage IIIA cancers, 8.20 in the stage IIIB cancers, and 21.95 in the stage IV cancers.

Pathological lymph node factor and EGFR concentrations

The mean EGFR concentrations were 24.05 ± 32.91 fmol/mg·p (range 0–151.8) in the 41 N0 cancers; 17.15 ± 14.23 fmol/mg·p (range 0.8–39.8) in the 11 N1 cancers; and 48.79 ± 58.59 fmol/mg·p (range 0–210.3) in the 22 N2 cancers. Significant differences were observed between the N0 and N2 cases ($P = 0.018$) and between the N1 and N2 cases ($P = 0.012$) (Figure 2). Cancers with mediastinal involvement (N2) had a significantly higher mean value (48.79 ± 58.59 fmol/mg·p) than cancers with no mediastinal involvement (N0 and N1) (22.59 ± 29.96 fmol/mg·p) ($P = 0.029$). The median values were 13.50 fmol/mg·p in the N0 cancers, 13.90 fmol/mg·p in the N1 cancers, and 31.1 fmol/mg·p in the N2 cancers.

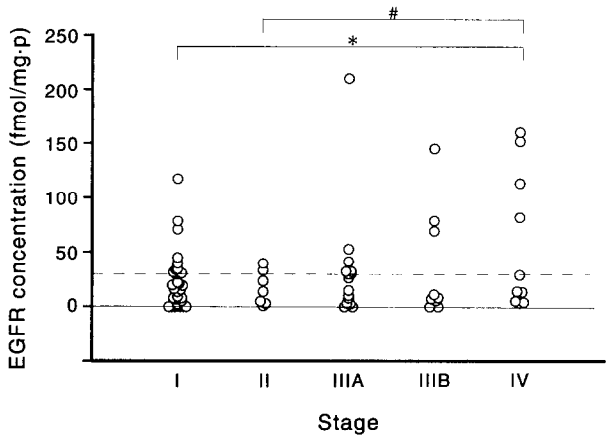

Figure 1. Pathological stage and epidermal growth factor receptor concentration. *$P = 0.049$, #$P = 0.040$; there were no significant differences between any other two groups ($P = 0.135 \sim 0.406$).

Pathological tumour size factor and EGFR concentrations

The mean EGFR concentrations were 26.49 ± 36.85 fmol/mg·p (range 0–160.1) in the 19 T1 tumours; 27.83 ± 38.80 fmol/mg·p (range 0–210.3) in the 40 T2 tumours; 38.38 ± 64.45 fmol/mg·p (range 2.7–151.8) in the 5 T3 tumours, and 43.93 ± 53.75 fmol/mg·p (range 0–145.3) in the 10 T4 tumours. There were no significant relationships between EGFR levels and pathological tumour size factors. The median values were 15.40 fmol/mg·p in the T1 cancers, 17.70 fmol/mg·p in the T2 cancers, 4.10 fmol/mg·p in the T3 cancers, and 9.75 fmol/mg·p in the T4 cancers.

DNA ploidy and EGFR concentrations

The mean EGFR concentrations were 23.40 ± 22.07 fmol/mg·p (range 0–78.5) in the 13 DNA diploid cancers; 28.41 ± 39.88 fmol/mg·p (range 0–160.1) in the 53 DNA aneuploid cancers; and 54.75 ± 70.02 fmol/mg·p (range 3.4–210.3) in the 8 DNA multiploid cancers, but no significant differences were found. Abnormally high concentrations of EGFR were observed in the cases with DNA aneuploidy (Figure 3). The median values were 16.20 fmol/mg·p in the DNA diploid cancers, 14.20 fmol/mg·p in the DNA aneuploid cancers, and 33.40 fmol/mg·p in the DNA multiploid cancers.

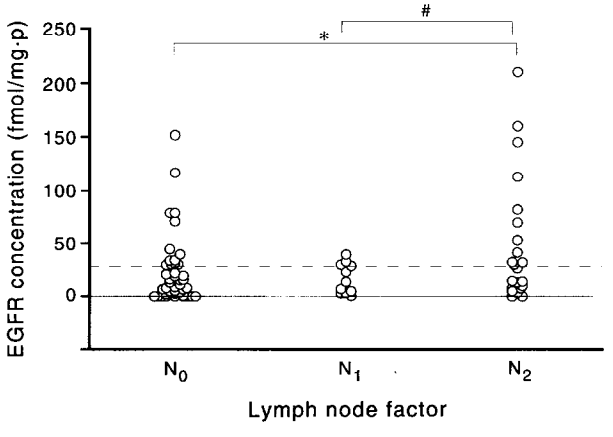

Figure 2. Pathological lymph node factor and epidermal growth factor receptor concentration. *$P = 0.018$, #$P = 0.012$; there was no significant difference between N0 and N1 ($P = 0.182$)

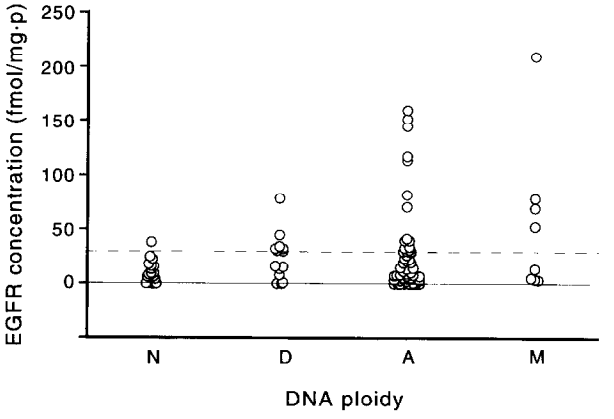

Figure 3. DNA ploidy and epidermal growth factor receptor concentration. N, normal lung tissue; D, DNA diploidy; A, DNA aneuploidy; M, DNA multiploidy; there were no significant differences between any two groups ($P = 0.077 \sim 0.461$).

Gender, age and EGFR concentrations

The mean EGFR concentration was 27.35 ± 41.21 fmol/mg·p in the 54 male patients (range 0–210.3) and 38.54 ± 43.91 fmol/mg·p in the 20 female patients (range 2.9–160.1). There was no significant relationship between EGFR concentrations and patients' gender. The median values were 12.40 fmol/mg·p in the males and 24.30 fmol/mg·p in the females.

No significant relationship was observed between EGFR concentrations and patients' age ($r^2 = 0.016$, data not shown).

DISCUSSION

EGFR levels in various cultured cells and tumour tissues have been measured and elevated levels of EGFR have been demonstrated, particularly in squamous cell carcinoma cell lines and tumour tissues [18, 19]. The presence of EGFR in lung cancer has also been shown [20–22]. Dittadi and associates [23] reported the results of the measurement of EGFR using a competitive radioligand binding assay in membrane preparations from 51 human NSCLC tissue and in normal tissue from the same patients. According to their report, EGFR concentrations in lung cancer tissues were significantly higher than in normal lung tissues. Because the distribution pattern in normal lung tissue was Gaussian, they deduced that EGFR in lung tissue could be physiologically expressed. On the basis of EGFR distribution in normal lung tissue samples, they calculated the normal/pathological cut-off point to be 12.9 fmol/mg·p. This is lower than the calculated cut-off point in the present study.

Many studies have shown that EGFR expression is higher in squamous cell carcinomas than in other histological types [21, 24]. No significant difference in EGFR concentrations between the squamous cell carcinomas and adenocarcino-

mas was found in the present study, but the mean level of EGFR concentrations was higher in squamous cell carcinomas.

In the present study, the relationship between DNA ploidy and EGFR concentration was investigated. There are some reports that have discussed this topic with regard to malignancies [25, 26]. Arai and colleagues showed that EGFR expression in aneuploid gastric cancers was significantly more frequent than in diploid cancers [25]. Chow and associates could not find a significant correlation between DNA content and EGFR expression in 56 cases of transitional cell carcinoma [26]. However, they emphasised the necessity for further evaluation. We found no report that discussed this topic with regard to lung cancer. In the present study, no significant relationship between DNA ploidy and EGFR concentrations was seen, but the mean concentration of EGFR was highest in the DNA multiploid cases and lowest in the DNA diploid cases, and the median value was higher in the DNA multiploid cancers than in the DNA diploid or aneuploid cancers. Abnormally high concentrations of EGFR were not observed in the cases with DNA diploidy.

Attempts to establish a relationship beween EGFR levels and prognosis or other clinical and pathological prognostic parameters in primary lung cancers have led to conflicting results. Tateishi and colleagues showed that the 5-year survival rates of patients with high EGF or TGFα levels were significantly worse only in the EGFR-positive cases [27]. Fontanini and associates suggested that EGFR was a useful indicator of nodal metastasis [28]. Veale and colleagues reported a higher expression of EGFR in stage III tumours using an immunohistochemical method [21], but were unable to confirm the results with a ligand binding assay [29]. Dittadi and associates found no significant relationships between EGFR concentrations or positivity and pathological stage, lymph node factor or tumour size factor, and found only a trend for a direct relation between receptor positivity and grading [23]. The present study did not demonstrate any correlations between EGFR positivity (cut-off point 30.3 fmol/mg·p) and other prognostic parameters (data not shown), but did show trends towards a direct relationship between EGFR concentrations and pathological stage or lymph node factor.

Only a few reports show a relationship between EGFR concentrations and prognosis in patients with lung cancer. Veale and colleagues demonstrated that patients with high EGFR concentration have a poor prognosis [30]. They suggested a prognostic cut-off point of 34.4 fmol/mg·p, which is almost the same as the calculated upper limit to the normal range in the present study. We did not have enough data to determine the relationship between EGFR concentration and long-term patient prognoses. However, 2 of 11 patients died from stage I lung cancer in the high EGFR group (EGFR $\geqslant$ 30.3 fmol/mg·p; median follow-up 21 months), while only 1 of 22 patients died from stage I lung cancer in the low EGFR group (EGFR < 30.3 fmol/mg·p; median follow-up 23 months).

In this study, some significant relationships were found between EGFR concentrations and prognostic parameters. A possible prognostic role for EGFR in lung cancers should be investigated.

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