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Original Article

The Role of Epidermal Growth Factor Receptor in Head and Neck Squamous Cell Carcinoma in Thai Patients

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Abstract

Introduction: **Epidermal growth factor receptor (*EGFR*), a member of the type 1 tyrosine kinase family of receptors, plays a crucial role in several types of cancer especially in squamous cell carcinoma of the head and neck (HNSCC).**

Objective: **This study aimed to determine the frequency of *EGFR* overexpression in HNSCC and to find the possible correlation with various clinical pathological parameters and patient outcomes.**

Materials and Methods: **Fresh cancerous tissues and matched normal mucosa were collected from 78 previously untreated HNSCC patients after obtaining informed consent. All patients had no detectable distant metastases at presentation. *EGFR* mRNA expression were examined by quantitative real-time RT-PCR analysis. Data were correlated with both clinicopathological characteristics and survival outcome.**

Results: **Overexpression of *EGFR* mRNA was found in 21 of 78 patients. *EGFR* expression was significantly correlated with overall survival in univariate analysis.**

Conclusions: ***EGFR* expression plays an important role in the pathogenesis and progression of HNSCC.**

Keywords: **Head and neck cancer, *EGFR*, Squamous cell carcinomas**

INTRODUCTION

Head and neck cancer is the fifth most common type of cancer worldwide and is a significant cause of morbidity and mortality with an estimate of 650,000 new cases and 350,000 cancer-related deaths every year.[1,2] Epidermal growth factor receptor (*EGFR*) is a member of the tyrosine kinase family of receptors.[3,4] Overexpression of *EGFR* mostly found in 90% of head and neck tumors.[5-7] Upregulation of this factor occurs early in the progression of dysplasia to HNSCC in the upper aerodigestive tract.[3,4] Overexpression of *EGFR* gene is frequently found in head and neck tumor and has been proposed to be due to gene amplification. Recent studies of *EGFR* mRNA, Jin et al. detected the *EGFR* mRNA level by RT-PCR showed that 49% of specimens overexpressed *EGFR* in tumor compared with normal mucosa.[8] Several studies report *EGFR* protein overexpressed in 25% to 95% of HNSCC tumors by immunohistochemical technique.[9] For example, Schartinger et al. demonstrated *EGFR*

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overexpression in 44.7% (51 of 114 cases) of HNSCC.[10] Several studies have investigated correlations between altered expression of *EGFR* in HNSCC and clinicopathological parameters or tumor behavior.[9] Although most studies suggested prognostic implications of *EGFR* expression in HNSCC, the sometimes contradictory results are currently under debate. Some authors reported correlation between *EGFR* overexpression with advanced tumor stage[11-13], tumor differentiation[13,14], nodal metastasis[11,12,14,15], disease-free survival[16,17], and overall survival[17,18]. In contrast, others studies in HNSCC showed no correlation between *EGFR* overexpression and tumor size[18-20], tumor differentiation[19-22], tumor site[19,21], tumor stage[15,18,19,21,23], nodal stage[18-20,22,23], clinical stage[14,18,23], tumor thickness[19,23], and survival.[14,19,23]

In the present study, we characterized the expression and prognostic value of *EGFR* receptor in Thai patients and their relationship with clinico-pathological characteristics.

MATERIALS AND METHODS

Patients and tissue samples

Total RNAs were extracted from frozen section of HNSCC tumors from 78 patients undergoing surgical resection for HNSCC at the Division of Head-Neck & Breast Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, from January 28, 2002 through January 4, 2004 after obtaining informed consent and following guidelines established by the Siriraj Ethical Committee on Research Board. All patients had no detectable metastases to distant organs at presentation. All patients will be selected by inclusion criteria: patients diagnosed with HNSCC (primaries of the oral cavity, oropharynx, hypopharynx, pharynx and larynx) with no prior chemo- or radio-therapy. Patients with recurrent disease, incomplete standard treatment, or lost to follow-up were excluded.

Reverse transcription

Tissue samples and head and neck cancer cell lines will be prepared for total RNA extraction using Trizol® reagent (Invitrogen). DNase treatment was employed, in order to eliminate DNA contamination in RNA samples. The cDNA synthesis will be performed using Omniscript RT Kit® (Qiagen).

Real time PCR

Real-time PCR was performed in the LightCycler PCR device (Roche Diagnostics, Mannheim, Germany). The Quantitect SYBR-Green PCR kit (Qiagen) together with 0.5 µmol/L of each primer was used as a master mix (total volume, 20 µl). Eighteen microlitres of master-mix were filled in the glass capillaries and 2 µl volume of cDNA was added as PCR template. Cycling condition were 95˚C for 15 min, 45 cycles of 95˚C 15 s, 57˚C 20 s, 72˚C 10 s). β-actin was used as a reference gene. A calibrator sample was included in every run and used for normalization of final results. Sequences of PCR primer sets for EGFR receptors were as follows: EGFR forward, TCCCAGTGCCTGAATACATA; EGFR reverse, TGGACAGTGTTGAGATACTCG, product size = 150 bps; β-actin forward, CACTCTTCCAGCCTT CCTTCC and β-actin reverse, CTGTGTTGGCGTA CAGGTCT, product size = 114 bps. An efficiency curve for each primer was constructed by using various dilutions of cDNA. After quantification, an efficiency curve was generated by Light cycle software and efficiency of each primer was calculated. The data were analyzed and compared using a relative quantification method:

$$\text{ratio} = \frac{(E_{\text{ref}})^{\text{CPSample}}}{(E_{\text{target}})^{\text{CPSample}}} \div \frac{(E_{\text{ref}})^{\text{CPCalibrator}}}{(E_{\text{target}})^{\text{CPCalibrator}}}$$

where ratio is relative amount of EGFR relative to β-actin; E = Efficiency of primer; cp = cycle threshold of PCR product

Statistical Analysis

All statistical analyses were performed using the SPSS statistical software system (SPSS for Windows, version 16.0). The association between the different clinicopathological and biological characteristics was studied by the Pearson χ^2 test. Survival was measured in months from the date of surgery to the date of death or to the last follow-up. Cancer specific survival curves and median survival times were calculated by the method of Kaplan and Meier, and differences in survivor function due to prognostic factors were calculated by the log-rank test. A P-value of less than 0.05 was considered statistically significant. The predictive values of various biological markers and clinicopathologic parameters were assessed with univariate and multivariate logistic regression analysis.

Table 1 Clinicopathological features of 78 patients with HNSCC

Parameters	Number of patients (%)
Age(years)	
<60	31 (40)
≥60	47 (60)
Median, range	64.5, 27-100
Gender	
Males	37 (47)
Females	41 (53)
Alcohol drinking	
Yes	44 (56)
No	33 (42)
Unknown	1 (2)
Smoking	
Yes	40 (51)
No	37 (47)
Unknown	1 (2)
Betel nut chewing	
Yes	26 (33)
No	52 (67)
Tumor Sites	
Oral cavity	72 (91)
Oropharynx	4 (6)
Oropharynx and Hypopharynx	2 (3)
Histological grade	
Well differentiated	36 (46)
Moderately differentiated	35 (44)
Poorly differentiated	4 (6)
Unknown	3 (4)
T stage	
T1	13 (16)
T2	18 (23)
T3	18 (23)
T4	28 (36)
Unknown	1 (2)
N stage	
N0	42 (54)
N1	8 (10)
N2	19 (24)
N3	2 (3)
Unknown	7 (9)
Overall stage	
1	13 (16)
2	10 (13)
3	12 (15)
4	32 (41)
Unknown	11 (15)
Perineural invasion	
Yes	25 (31)
No	42 (54)
Unknown	11 (15)
Perivascular invasion	
Yes	16 (21)
No	53 (68)
Unknown	9 (11)
Post-operative radiotherapy	
Yes	44 (56)
No	32 (41)
Unknown	2 (3)
Post-operative chemotherapy	
Yes	5 (6)
No	70 (90)
Unknown	3 (4)

RESULTS

Clinicopathological features of 78 patients with HNSCC

In each case, a portion of tumor was resected near the advancing edge of the tumor, avoiding its necrotic center. After excision, the tissues were immediately snap-frozen and stored in liquid nitrogen until use. The adjacent tissues were submitted for histopathological study which revealed that most of the cells were malignant. Tumors were staged according to the TNM classification 5th edition and graded as: well, moderately and poorly differentiated. The T stage was evaluated according to tumor size in the case of tumors from the oral cavity or the oropharynx, and of tumor size and extensiveness in the case of the tumor from the hypopharynx. The mode of cancer invasion was divided into perineural and perivascular invasion (Table 1).

Expression of EGFR mRNA in HNSCC tissues

To examine the expression of *EGFR* in head and neck cancer patients, I performed quantitative real time RT-PCR analysis of *EGFR* mRNA derived from head and neck cancer tissues (n = 78) and normal adjacent mucosae (n = 17) of Thai HNSCC patients.

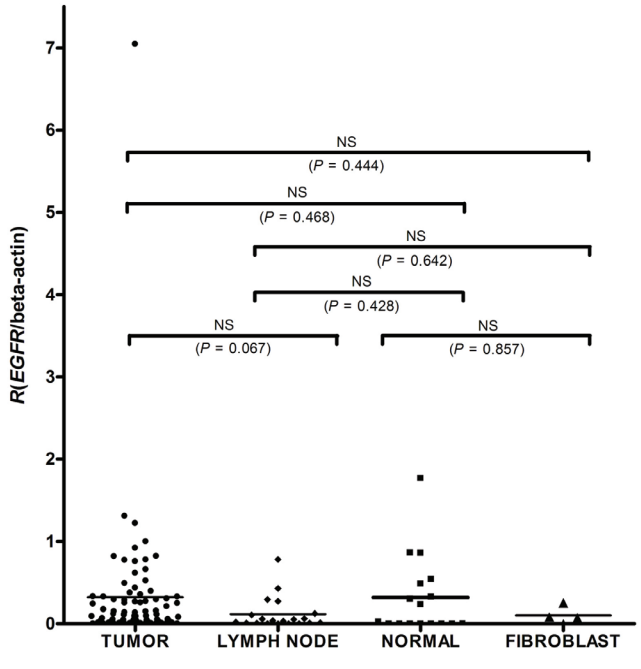

Figure 1 *EGFR* mRNA expression by real time RT-PCR in HNSCC tissue samples.

Table 2 Clinicopathological parameters of HNSCC according to overall survival

Parameters	Dead (%)	Alive (%)	Odds ratio	95% CI	P value
Age(years)					
<60	12 (39)	19 (61)			
≥60	27 (60)	18 (40)	2.375	0.931-6.062	0.070
Gender					
Females	15 (43)	20 (57)			
Males	24 (59)	17 (41)	1.882	0.755-4-692	0.275
Alcohol drinking					
No	14 (44)	18 (56)			
Yes	25 (57)	19 (43)	2.000	0.796-5.024	0.321
Smoking					
No	15 (42)	21 (58)			
Yes	24 (60)	16 (40)	2.100	0.840-5.243	0.240
Betel nut chewing					
No	26 (50)	26 (50)			
Yes	13 (57)	10 (43)	1.300	0.484-3.490	0.736
Tumor Sites					
Oral cavity	35 (69)	16 (31)			
Oropharynx and Hypopharynx	4 (16)	21 (84)	0.087	0.026-0.296	0.216
Histological grade					
Well differentiated	28 (62)	17 (38)			
Moderately-Poorly differentiated	31 (61)	20 (39)	0.941	0.413-2.146	0.913
T stage					
T1-T2	9 (26)	26 (74)			
T3-T4	30 (65)	16 (35)	5.417	2.051-14.302	0.004
N stage					
N0	18 (45)	22 (55)			
N1-N3	21 (58)	15 (42)	1.711	0.689-4.249	0.024
Overall stage					
1-2	6 (26)	17 (74)			
3-4	33 (62)	20 (38)	4.675	1.582-13.818	0.002
Perineural invasion					
No	21 (40)	31 (60)			
Yes	18 (75)	6 (25)	4.429	1.508-13.005	0.007
Perivascular invasion					
No	28 (47)	32 (53)			
Yes	11 (69)	5 (31)	2.514	0.718-8.121	0.105
Post-operative radiotherapy					
No	11 (34)	21 (66)			
Yes	28 (64)	16 (36)	3.341	1.287-8.670	0.009
Post-operative chemotherapy					
No	36 (51)	35 (49)			
Yes	3 (60)	2 (40)	1.458	0.230-9.263	0.645
EGFR mRNA					
Negative	24(44)	31 (56)			
Positive	15 (71)	6 (29)	3.229	1.090-9.570	0.034
HER2 DNA					
Negative	12 (41)	17 (59)			
Positive	3 (43)	4 (57)	0.438	0.075-2.552	0.943
HER2 mRNA					
Negative	23 (51)	22 (49)			
Positive	16 (52)	15 (48)	1.020	0.409-2.548	0.966
HER2 protein					
Negative	13 (68)	6 (32)			
Positive	12 (43)	17 (57)	0.326	0.096-1.101	0.071

EGFR mRNA was examined using SYBR green dye and the fluorescence signal was detected by the Light Cycler instrument. Relative mRNA expression levels of the *EGFR* in head and neck cancer tissue were normalized to the level of β-actin mRNA expression in the respective sample. I decided to use the value above mean of fibroblast + 2 SD as cut of point to show *EGFR* expression positivity. The *EGFR* mRNA were detected by real time RT-PCR in 27% (21 of 78) of tumor, in 45% (9 of 20) of metastatic lymph node, in 35% (6 of 17) of normal adjacent mucosae and not found EGFR positive in normal fibroblast (0 of 4). The mean of mRNA expression of *EGFR* was greater in tumor (0.3261), metastatic lymph node (0.2908) and normal adjacent mucosae (0.3174) when compared with normal fibroblast (0.1016). However, the expression levels among groups were not significantly different (Figure 1).

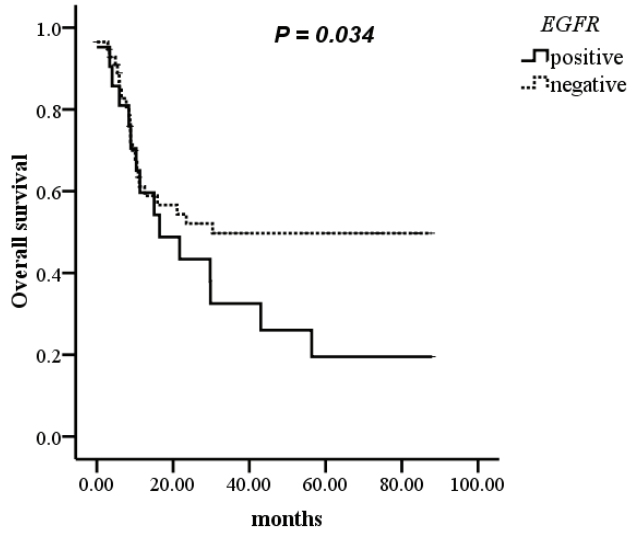

Figure 2 Kaplan-Meier curves for the overall survival of HNSCC patients were calculated according to EGFR mRNA expression (positive versus no).

Correlations between clinicopathological parameters and overall survival

Overall survival was measured from diagnosis to date of death/date of last follow-up status. Survival curve was calculated by the Kaplan-Meier method and groups compared by the univariate analysis. As of 3 January 2011, 38 patients (48.7%) were alive, whereas 40 patients (51.3) were dead. As shown in Table 2, statistically significant correlations were demonstrated between overall survival and tumor stage (P = 0.004), overall stage (P = 0.002), perivascular invasion (P = 0.007), post-operative radiotherapy (P = 0.009) or *EGFR* mRNA (P = 0.034; Figure 2). However, other clinical variables including age, gender, alcohol drinking, smoking, betel nut chewing, tumor site, histological grade, nodal stage, perineural invasion, post-operative chemotherapy, had no significant association with overall survival.

Correlations between level of EGFR mRNA and clinicopathological parameters

The relationship between the level of EGFR mRNA expression in HNSCC tumors (n = 78) and clinicopathological parameters were analyzed. As shown in Table 3, *EGFR* mRNA expression in HNSCCC tumors showed no statistically significant correlation with clinicopathological parameters (age, gender, alcohol drinking, smoking, betel nut chewing, tumor site, histological grade, tumor stage, nodal stage, overall stage, perivascular invasion, perineural invasion, post-operative radiotherapy, post-operative chemotherapy, recurrent, last follow-up).

DISCUSSION

The present results showed that the mean mRNA levels of *EGFR* among primary tumors, metastatic lymph nodes and normal adjacent mucosae did not differ significantly. In addition, *EGFR* mRNA in tumor showed overexpression around 27% (21/78). The percentage of this results was less than previous report that showed 49% of specimens overexpressed *EGFR* in tumor compared with normal adjacent mucosae.[8] The mean mRNA levels of *EGFR* from normal adjacent mucosae were not different and tend to be higher than those of primary tumor. These results may be explained that some normal adjacent mucosae from patients exposed to carcinogen (smoking and alcohol) are subjected to genetic change so called "field can-cerization" leading to aberration in growth factor receptors. Other studies also found *EGFR* gene overexpression in normal mucosa of patients. For example, Grandis et al. showed *EGFR* mRNA and protein levels in tumor tissues and histological normal mucosae of HNSCC patients were greater than in control normal mucosae from patients without cancer[24]. Jin et al. also detected *EGFR* mRNA

Table 3 Correlation between *EGFR* mRNA expression and clinicopathological parameters

Parameters	EGFR mRNA expression		P valuea
	Positive	Negative	
Age(years)			0.081
<60	5	26	
≥60	16	31	
Gender			0.623
Males	12	29	
Females	9	28	
Alcohol drinking			0.851
Yes	13	31	
No	8	26	
Smoking			0.853
Yes	10	30	
No	11	27	
Betel nut chewing			0.588
Yes	8	18	
No	13	39	
Tumor Sites			0.630
Oral cavity	19	53	
Oropharynx	2	2	
Oropharynx and Hypopharynx	0	1	
Histological grade			0.302
Well differentiated	9	27	
Moderately-Poorly differentiated	10	29	
Unknown	2	1	
T stage			0.135
T1-T2	25	26	
T3-T4	16	31	
N stage			0.853
N0-N1	14	36	
N2-N3	6	15	
Unknown	1	6	
Overall stage			0.632
1-2	5	18	
3-4	14	30	
Unknown	2	9	
Perineural invasion			0.381
Yes	8	17	
No	12	30	
Unknown	1	10	
Perivascular invasion			0.156
Yes	5	11	
No	16	37	
Unknown	0	9	

in both squamous cell carcinomas specimens and normal mucosa adjacent to carcinoma.[8] This finding suggested that the normal adjacent mucosae in cancer patients may not be an ideal normal control.

Several studies have demonstrated the relationship between the *EGFR* overexpression and adverse clinicopathological parameters in HNSCC, but the results in Thai patients were unknown. The present studies showed that *EGFR* mRNA overexpression in HNSCC tumors showed no statistically significant correlation with clinicopathological parameters. Whereas, other studies demonstrated correlation between *EGFR* expression with tumor size[13], tumor stage[11,12], nodal stage[11,14], lymph node metastasis[12,15] and tumor differentiation.[13,14]

The univariate and multivariate analyses were also used to evaluate the correlation between clinicopathological parameters and overall survival. The

results showed statistically significant correlations between overall survival and tumor stage, overall stage, perivascular invasion, post-operative radiotherapy or *EGFR* mRNA. In addition, multivariate analysis of the eight categories demonstrated that tumor stage, nodal stage and overall stage were independent predictors for overall survival. Taken together, similar findings were observed in previous studies that *EGFR* overexpression correlated with overall survival.[17,18]

In conclusion, the clinical study of *EGFR* expression showed significantly correlation with overall survival.

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