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Short Communication

EGFR mRNA is Upregulated, but Somatic Mutations of the Gene are Hardly Found in Renal Cell Carcinoma in Japanese Patients

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Purpose. Heterozygous somatic mutations of epidermal growth factor receptor (EGFR) in exons 18, 19, and 21 were recently reported to be associated with response to gefitinib in patients having nonsmall cell lung cancer. Such mutations are more frequently found among Japanese than Europeans. In this work, the frequency of mutations was investigated in renal cell carcinoma (RCC) samples obtained from Japanese subjects to examine the potential of gefitinib as a therapeutic agent for RCC.
Methods. Nineteen patients with RCC, who gave written informed consent, were enrolled in this study. mRNA expression levels of EGFR were measured in RCC and its adjacent noncancerous renal tissue via the real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) method. Somatic mutations were determined using genomic DNA extracted from RCC by direct sequencing method.
Results. mRNA expression was confirmed to be about 19 times higher in RCC than in adjacent noncancerous renal tissues, but no such mutations were detected in both.
Conclusion. Results from this study do not support the validity of further clinical trials on gefitinib for RCC with genotyping even in Japanese patients, although EGFR plays a key role in tumor progression.

KEY WORDS: EGF receptor; renal cell carcinoma; somatic mutation.

INTRODUCTION

During the second half of the 20th century, considerable efforts were made to ensure higher levels of efficacy and safety in cancer chemotherapy. At present, however, the therapeutic index of anticancer drugs remains narrow, and responses are partial and often disappointingly brief, strongly suggesting the limitation of traditional cytotoxic chemotherapy. Recent advances in research on signaling pathways in tumor growth and progression have evoked a novel strategy of molecular targeting for cancer chemotherapy, and this concept has been realized with the success of imatinib mesilate (Glivec®) for chronic myelogenous leukemia (CML), which targets Bcr-Abl, a constitutively activated tyrosine kinase in CML (1,2). In solid tumors, research into signal transduction has shown the importance of members of the human epidermal growth factor receptor (HER) family of transmembrane tyrosine kinases consisting of HER1–4 (ErbB1–4) (3–6). It has been demonstrated that effective treatment can be provided with trastuzumab (Herceptin®), an anti-HER2 antibody, for breast cancer with amplification of the *HER2* gene (7,8).

Subsequently in 2002, an inhibitor of EGFR tyrosine kinase (HER1, ErbB1), gefitinib (Iressa®), was approved in

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ABBREVIATIONS: CML, chronic myelogenous leukemia; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; IDEAL, Iressa dose evaluation in advanced lung cancer; INTACT, Iressa NSCLC trial assessing combination treatment; NSCLC, nonsmall cell lung cancer; PCR, polymerase chain reaction; RCC, renal cell carcinoma; RT, reverse transcription.

Japan for treatment of nonsmall cell lung cancer (NSCLC), based on a phase I trial (9) and phase II trials (IDEAL1&2) (10,11). The drug has subsequently gained global approval. In IDEAL studies, objective responses were more likely to be found in female than male patients (19 *vs.* 3%) and in patients with adenocarcinoma NSCLC than those with other histological types (13 *vs.* 4%) (10,11), and the response rate was higher in Japanese patients than in a predominantly European-derived population (27.5 *vs.* 10.4%) (10). However, even among these selected subgroups, only a minority showed a response to gefitinib, and the response was weaker compared to imatinib mesilate or trastuzumab. Moreover, recently conducted phase III trials (INTACT1&2) have proven that, in combination with platinum-based chemotherapy, gefitinib provides no additive advantage (12,13).

The level of EGFR expression in the tumor was not found to be correlated with response to gefitinib (3,6), and there was no dose–response correlation in IDEAL trails (10,11). The molecular mechanisms underlying sensitivity to gefitinib remain unknown. Very recently, Lynch *et al.* (14) and Paez *et al.* (15) reported that heterozygous somatic mutations of EGFR, i.e., in-frame deletions and missense substitutions in exons 18, 19, and 21, which correspond to the ATP-binding pocket, are strongly associated with response to gefitinib in patients with NSCLC. Lynch *et al.* (14) also reported that no such mutations were found in 95 primary tumors of the breast, colon, kidneys, pancreas, and brain (no ethnic information was provided). In agreement with the findings of two IDEAL trials (10,11), Paez *et al.* (15) likewise indicated that these mutations are more likely to be found in women than in men (20 *vs.* 9%), in adenocarcinoma than in other types of cancers (21 *vs.* 2%), and in Japanese patients than in U.S. patients (26 *vs.* 2%). These findings indicate that these mutations induce a gain of EGFR function. Sordella *et al.* (16) recently showed that these mutations activate antiapoptotic Akt and STAT5 signaling pathways. However, we are still uncertain as to why these mutations are NSCLC-specific and found in a specific population. In this study, the frequency of somatic mutations of EGFR was investigated as well as the upregulated expression of EGFR mRNA in samples of renal cell carcinoma (RCC) obtained from 19 Japanese patients to examine the potential of gefitinib as a therapeutic agent for RCC. Epidermal growth factor receptor is one of the major growth factors expressed in RCC, but controversy exists concerning the rationale for using EGFR inhibitors in patients with advanced RCC, because of insufficient clinical efficacy (17).

MATERIALS AND METHODS

Human RCC and Adjacent Noncancerous Renal Tissues

Renal cell carcinoma samples were obtained with adjacent noncancerous renal tissues as surgical samples from 19 patients, who were diagnosed with primary RCC at Kobe University Hospital, Japan (nine males and ten females). The average age was 61.0 ± 13.1 years (range: 29–81). Blood samples were also obtained from these patients in advance. All samples were immediately frozen and stored at −80°C until the assay. Informed consent was obtained from all patients prior to their participation in the study. The protocol was approved by the institutional review board of Kobe University Hospital, Kobe University, Japan.

EGFR mRNA Levels in RCC and Adjacent Noncancerous Renal Tissue

Epidermal growth factor receptor mRNA expression was determined in 18 of 19 pairs of samples via the real-time quantitative reverse transcription (RT)-polymerase chain reaction (PCR) method as previously described (18,19). Briefly, total RNA was extracted from the samples using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and an RNase-Free DNase Set (Qiagen) according to the manufacturer's directions. In each run of the assay, the mRNA levels of EGFR and β-actin were analyzed using samples serially diluted fivefold from the authentic sample, and the levels of EGFR mRNA were expressed relative to the concentration of actin mRNA. β-Actin was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. The primer pairs and *TaqMan* probe for EGFR mRNA (Genbank accession no. NM_005228), designed using the Primer Express 1.0 program (Applied Biosystems, Foster City, CA, USA) for covering exons 26 to 27, were as follows; forward primer, 5′-TCGCAAAGGGCATGAACTACTT-3′; reverse primer, 5′-TTGACATGCTGCGGTGTTTT-3′; and *TaqMan* probe, 5′-TGCACCGCGACCTGGCAGC-3′. The primers and *TaqMan* probe for β-actin mRNA (Genbank accession no. NM_001101), designed using the Primer Express 1.0 program, were as follows: forward primer, 5′-TCGTCATACTCCTGCTTGCTGAT-3′; reverse primer, 5′-GGCACCCAGCACAATGAAG-3′; and *TaqMan* probe, 5′-AGTACTTGCGCTCAGGAGGAGCAATGATC-3′. The primers and *TaqMan* probe were synthesized by Hokkaido System Science (Sapporo, Japan).

Table I. Epidermal growth factor receptor somatic mutations

Exon	Type	Nucleotide	Amino acid	References
18	Substitution	2155G>A	G719S	(14,15)
19	Deletion	2235_2249delGGAATTAAGAGAAGC	E746_A750del	(14,15)
19	Deletion	2236_2250delGAATTAAGAGAAGCA	E746_A750del	(15)
19	Deletion/Substitution	2239_2247delTTAAGAGAA, 2248G>C	L747_E749del, A750P	(15)
19	Deletion/Substitution	2240_2257delTAAGAGAAGCAACATCTC	L747_S752del, P753S	(14,15)
19	Deletion/Substitution	2238_2255delATTAAGAGAAGCAACATC, 2237A>T	L747_S752del, E746V	(15)
19	Deletion	2240_2251delTAAGAGAAGCAA	L747_T751del	(14)
19	Deletion	2254_2277delTCTCCGAAAGCCAACAAGGAAATC	S752_I759del	(15)
21	Substitution	2573T>G	L858R	(14,15)
21	Substitution	2582T>A	L861Q	(14)

EGFR Genotyping

Table I lists the mutations examined in this study. DNeasy tissue kits (Qiagen) were used to extract genomic DNA from 19 paired samples of blood, RCC, and adjacent noncancerous renal tissue for PCR to amplify exons 18, 19, and 21 of the *EGFR* gene. The reaction mixture (25 μl) contained 30 ng of DNA, 10× ExTaq buffer, 0.5 mM of dNTP mixture, 1.25 U of ExTaq polymerase (Takara Bio, Shiga, Japan), and 0.4 μM of the forward and reverse primers. The reaction profile was as follows: denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 1 min, and finally, 72°C for 3 min. For amplification of exon 18, nested PCR was performed using 1.5 μL of reaction mixture. Primer pairs used were as follows: exon 18-initial, 5′-TCAGAGCCTGTGTTTCTACCAA-3′ (forward) and 5′-TGGTCTCACAGGACCACTGATT-3′ (reverse); exon 18-nested, 5′-TCCAAATGAGCTGGCAAGTG-3′ (forward) and 5′- TCCCAAACACTCAGTGAAACAAA-3′ (reverse); exon 19, 5′-AAATAATCAGTGTGATTCGTGGAG-3′ (forward) and 5′-GAGGCCAGTGCTGTCTCTAAGG-3′ (reverse); exon 21, 5′-GCAGCGGGTTACATCTTCTTTC-3′ (forward) and 5′-CAGCTCTGGCTCACACTACCAG-3′ (reverse). The PCR products were then treated using Applied Biosystems Version 1.1 BigDye terminator chemistry (Applied Biosystems), followed by PCR as follows: 96°C for 1 min, then 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The reaction mixtures were purified with a CentriSep column (Princeton Separations, Adelphia, NJ, USA), and analyzed on an Applied Biosystems 3100 Avant Genetic Analyzer (Applied Biosystems). All sequence chromatograms were read in both sense and antisense directions.

STATISTICAL ANALYSIS

Values are given as mean ± standard deviation. The statistical significance of differences between mean values was calculated using paired Student's *t* test. A *P* value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Several decades after Cohen's pioneering studies, the growth factor signaling pathways have attracted a great deal of attention as attempts continue to develop novel targeted anticancer drugs, because of the fundamental roles played by these pathways in cell proliferation, cell differentiation, metastasis, and the survival of tumor tissues. A member of the HER family of tyrosine kinases, EGFR has been a main focus of research, because it is frequently overexpressed in various types of solid tumors, including cancers of the breast and lung, head and neck cancer, bladder carcinoma, colorectal cancer, ovarian carcinoma, and prostate cancer. Clinical investigations have proven that increased EGFR expression is associated with a poorer clinical outcome (20). Increased expression of EGFR is accompanied by the production of its ligands, EGFR, and TGF-α, resulting in the consecutive activation of the receptors via autocrine mechanisms (20). Here, the expression was confirmed to be upregulated in RCC compared to adjacent noncancerous renal tissue (EGFR mRNA/β-actin mRNA: 0.294 ± 0.209 *vs.* 0.0284 ± 0.0297, $p < 0.001$) (Fig. 1). The RCC/adjacent noncancerous renal tissue ratio was 18.8 ± 22.9 (1.11–84.5), consistent with Northern blotting results (21–24). As Mendelsohn and Baselga (20) proposed about 20 years ago, upregulated EGFR expression in RCC may be a target in chemotherapy for cancer.

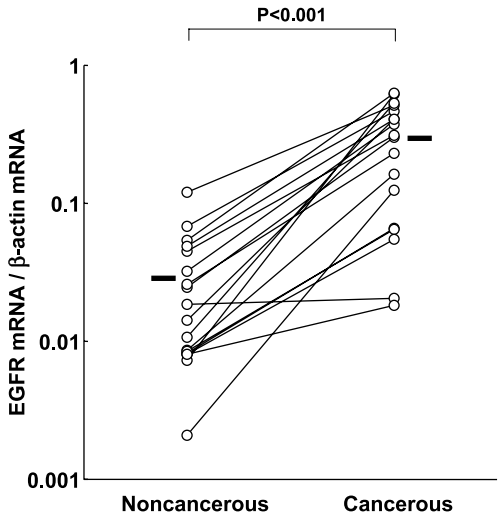

Fig. 1. Relative concentrations of epidermal growth factor receptor (EGFR) mRNA in renal cell carcinoma and adjacent noncancerous renal tissue obtained from 18 Japanese patients. EGFR mRNA expression was determined by real-time quantitative RT-PCR with β-actin as an internal control. Each bar represents the mean value, which was 0.294 ± 0.209 and 0.0284 ± 0.0297, respectively, with a statistically significant difference (*$p < 0.001$).

Metastatic RCC is one of the cancers for which successful therapy remains elusive, and although a diagnosis can now be made earlier and extensive efforts have been expended to develop a more effective therapy, the clinical outcome remains poor (17). Cytokine-based immunotherapy with interleukin-2 or interferon-α after resection of the primary site is the most readily available treatment for metastatic RCC, but only 15–20% of patients respond to this type of treatment (17), and various types of agents are now being tested, including imatinib mesilate, gefitinib, and other molecular targeting agents (25). Overexpression of EGFR and its ligands, EGF and TGF-α, frequently occurs in RCC, and autocrine/paracrine signaling is associated with the development and progression of metastasis in RCC (17). Studies *in vitro* and in animals had shown that EGFR inhibitors are promising for RCC (26–31). However, as these agents only showed minimal effects in clinical trials—e.g., gefitinib (32–33) and the anti-EGFR monoclonal antibody, C225 (34), and ABX-EGF (35)—considerable controversy has consequently erupted. The reports by Lynch *et al.* (14) and Paez *et al.* (15) on NSCLC have shed light on the difference in response to EGFR inhibitors between experimental RCC and clinical RCC. These reports provided a milestone for selecting RCC patients more likely to respond to gefitinib in addition to NSCLC patients. Thus, we examined the somatic mutations of EGFR in RCC samples obtained from 19 Japanese patients, together with samples from adjacent normal tissue and blood. However, we did not find any mutations in RCC, normal tissue, and blood. In addition, we could not find any novel mutations in exons 18, 19, and 20, showing that the frequency

of EGFR somatic mutations in Japanese patients with RCC is less than 5%. Although it is unknown whether the association of mutation with sensitivity in NSCLC is similar to that in RCC, it could be speculated based on our findings that clinical efficacy is insufficient in RCC even among Japanese patients. This observation is consistent with the recent clinical studies (32–33), showing that gefitinib had minimal effect on U.S. patients. In RCC, the mutation of von Hippel–Lindau (VHL) has been reported as a cause of cancer (36). It targets the hypoxia-inducible factor (HIF) for ubiquitin-mediated degradation. Mutations of VHL result in the accumulation of HIF leading to increased transcription of cancerous signals including EGFR, vascular endothelial growth factor, transforming growth factor-α, and so forth. The EGFR upregulation in this study could be attributable to such factors as VHL mutations.

We found large interindividual variability, but no rational explanation for this has been available so far. Also, we could not find any association with stage, age, and gender, which could be due to variability in cancerous signals among patients. However, it is noted that response to gefitinib was not associated with EGFR expression levels in the clinical study of gefitinib in NSCLC (3,6). Collectively, gefitinib is not likely to exhibit clinical effects even in patients with higher EGFR levels.

In conclusion, the present results do not support the validity of further clinical trials on gefitinib for RCC with genotyping even in Japanese patients, although EGFR upregulation is confirmed in RCC by real-time quantitative RT-PCR method.

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