Expression and Mutation Statuses of Epidermal Growth Factor Receptor in Thymic Epithelial Tumors

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Background: Epidermal growth factor receptor (EGFR) gene mutations have been reported to correlate with the sensitivity to the tyrosine kinase inhibitor treatment for advanced lung cancers. Since several reports have shown that invasive thymoma overexpress the EGFR protein, we examined the EGFR expression and mutation statuses in thymoma and thymic carcinoma tissues.

Methods: EGFR mutation statuses from 99 thymic epithelial tumor samples were evaluated by a rapid and sensitive TaqMan assay using Applied Biosystems 7500 real-time PCR system. Probes were designed according to the 13 different EGFR mutations reported previously in lung cancers. A total of 38 thymoma samples were directly sequenced for the EGFR gene. Protein expressions were evaluated for 56 thymic epithelial tumors by immunohistochemistry.

Results: EGFR gene mutations were not detected in any of the thymoma and thymic cancer samples using TaqMan PCR assay. Of the 38 samples 3 showed a heterozygous silent mutation without changes in the protein, a G to A transition at the nucleotide 2361 in exon 18. EGFR expression was significantly higher in invasive thymomas (stages III–IV, 15/19 were positive) than in early stage thymomas (stages I–II, 7/33 were positive) (P < 0.0001). All four carcinomas and all seven B3 thymomas showed EGFR positive staining.

Conclusions: Although EGFR mutation at the tyrosine kinase domain is unlikely to be a therapeutic target for thymoma, the information about EGFR expression would contribute to the further identification of the therapeutic target for advanced thymomas.

Key words: EGFR – thymic cancer – thymoma – mutation

INTRODUCTION

Thymoma is one of the common neoplasms in the anterior mediastinum. Thymoma usually grows slowly when it is at early stages. In these patients, long-term survival is common. On the other hand, many studies have demonstrated worse prognosis for patients with invasive thymomas (1–4). As complete resection is often impossible for advanced-stage thymoma, multimodal treatment is necessary (3–6). However, consensus has not been achieved for the multidisciplinary treatment for the advanced thymoma. On the other hand, molecular target therapy is one of the most promising fields for cancer treatment. Epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane glycoprotein with tyrosine kinase domain. EGFR gene or protein is overexpressed in many malignancies (7–9). Since EGFR tyrosine kinase inhibitor was approved as a third-line therapy for non-small cell lung cancer (NSCLC) patients, phase II and phase III trials have been underway for many other cancers. Recently, we and others demonstrated EGFR gene mutations around the ATP binding pocket of the tyrosine kinase domain in NSCLC patients. These EGFR gene mutations have been reported to correlate with the sensitivity to the tyrosine kinase inhibitor treatment for advanced NSCLC (10–12). Several reports have shown overexpression of EGFR protein in invasive thymomas by immunohistochemistry (13–16). We have also found elevated levels of EGFR in serum specimens from patients with advanced-stage thymomas (17). More recently, gene amplification of EGFR has been reported to correlate with clinical stage of thymoma (18). However, there have not been studies that explored EGFR gene mutations in clinical thymoma specimens. In the present study, we examined the EGFR mutation status in thymoma and thymic carcinoma tissues by the Applied Biosystems 7500 Real Time PCR system. This TaqMan PCR assay is a rapid and sensitive method for the detection of EGFR mutations with high throughput (19,20). Probes were designed according to the 13 different EGFR mutations reported previously in NSCLC.
MATERIALS AND METHODS

PATIENTS

Primary thymic tumor samples were obtained from 99 patients who had undergone operation at the Department of Surgery II, Nagoya City University Medical School, between 1993 and 2003. Patient's age ranged from 20 to 81 years (mean: 55.0 years). There were 48 females and 51 males. Clinical staging was based on Masaoka's staging system (1) and histological classification on WHO thymic epithelial tumor classification (21). The distribution of patients was as follows: stage I in 33, stage II in 19, stage III in 11, stage IVa in 14, stage IVb in 6, thymic cancer in 8 and unknown 8. And by WHO classification, none was in type A, 28 in type B, 21 in type B2, 8 in type B3, 8 in type C and 23 unknown (Table 1). Among 99 patients, 10 were treated with neoadjuvant (9 treated with steroid-plus and 1 treated with chemotherapy) and 3 were treated with adjuvant chemotherapy. The study was reviewed by the Institutional Review Broad and written consent was obtained from all the patients.

GENOTYPING BY TaqMAN PCR ASSAY

We extracted genomic DNA from 99 frozen thymic epithelial tumor samples by the protocol of the kit (Wizard® SV Genomic DNA Purification System: Promega), and then performed TaqMan PCR assay using the Applied Biosystems 7500 Real Time PCR System. The primers and ABI PRISM TaqMan MGB probes were designed with Primer Express 2.0 software (Applied Biosystems). The sequence of the allele-specific probes and the primer used in the TaqMan PCR assay are shown in Table 2. PCR cycling conditions were as follows: 1 cycle at 94°C for 10 min, followed by 40 cycles at 95°C for 15 s and 58°C for 1 min. The results were analyzed on Applied Biosystems 7500 Real Time PCR system using allelic discrimination assay program.

DIRECT SEQUENCING FOR EGFR

Total RNA was extracted from 38 thymoma tissues using the Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturers’ instructions. RNA concentration was determined using a spectrophotometer and adjusted to a concentration of 200 ng/ml. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 μg oligo (dT)12-16 (Amersham Pharmacia Biotech Inc. Piscataway, NJ) . The reaction mixture was incubated at 42°C for 50 min and then at 72°C for 15 min. We then used 1 μl of each DNA for PCR analyses. The PCRs were performed using the LA-Taq kit (Takara Bio Inc, Shiga, Japan) in a 25 μl reaction volume. The primer sequences for the EGFR gene for kinase domain (exon 18–21) were as follows: the forward primer, 5'-CTCTTACACCCACTTGAGAGAA-3' and the reverse primer, 5'-CATCCACCTGATAGGCACCTT-3' (572 bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 s, 60°C for 40 s and 72°C for 45 s. The products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA). Amplified cDNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide and photographed under ultraviolet transillumination. These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review.

IMMUNOHISTOCHEMISTRY

Mouse monoclonal antibody against EGFR (clone EGFR.113) was purchased from Novocastra Laboratorie Ltd. (Newcastle, United Kingdom). From paraffin tissue blocks from thymic epithelial tumors 4 μm sections were made. The slides were treated with xylenes and then dehydrated in alcohol. Endogenous peroxidase was blocked with 0.3% H2O2. For antigen retrieval, tissue sections were boiled in a stainless steel pressure cooker for 5 min in 10 mM citrate buffer (pH 6.0). After blocking with Block Ace Solution (Dako Japan Co., Kyoto, Japan), the slides were incubated with the monoclonal antibody against EGFR at 1:20 dilutions for overnight at 4°C. The Envision-plus Kit and 3,3-diaminobenzidine (DAB) substrate were used to visualize the antibody binding, and the sections were counterstained with hematoxin. More than 10% staining was considered as positive staining.

RESULTS

TaqMAN PCR ASSAY FOR DETECTION OF THE EGFR GENE MUTATION

We used allelic discrimination assay using the Applied Biosystems 7500 Real Time PCR system to detect whether the sample DNA contained any of the 13 reported EGFR mutations. Each
<table>
<thead>
<tr>
<th>Mutation no.</th>
<th>Probe name</th>
<th>Nucleotide (Forward/Reverse)</th>
<th>Amino acid</th>
<th>Primer sequence (Forward)</th>
<th>TaqMan probe</th>
<th>Primer sequence (reverse)</th>
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</table>
of the 13 probes has been shown to anneal only to the correct sequence. EGFR gene mutations were not detected in any of the 99 thymoma or thymic cancer samples (Fig. 1).

**DIRECT SEQUENCING OF THE EGFR GENE**

We next sequenced the cDNA from 38 thymoma for the EGFR gene. Of the 38 samples 3 showed a heterozygous silent mutation without change in the protein, a G to A transition at the nucleotide 2361 in exon 18. Three patients were as follows: case one; an 81-year-old man with stage III thymoma, case two; a 56-year-old man with stage I thymoma, case three; a 53-year-old woman with stage I thymoma. We could not identify any clinical characteristics that can identify the three cases (Fig. 2). No other EGFR mutations were found.

**EGFR PROTEIN EXPRESSION**

Immunohistochemical (IHC) approach was used to study the relative amount of EGFR protein expression in 56 thymic epithelial tumors. In 26 of 56 tumors, more than 10% of tumor cells were stained positive for EGFR (Fig. 3). Frequency of EGFR positive tumors according to the stages of thymomas were stage I, 3/19; stage II, 4/14; stage III, 5/8; and stage IV, 10/11. EGFR expression was significantly higher in invasive thymomas (stage III–IV, 15/19 were positive) than in early stage thymomas (stage I–II, 7/33 were positive) \( (P < 0.0001) \).

Frequency of EGFR positive sections according to the WHO classification of thymic epithelial tumors were AB, 4/22; B1, 1/7; B2, 8/14; B3, 7/7; others, 2/2; and C, 4/4. All four carcinomas and all seven B3 thymomas were EGFR positive.

**DISCUSSION**

In the present study, we were unable to detect any EGFR missense mutations in thymoma specimens from Japanese patients. Kosaka et al. (22) demonstrated that 40% of Japanese NSCLC patients had EGFR mutations, a ratio which was significantly higher than the EGFR mutation ratio from Caucasian NSCLC patients (10–12). Many studies have demonstrated that the EGFR mutation ratio was higher in female NSCLC patients (11,12,22). Although, 48.5% (48/99) were females in our analysis, there was no EGFR mutation found from their thymoma specimens. Lee et al. reported that they found only 1 EGFR mutation out of 93 breast carcinoma tissues, but no EGFR mutation was detected in the other 536 samples, including 98 colon, 185 gastric and 73 hepatocellular carcinomas (23). These data suggest that the EGFR kinase domain very rarely mutates in common human cancers other than NSCLC.

Our data showed that invasive thymoma (stage III and IV) had higher EGFR expressions by immunohistochemistry, in agreement with previous reports (13–16). If thymoma is completely encapsulated (Masaoka stage I) or has only microscopic invasion into surrounding fatty tissue (stage II), surgical resection is the first choice treatment. In such cases, recurrence incidence is low, for example 3% with stage I and 13% with stage II (24). But for invasive thymoma, local recurrence has been observed in 27% with stage III and 54% with stage IV (24). Therefore additional therapy might be necessary.

*Figure 1.* Allelic discrimination assay. Common mutations in NSCLC, G719S (serine for glycine at codon 719 within exon18), L858R (leucine to arginine of 858) Del 1a(in-frame deletion: 2235–2239) and Del 4(in-frame deletion: 2240–2257) are shown.
Radiotherapy, chemotherapy (4–6,25), steroid-pulse treatment (26) and so on are usually advocated. However, there is no general consensus for improving the prognosis other than total resection of thymoma, as reviewed by Henja et al. (27) on nonsurgical management of thymoma. Thus we tried to probe the treatment using tyrosine kinase antagonists which looks promising in other neoplasia (28).

Many studies have indicated that invasive thymoma has high ratio of EGFR protein expression by immunohistochemistry (14–16), whereas EGFR expression was not detected in control thymuses (14). Diana et al. (18) indicated that invasive and advanced-stage thymoma had higher EGFR gene amplification. Currently, EGFR gene mutations are detected in NSCLC patients, and those with EGFR mutation show a good clinical response to tyrosine kinase inhibitors (gefitinib or erlotinib). If EGFR mutations were detected in thymoma, these tyrosine kinase inhibitors would be added to adjuvant therapy. But we could not find any EGFR missense mutations with TaqMan PCR assay and direct sequencing in any of the thymic tumors we studied.

Figure 2. Direct sequencing of the EGFR gene in thymoma samples. All three panels show a heterozygous silent mutation, a G to A transition at the nucleotide 2361 (arrows). (A) an 81-year-old man with stage III thymoma, (B) a 56-year-old man with stage I, type B3 thymoma, (C) a 53-year-old woman.

Figure 3. EGFR protein expression by immunohistochemistry. Right; EGFR positive section (WHO; type B3, stage IV thymoma). Left; EGFR negative section (WHO; type B1, stage I thymoma).
If the correlation between EGFR expression and thymoma, and the mechanism, is clarified as to what brings about the EGFR expression, EGFR inhibitor may be added to new adjuvant therapy. Although EGFR mutation at tyrosine kinase domain is unlikely to be a therapeutic target for thymoma, the information about EGFR expression would contribute to further development of therapy for advanced thymomas. Cetuximab might be one of the candidates for the treatment of advanced thymomas with increased EGFR expression. Although published preclinical reports suggest that EGFR expression might be needed for cetuximab activity (29,30), recent clinical evidence indicates that response to cetuximab treatment is independent of the relative degree of EGFR expression level in tumors (31).

Acknowledgment

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References