

Correlation of Epidermal Growth Factor Receptor Mutation, Immunohistochemistry, and Fluorescence in Situ Hybridization in Esophageal Squamous Cell Carcinoma

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Background: The epidermal growth factor receptor (EGFR) has become a promising target for novel anticancer therapy. Evaluation of its biological profiles including gene mutation, amplification, and protein expression in esophageal squamous cell carcinoma (ESCC) is essential to establish the EGFR molecular feature(s) suitable to select patients in anti-EGFR therapy.

Material and Method: The subjects' specimens of ESCC at Songklanagarind Hospital were obtained and investigated for EGFR protein expression and gene amplification. Polymerase chain reaction (PCR) was performed to amplify the EGFR DNA product. The mutational status of EGFR exons 19 and 21 was analyzed using direct sequencing. The entire biological profiles of the EGFR were then correlated.

Results: There were 48 eligible ESCC specimens. No somatic mutation in the tyrosine kinase domain of EGFR was detected. A high level of EGFR protein was exhibited in 22 patients (46%). Twenty-three patients (48%) had shown a high gene copy numbers. However, no direct correlation between EGFR protein and gene status was observed.

Conclusion: EGFR mutations in the tyrosine kinase domain of exons 19 and 21 were absent in ESCC, whereas, protein overexpression and gene amplification was prevalent. Therefore, selection of ESCC patients for studies with anti-EGFR agents based on protein expression or gene copy number, not gene mutation, is rational.

Keywords: Epidermal growth factor receptor, Mutation, Immunohistochemistry, Fluorescence in situ hybridization, Esophageal, Squamous cell carcinoma

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Esophageal cancer remains one of the most devastating cancers, of which 3.2 male and 0.9 female per 100,000 population are estimated worldwide each year⁽¹⁾. Tumor classification contains two distinct

histological subtypes: adenocarcinoma, which is prevalent in Western countries and squamous cell carcinoma found in Asia, East Europe, and South America. Esophageal carcinoma is prevalent in Southern Thailand where the incidence rate is higher than in other parts of the country (6.1 in males and 2.5 in females per 100,000 population)⁽¹⁾ and most of the patients have esophageal squamous cell carcinoma (ESCC). Due to the difficulty of undertaking radical

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surgery in locally advanced esophageal cancer, non-surgical treatments such as chemotherapy and radiotherapy have been considered a standard option in unresectable patients. However, the epidermal growth factor receptor (EGFR), which plays a critical role in cellular proliferation, differentiation, and survival⁽²⁾, has been known to have a high expression correlated with resistance to chemoradiotherapy and a poor prognosis in various kinds of human cancers⁽³⁾. The new treatment modalities such as molecular targeted therapies have been applied to a number of solid tumors including esophageal cancer. Among the rapidly progressing data on molecular therapy, EGFR tyrosine kinase inhibitors (EGFR-TKIs) are the most investigated⁽⁴⁾.

Exon 18 through 21 of the EGFR gene encompasses most of the tyrosine kinase binding domain⁽⁵⁾ and EGFR mutation are restricted in these four exons. Furthermore, there are reports showing that the deletion in exon 19 and the point mutation of codon 858 in exon 21 covers about 90% of cases with the EGFR-TK mutation⁽⁶⁾. An application of anti-EGFR for cancer therapy has been reported with an impressive efficacy in multiple tumors, especially in non-small cell lung cancers (NSCLC)^(7,8). Interestingly, a preclinical study has demonstrated that levels of EGFR expression did not always correlate with gefitinib, an antitumor growth agent, but mutations in exons 19 and 21 of the EGFR gene were associated with sensitivity to EGFR-TKIs in NSCLC patients^(9,10). From a clinical point of view, however, other investigators demonstrated no significance of the EGFR mutation on the survival outcome in NSCLC patients who had received EGFR-TKIs^(11,12). Due to the heterogeneity of the significance of EGFR profiles as predictive markers to EGFR targeted therapy, patients should not be selected for treatment based only on the EGFR profiles⁽¹³⁾. Thus, the present study was carried out to analyze the mutations within the EGFR-TK domain in exons 19 and 21 of ESCC and to evaluate the correlation of the status of gene mutation with gene copy number and protein expression. Ultimately, the aim was to identify appropriate markers for EGFR status to select patients for clinical trials of anti-EGFR therapy in ESCC.

Material and Method

Specimens

At Songklanagarind Hospital, the specimens were obtained from patients with ESCC, who had undergone esophagectomy and who had been investigated for EGFR protein expression and gene

amplification. A semiquantitative scoring system was used for EGFR protein interpretation as previously published⁽¹⁴⁾. In brief, the percentage of EGFR positive tumor cells (0-100%) was multiplied by the dominant intensity staining pattern (1 = negative or trace; 2 = weak; 3 = moderate; 4 = intense), scores ranging from 0-400. Scores > 200, 201-300, and 301-400 were classified as negative/low (Fig. 1a), intermediate, and high levels (Fig. 1b) of EGFR protein expression respectively. In addition, the gene copy number was scored and characterized as balanced disomy (Fig. 1c), balanced trisomy or balanced polysomy when the chromosome numbers per nucleus were 2, 3 or more copies, respectively, and the ratio gene/chromosome copy number per cell was 0.9-1.2. Gene amplification (Fig. 1d) was assessed by multiple, clustered copies of the gene in which any chromosomal status with the gene/chromosome ratio was 1.3 or higher, as previously published⁽¹⁴⁾. Balanced polysomy was also recognized as gene overrepresentation. The EGFR gene copy number was considered to be at a low level when a FISH pattern revealed either balanced disomy or trisomy and at a high level when gene overrepresentation or amplification was observed.

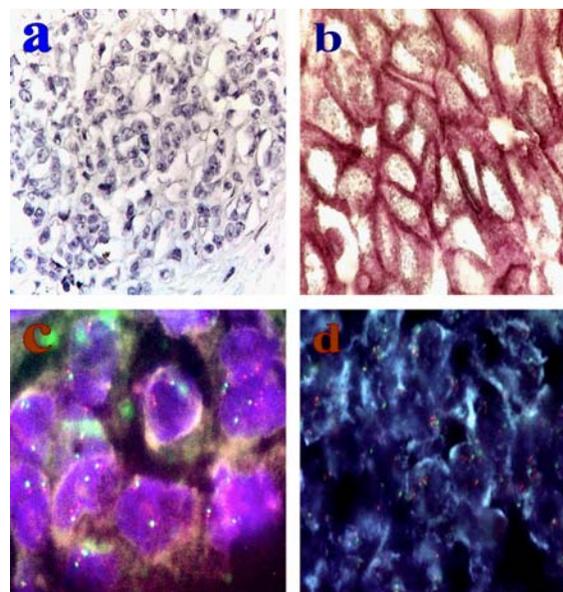


Fig. 1 Immunohistochemical analysis of EGFR protein expressions (above) and amplification of the EGFR gene by FISH (below); (a) the negative/low (x 40) protein expression, (b) high protein expression (x100), (c) balanced disomy (BD) and (d) gene amplification (GA)

Laboratory procedures

EGFR gene sequencing and analysis

Formalin-fixed, paraffin-embedded tissue blocks with > 80% of tumor cells were dissected at 5 µm thickness for the sectioned slides. DNA extraction was performed using a QIAamp DNA Kit. PCR amplification (initial denaturation at 95°C for 7 minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute with a final extension step of 7 minutes at 72°C, then maintenance of the reaction at 4°C for 2 minutes after cycling) was used to amplify exons 19 and 21 of the EGFR gene. The following oligo-nucleotide primers were used: Exon 19 forward, 5'- tggatcccagaagtgagaaag - 3'; reverse, 5'-gcaaagcagaaactcacatcgag - 3'; Exon 21 forward, 5'- taccgtggtgaaaacaccgc - 3' and reverse, 5'- ccttactttgctctcttctgc - 3'. PCR products were separated and visualized by electrophoresis in 2% agarose gel and ethidium bromide stained to check the quality of the PCR products. Mutations were detected by direct sequencing of the purified amplified DNA products in both the sense and antisense directions using the BigDye® Terminator Ready Reaction Cycle Sequencing kit v3.0 (ABI Prism, Foster City, CA) following the manufacturer's instructions. Sequencing reactions were analyzed on an ABI Prism 377 sequencing analysis system. Specimens of non-small cell lung cancer were used as positive and negative controls for an EGFR gene mutational analysis.

Statistical analysis

Data was analyzed with SPSS 11.5.0 software (Chicago, Ill, USA). EGFR gene mutation, amplification, and protein expression were compared using the Chi-square test. A probability of < 0.05 was taken to indicate statistical significance.

Results

Agarose gel electrophoresis demonstrating EGFR PCR products around the 100 bp ladders is shown in Fig. 2. Among 48 ESCC specimens evaluated, no somatic mutations in the tyrosine kinase domain of the EGFR exons 19 or 21 were detected (Fig. 3). These specimens revealed that 22 patients (46%) expressed EGFR protein at the high level (scores 301-400). The EGFR gene was amplified in seven patients (15%) and overrepresented in 16 patients (33%), making 23 patients (48%) with a high gene copy numbers (FISH patterns BP+GA). Nine patients (19%) who had a low level of EGFR protein expression also had a low gene copy numbers (FISH patterns BD+BT). While 16

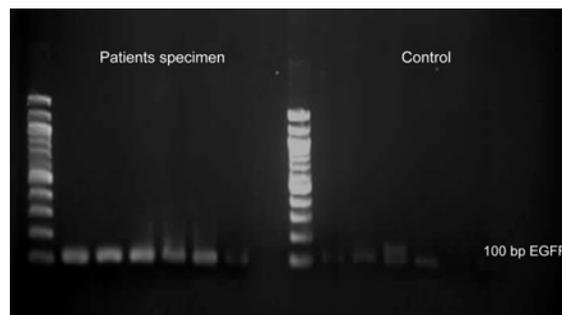


Fig. 2 Agarose gel electrophoresis and ethidium bromide stained of EGFR from patients with ESCC (left) and control (right)

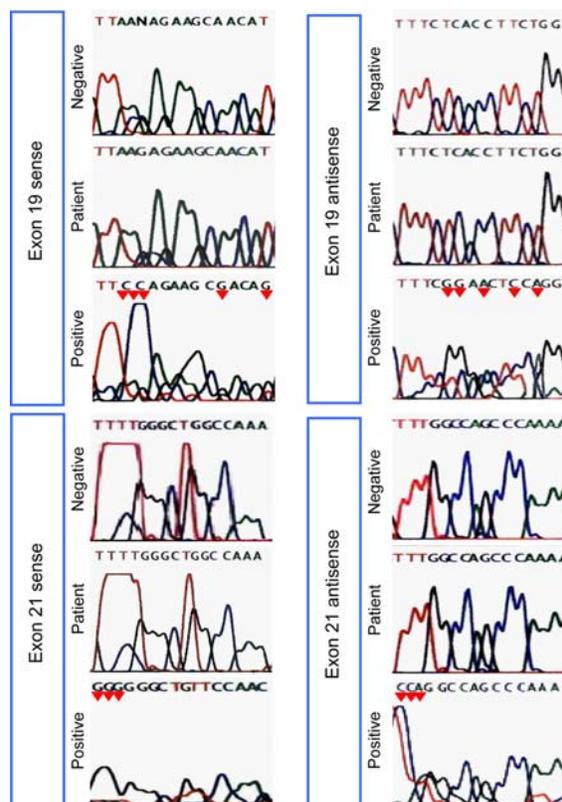
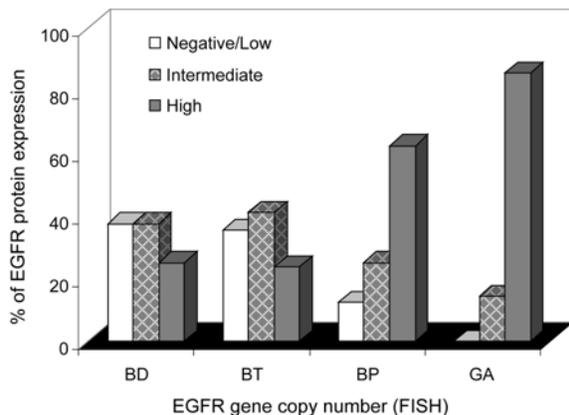


Fig. 3 Comparison of the negative and positive controls from NSCLC with the results of EGFR exons 19 and 21 (sense and antisense directions) of patients with ESCC. The positive control specimens from NSCLC revealed heterozygous missense or frame-shift mutation (red triangles), whereas no such mutations were found in ESCC



FISH = fluorescence in situ hybridization, BD = balanced disomy, BT = balanced trisomy, BP = balanced polysomy, GA = gene amplification. Classification of low and high FISH pattern is BD+BT and BP+GA, respectively. No statistical significance of correlation between the gene copy number and protein expression was demonstrated

Fig. 4 EGFR protein expression and gene copy number in 48 esophageal squamous cell carcinoma patients

patients (33%) who had high level protein expression had a high gene copy number (Fig. 4). However, there was no significant correlation found between EGFR protein expression and gene copy number.

Discussion

EGFR, also known as HER1 or ErbB, is a 170 kDa receptor transmembrane glycoprotein encoded by 28 exons located on chromosome 7p12. There are three functional domains of EGFR: an extracellular domain (containing of two EGF binding sites); a hydrophobic transmembrane domain and a cytoplasmic domain (tyrosine kinase (TK) and additionally a carboxyl autophosphorylation region)^(15,16). EGFR plays an important role in intracellular signal transduction, and controls growth as well as proliferation, metastasis, and angiogenesis in several tumors including lung, gastrointestinal, head and neck cancers⁽⁴⁾. EGFR targeting agents are composed of two major substance categories, which are monoclonal antibodies against the EGFR extracellular domain and the EGFR-TKIs. These anti-EGFR agents are potential therapeutic agents against EGFR-expressing tumors including esophageal carcinoma. A number of EGFR biological markers, including protein expression and gene copy number, have been studied in esophageal cancer to identify the appropriate markers that may predict

outcome of treatment with anti-EGFR agents or to indicate the prognosis of a patient⁽¹⁴⁾. Recently, somatic mutations within the EGFR-TK domain, particularly in exons 19 and 21, have been reported to be a significantly predictive profile in NSCLC patients who have received EGFR-TKIs^(9,10). However, EGFR mutation, amplification or polysomy, and over-expression have heterogeneously been demonstrated to associate with sensitivity and benefits to EGFR inhibitor treatment in different ways^(11,12). EGFR protein expression and polysomy or amplification of EGFR gene for instance have been found to be predictive markers for responsiveness to erlotinib, an oral EGFR-TKIs, in NSCLC patients, whereas EGFR gene mutation has not⁽¹¹⁾. However, none of the EGFR protein or gene status was associated with survival in that study. On the other hand, Cappuzzo et al⁽¹²⁾ have demonstrated that in patients who received gefitinib, another EGFR-TKIs, as a treatment for NSCLC, the amplification or high polysomy and mutations of the EGFR gene as well as high protein expression were all associated with better response but only a high EGFR gene copy number was significantly associated with survival outcome. Consequently, further investigations in other cancers potentially responsive to anti-EGFR therapeutic agents need to be conducted to identify appropriate markers to select patients for treatment.

In the present study, the PCR method was used to identify the previously-established mutations of exon 19 and 21 of EGFR from 48 ESCC specimens with known status of EGFR protein expression and gene copy number. Forty-six percent of ESCC had high-level EGFR expression, whereas, 15% revealed gene amplification. However, no mutations in exons 19 or 21 of the TK domain of the EGFR gene was detected implying that mutations within such a region are not likely to be a major responsible factor in ESCC tumorigenesis among Thai patients. Mutation analysis for EGFR in ESCC has been previously reported by a group from Japan. Of the 40 primary ESCC specimens evaluated, 15 with EGFR gene amplification and 25 without amplification, no mutations were detected in exons 19 and 21⁽¹⁷⁾. The authors' findings also agree with two recently published reports describing EGFR mutation analyses in esophageal cancer, though mainly adenocarcinoma histology, where no EGFR mutation was detected^(18,19). In the first paper from the Netherlands, 26 esophageal tumors in advanced esophageal cancer patients, who had been administered gefitinib as a second line therapy, were evaluated for EGFR gene mutations in exons 18 to 21. No activating

mutation in respective regions was detected, whereas, in female patients, squamous cell carcinoma histology, and a high level of EGFR expression were demonstrated to be associated with a higher disease control rate by gefitinib⁽¹⁸⁾. Dragovich et al⁽¹⁹⁾ have recently demonstrated no somatic mutations involving the EGFR exons 18, 19, or 21 in 54 unresectable or metastatic gastroesophageal junction and gastric adenocarcinoma patients who received erlotinib as a first line drug. However, modest activity of erlotinib was confirmed in patients with gastroesophageal junction adenocarcinoma stratum.

As previously investigated, EGFR protein expression is not always caused by gene amplification. High expression of EGFR protein did not show statistical correlation to EGFR gene amplification⁽¹⁴⁾. In addition, the presented data revealed that no EGFR mutations were detected in ESCC specimens with either high protein expression or gene amplification. The results also confirmed no correlation between the mutation of exons 19 and 21 of EGFR gene and protein or gene copy number.

EGFR mutation has also been evaluated in several other tumor types as a potential marker to predict response and estimate survival for cancer patients receiving anti-EGFR therapy. Nevertheless, at present, the authors are still unable to select patients for EGFR-TKIs treatment based only on their biological profiles of EGFR due to the heterogeneity of the clinical significance of EGFR profiles in various tumor types. For example, in glioblastoma multiforme (GBM), the most aggressive histological entity of primary brain tumor, 10%-20% of patients respond to EGFR-TKIs despite no somatic mutations in the EGFR kinase domains being detected, supporting that activity of anti-EGFR agents in GBM occurs through alternative mechanism⁽²⁰⁻²²⁾. Moreover, data from clinical trials of GBM in which the EGFR gene is commonly amplified showed that the EGFR gene amplification does not correlate with responsiveness to EGFR-TKIs⁽²⁰⁾. EGFR profiles, therefore, should be used as markers of disease rather than indicators for selection of anti-EGFR treatment. In ESCC, however, due to the high prevalence of EGFR expression and modest level of gene amplification, both EGFR biological features should be considered as potential markers of anti-EGFR activity to be further investigated in clinical trials of ESCC.

In conclusion, a study of the EGFR gene mutation was performed on ESCC patients from a single institution and no mutations in exons 19 or 21 of

EGFR gene were found. EGFR overexpression and amplification do not correlate with EGFR mutation in Thai patients with ESCC; therefore, appropriate markers of EGFR status to select patients for anti-EGFR therapy need to be further evaluated to improve treatment outcomes, and survival for ESCC patients.

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ความสัมพันธ์ของการกลายพันธุ์ การแสดงออกของโปรตีน และการเพิ่มจำนวนจีนของ epidermal growth factor receptor ในโรคมะเร็งหลอดอาหารชนิด squamous cell

ภัทรพิมพ์ สรรพวีรวงศ์, สุภาพร สุวิวัฒน์, สมเกียรติ สรรพวีรวงศ์, พุฒิศักดิ์ พุทธิวิบูลย์, วิญญู มิตรานันท์

ภูมิหลัง: Epidermal growth factor receptor (EGFR) เป็นเป้าหมายที่สำคัญในการรักษาโรคมะเร็งในปัจจุบัน การศึกษาลักษณะต่าง ๆ ทางชีวภาพของ EGFR ได้แก่ การกลายพันธุ์ของจีน การเพิ่มจำนวนจีน และการแสดงออกของโปรตีน ในโรคมะเร็งหลอดอาหารชนิด squamous cell จึงมีความจำเป็นเพื่อที่จะระบุคุณสมบัติในระดับโมเลกุลของ EGFR เพื่อสามารถคัดเลือกผู้ป่วยที่เหมาะสมในการรักษาด้วยยาต้าน EGFR ต่อไป

วัตถุประสงค์และวิธีการ: ศึกษาการกลายพันธุ์ของจีน EGFR โดยใช้ชิ้นเนื้อทางพยาธิวิทยาของผู้ป่วยมะเร็งหลอดอาหารชนิด squamous cell ของโรงพยาบาลสงขลานครินทร์ที่ผ่านการตรวจวิเคราะห์การแสดงออกของโปรตีน และการเพิ่มจำนวนจีน มาทำการเพิ่มจำนวนดีเอ็นเอของ EGFR โดยวิธี polymerase chain reaction (PCR) จากนั้นทำการแยกสกัด และวิเคราะห์การกลายพันธุ์ของ exon 19 และ 21 ด้วยวิธี direct sequencing แล้วนำลักษณะต่าง ๆ ของ EGFR มาศึกษาวิเคราะห์ถึงความสัมพันธ์ระหว่างกัน

ผลการศึกษา: จากชิ้นเนื้อของผู้ป่วยมะเร็งหลอดอาหารชนิด squamous cell จำนวน 48 ราย ไม่พบว่ามี การกลายพันธุ์ของจีน EGFR ที่ตำแหน่ง tyrosine kinase domain การแสดงออกของโปรตีน EGFR ในระดับสูง พบในผู้ป่วยจำนวน 22 ราย (ร้อยละ 46) ส่วนการเพิ่มจำนวนของจีน EGFR ในระดับสูงพบในผู้ป่วยจำนวน 23 ราย (ร้อยละ 48) อย่างไรก็ตามไม่พบความสัมพันธ์ทางสถิติของการแสดงออกของโปรตีน EGFR และการเพิ่มจำนวนของจีน

สรุป: ในผู้ป่วยมะเร็งหลอดอาหารชนิด squamous cell ไม่พบการกลายพันธุ์ที่ตำแหน่ง exon 19 และ 21 ใน tyrosine kinase domain ของจีน EGFR ในขณะที่พบการแสดงออกของโปรตีนและการเพิ่มจำนวนของจีนในระดับสูง ดังนั้นการคัดเลือกผู้ป่วยในการศึกษาสำหรับยาต้าน EGFR ต่อไปควรพิจารณาลักษณะการแสดงออกของโปรตีน EGFR และจำนวนของจีนมากกว่าการกลายพันธุ์ของจีน