Heterozygous mutation (G/G→G/A) at nt 2607 of the EGFR gene is closely associated with increases in EGFR copy number and mRNA half life, but impaired EGFR protein synthesis in squamous cell carcinomas of the head and neck – implication for gefitinib efficacy

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Abstract. Gefitinib (ZD1839, Iressa®) is an orally active agent that inhibits the tyrosine kinase activity of epidermal growth factor receptor (EGFR). Gefitinib has shown a high efficacy in patients with non-small cell lung carcinoma and specific mutations in the ATP-binding pocket of EGFR. These mutations, however, are extremely rare in squamous cell carcinomas of the head and neck (SCCHN). We previously showed that SCCHN cell lines with a heterozygous mutation (G/G→G/A) at nucleotide 2607 of EGFR are more sensitive to gefitinib than wild-type cell lines (79.5% higher IC_{50}). To determine the relationship between the G/G and G/A genotypes, we assessed the *EGFR* gene copy number and the *EGFR* mRNA half-life in 16 different SCCHN cell lines. Fluorescence *in situ* hybridization showed that the *EGFR* copy number was significantly higher in the nine 2607G/A cell lines than in the seven 2607G/G cell lines (3.59±2.14 vs. 1.58±0.32 copies, a 2.27-fold difference). Similarly, the half life of *EGFR* mRNA was 2.24-fold higher in cell lines with the 2607G/A genotype than in those with wild-type. By contrast, the EGFR protein levels were inversely correlated with mRNA abundance. These findings suggest that sensitivity to gefitinib of cells with the heterozygous mutation (2607G/G→G/A) was closely associated with gene amplification, the prolongation of mRNA half-life and a decrease in EGFR protein.

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Introduction

Epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein receptor that exhibits tyrosine kinase activity, which regulates cell growth. EGFR expression was frequently observed in squamous cell carcinomas of the head and neck (SCCHN) (1). Since EGFR signaling was found to control not only cell growth, but also angiogenesis and DNA repair (2,3), it may be a molecular target in patients with SCCHN (4,5).

Gefitinib (ZD1839, Iressa®), an anilinoquinazoline-based inhibitor specific for EGFR tyrosine kinase, was shown to have clinical efficacy in patients with non-small cell lung carcinoma (NSCLC) (1). Since mutations in the tyrosine kinase domain of the *EGFR* gene were reported to increase the binding affinity of gefitinib, the presence of EGFR mutations in this domain is predictive of the clinical benefits of gefitinib treatment for NSCLC patients (6,7). Gefitinib has also shown clinical efficacy in some patients without EGFR mutations (4,5). Thus, in such patients, *EGFR* gene amplifications are critical for gefitinib efficacy (6,7). Previous studies have shown that mutations in the kinase domain of the *EGFR* gene occur less frequently in SCCHN than in NSCLC (1,4). The clinical efficacy of gefitinib is due not only to its direct action on EGFR tyrosine kinase, but on its indirect action through activation of the immune system (9). For example, to demonstrate the direct action of EGFR tyrosine kinase, it was shown that SCCHN cell lines with heterozygous mutations

Table I. QPCR primer sets.

Genes	Sequence (5'→3')
EGFR	Forward: TTCGATGATCAACTCACGGAAC Reverse: GCCACCCATATGTACCATCGAT
β-actin	Forward: CAGCTGAGAGGGAAATCGTG Reverse: GCTGGTTGCCAATAGTGAATG

(G/G→G/A) at nucleotide 2607 of *EGFR* are more sensitive to gefitinib than wild-type *EGFR* (4). To further investigate these observations, the mechanism by which the G/A mutation confers sensitivity to gefitinib was examined.

Materials and methods

Cell lines and cell culture. The 16 human SCCHN cell lines and their sites of origin included YCU-M862, YCU-M911 and KCC-M871, from tumors of the mesopharynx; YCU-H891, from a tumor of the hypopharynx; KCC-L871 and YCU-L891, from tumors of the larynx; KCC-T871, YCU-T891, YCU-T892 and KCC-T873, from tumors of the tongue; KCC-TCM902, KCC-TCM903 and KCC-TCM901, from metastatic tumors of 3 tongue carcinomas; KCC-OR891, from tumors of the oral floor; and KCC-MS871 and YCU-MS861, from tumors of the maxillary sinus. The cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and cultured at 37˚C in a humidified atmosphere of 5% CO_2/95% air.

Fluorescence in situ hybridization (FISH). The cells were fixed in carunor solution, placed on glass slides and hybridized with LSI EGFR Dual Color Probe (Abbott Molecular, CA, USA) (1,2). The signals were counted for 60 cells in each cell line and data were expressed as mean ± SD.

mRNA half life. Each cell line was treated with 5 μg/ml actinomycin D (Wako Pure Chemical Industries, Osaka, Japan) and total RNA was purified using Isogen RNA purification kit (Nippon Gene, Tokyo, Japan). Total RNA was then reverse-transcribed to cDNA using avian myeloblastosis virus (AMV) reverse transcriptase (Takara, Tokyo, Japan) and amplified by real-time quantitative PCR (Stratagene® MX3000P; Agilent Technologies, Santa Clara, CA, USA) with Stratagene Brilliant II Fast SYBR® Green QPCR Master Mix (Agilent Technologies). The specific primer sets are shown in Table I. The amplification protocol consisted of 30 cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 1 min and extension at 72˚C for 30 sec.

Statistical analysis. The groups were compared using paired Student's t-tests and Chi-square tests. Linear regression analysis was used to compare the *EGFR* mRNA half life with the *EGFR* gene copy number, the steady state levels of *EGFR* mRNA and the EGFR protein levels. P<0.05 was considered to be statistically significant.

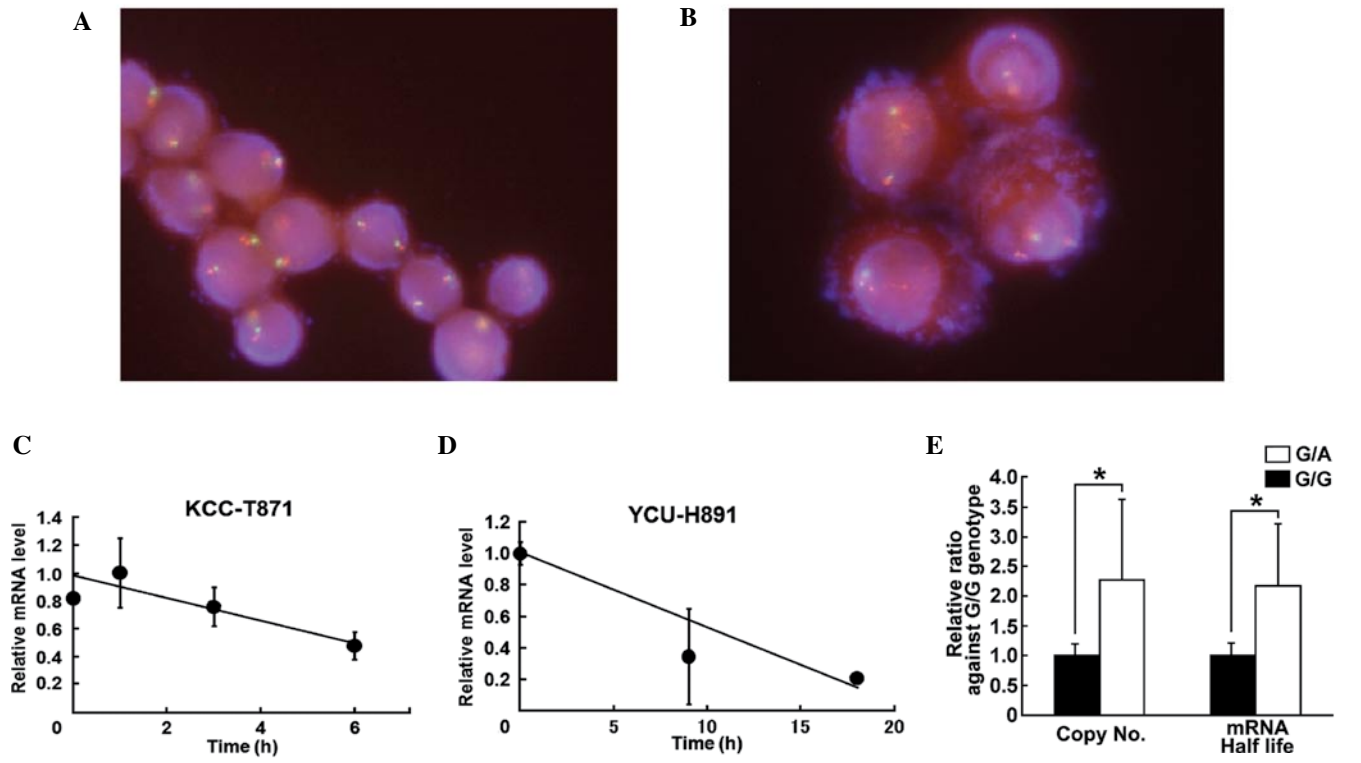

Figure 1. Status of the *EGFR* gene in SCCHN cell lines carrying the G/A and G/G genotypes at codon 2607. (A and B) Number of copies of the *EGFR* gene, as determined by FISH. Representative results are shown. (A) YCU-MS861, 2.02 copies/cell. (B) KCC-TCM903, 1.13 copies/cell. (C and D) EGFR mRNA half-life, determined following the addition of actinomycin D (5 μg/ml). Representative results are shown. (C) KCC-T871, 6 h. (D) YCU-H891, 13 h. (E) Average mRNA half life in the seven cell lines with the G/G genotype and the nine cell lines with the G/A genotype. Error bars indicate SD. *P<0.05.

Table II. A summary of EGFR protein, mRNA, gefitinib IC_{50} value, SNP, FISH copy number ratio and mRNA half life.

Cell line	EGFR protein[a]	*EGFR* mRNA	IC_{50} value[a]	Genotype at 2607[b]	FISH X/Y	mRNA half life (h)
YCU-MS861	0.15	0.20	66.3	G/G	1.13	4.3
YCU-M862	1.85	0.59	64.8	G/G	1.33	5.4
KCC-M871	0.46	0.10	52.1	G/G	1.47	3.9
KCC-L871	0.58	0.04	58.0	G/G	1.54	5.6
YCU-T892	2.25	4.43	64.8	G/G	1.68	3.0
YCU-M911	0.95	0.55	61.4	G/G	1.82	5.2
KCC-T873	2.96	0.85	85.0	G/G	2.12	5.6
KCC-TCM903	0.38	0.25	51.4	G/A	2.02	6.0
KCC-T871	0.60	0.09	44.4	G/A	2.55	6.0
KCC-TCM901	0.77	0.25	43.9	G/A	2.80	5.8
YCU-H891	0.21	2.38	69.0	G/A	2.89	13.0
YCU-L891	0.91	1.14	52.2	G/A	2.89	8.5
YCU-T891	0.56	2.46	56.0	G/A	2.89	18.0
KCC-MS871	0.23	1.35	41.6	G/A	3.53	15.0
KCC-TCM902	0.25	1.16	50.2	G/A	3.60	11.2
YCU-OR891	0.47	0.16	53.6	G/A	9.16	8.5
Average	0.85	1.00	57.2		2.71	7.8

[a]Data from Taguchi *et al* (1). [b]SNP, single nucleotide polymorphisms.

Table III. Heterozygous EGFR mutants contain a high copy number of EGFR.

Copy no.	G/G	G/A
≥2	1	9
<2	6	0

Chi-square test, p=0.00044.

Results

EGFR copy number. The 16 SCCHN cell lines were divided into two groups: those heterozygous for the mutation (G/A) at nucleotide 2607 of the *EGFR* gene (9 cell lines) and those with the wild-type (G/G) sequence (7 cell lines). The mean number of *EGFR* copies in the cell lines was 3.59±2.14 and 1.58±0.32, respectively (Fig. 1, Table II) (4), or 2.27-fold higher in cell lines with the mutant sequence. It was found that 10 of the 16 cell lines had ≥2 copies of the *EGFR* gene, whereas the other 6 had <2 copies. The Chi-square test showed a statistically significant correlation between the G/A mutation and *EGFR* copy number (Table III). The *EGFR* copy number was significantly higher in YCU-OR891 than in the remaining cell lines.

Association between EGFR copy number and mRNA half life. Real-time qPCR analysis revealed that the *EGFR* gene copy number was positively correlated with the steady state levels of *EGFR* mRNA and the *EGFR* mRNA half life (Fig. 2C). Notably, the mRNA half life was inversely correlated with EGFR protein concentration (Fig. 2A).

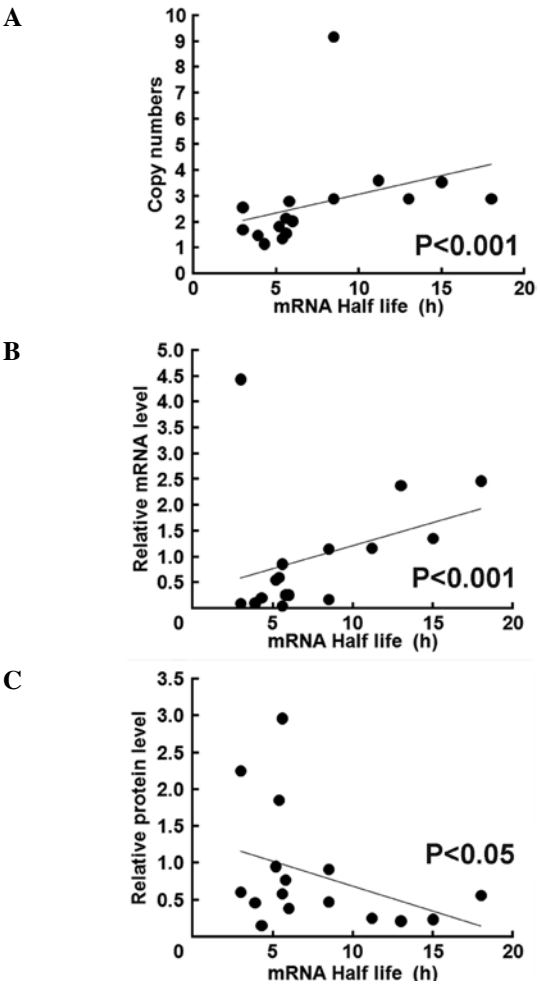

Figure 2. Linear regression analysis between *EGFR* mRNA half life and (A) *EGFR* gene copy number, (B) the steady state levels of *EGFR* mRNA and (C) EGFR protein levels. *EGFR* mRNA levels were determined following cell culture with actinomycin D (5 μg/ml). The EGFR protein levels were from our previously published results (4).

Discussion

The efficacy of gefitinib in patients with NSCLC was thought to depend on specific *EGFR* gene mutations as opposed to the concentration of EGFR protein. In particular, mutations in the tyrosine kinase domain, consisting of exons 18-21, were regarded as critical for a response to gefitinib. Dominant mutations or deletions of 2-15 nucleotides between codons 740 and 753 in exon 19 were found to increase the tyrosine kinase inhibitory activity of gefitinib due to conformational changes at the ATP-binding site (4). We found that a number of SCCHN cell lines have the G/A genotype, consisting of a heterozygous G→A transition at nucleotide 2607 in exon 20 of the *EGFR* gene (4). Cell lines with the G/A genotype were significantly more sensitive to gefitinib (lower IC_{50} values) than cell lines with the wild-type G/G genotype (P=0.016). We then focused on the relationship between gene expression and sensitivity to gefitinib *in vitro*. The FISH assay showed that cell lines carrying the G/A mutation have a significantly higher *EGFR* copy number than wild-type cell lines. Moreover, the steady state levels of *EGFR* mRNA were higher in cells with the G/A genotype than in those with the G/G genotype, suggesting a close correlation between the *EGFR* copy number and the steady state levels of *EGFR* mRNA. Notably, concentrations of EGFR protein were inversely correlated with the *EGFR* mRNA half life and steady state levels.

The G/A mutation proved to be synonymous, which may be due to impaired translation as opposed to protein instability. Generally, synonymous mutations are thought to be silent. However, cells with the G/A mutation produce less EGFR protein than those with the G/G wild-type, thereby suggesting that the mutation affected the translation rate. A previous study showed that a synonymous GAA to GAG mutation resulted in a 3-fold difference in the rate of translation due to the binding properties of tRNA to each codon (10). Moreover, a silent synonymous mutation in the *MDR1* (multidrug resistance 1)/*ABCB1* gene, which encodes P-glycoprotein, does not alter the mRNA or protein levels, but affects protein conformation, thereby altering the interaction between the substrate and inhibitor (11). Thus, synonymous mutations may affect mRNA structure and stability, the kinetics of translation and alternative splicing (12). We found that the heterozygous G/G→G/A mutation at codon 2607 of the *EGFR* gene was correlated with gene amplification, prolongation of *EGFR* mRNA half life and an increase in the steady state concentrations of *EGFR* mRNA. Additionally, the mutation was inversely correlated with the EGFR protein level, suggesting that this mutation affects translation efficacy. Although cell lines carrying the G/A mutation were more sensitive to gefitinib, we did not observe a significant correlation between gefitinib IC_{50} and the EGFR molecular status, including the copy number and the mRNA half life, due to the narrow range of IC_{50} values in a limited number of cell lines. However, combined with our previous findings, which demonstrated that G/A mutants exhibited a higher sensitivity to gefitinib, the down-regulation of EGFR protein expression, possibly due to a reduced translation efficacy, may be closely associated with gefitinib efficacy. Since the efficacy of gefitinib is thought to be independent of the EGFR protein concentration, but dependent on EGFR mutations in the tyrosine kinase domain, the structure of EGFR protein may have been altered by the synonymous mutation. Further studies are required to clearly determine the relationship between the efficacy of gefitinib and the protein structure of G/A mutants of the EGFR protein.

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