Somatic Mutations of *EGFR* Gene in Squamous Cell Carcinoma of the Head and Neck

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Abstract

Purpose: Recently, the kinase domain mutations of *epidermal growth factor receptor* (*EGFR*) gene have been identified in non–small-cell lung cancer, and these mutations have been related to the clinical response to the tyrosine kinase inhibitor gefitinib. Gefitinib treatment has also shown clinical benefits in squamous cell carcinoma of the head and neck (SCCHN). The aim of this study was to explore the possibility that SCCHN harbored the *EGFR* mutations.

Experimental Design: In this study, we analyzed *EGFR* gene in 41 SCCHN for the detection of the somatic mutations by PCR-single-strand conformational polymorphism analysis.

Results: Overall, we detected three *EGFR* mutations (7.3%), and all of the mutations were the same in-frame deletion mutation in exon 19 (E746_A750del).

Conclusion: These data indicated that in addition to non–small-cell lung cancer, SCCHN harbors the *EGFR* gene mutations, and suggested the rationale for the clinical applicability of gefinitib to SCCHN patients.

The epidermal growth factor receptor (EGFR), a receptor protein tyrosine kinase, regulates a number of cellular functions, including proliferation and survival, that are also crucial in tumorigenesis, thus making EGFR a promising target for the cancer therapies (1). Gefitinib (Iressa), which disrupts EGFR kinase activity by binding the ATP pocket within the kinase domain, is an orally active EGFR tyrosine kinase inhibitor (2). Gefitinib has been shown to reduce cellular proliferation in many tumor cell lines and tumor xenografts (3, 4). In the clinical trials, the clinical benefits of gefitinib have been identified mainly in non–small-cell lung cancer (NSCLC) patients (5, 6). However, because not all NSCLC patients exhibit the same response, there is considerable interest in prognostic indicators that might predict the response to gefitinib (5, 6). Recent studies showed that the kinase domain mutations of *EGFR* gene in the NSCLC tissues could predict significant clinical responses to gefitinib (7–9). All of the *EGFR* mutations affect amino acids near the ATP-binding pocket that is targeted by gefitinib. In addition to NSCLC, there is evidence that gefitinib could be a potential agent for the treatment of other tumors, including squamous cell carcinoma of the head and neck (SCCHN; ref. 10). In the preclinical studies, antitumor activity was observed in the SCCHN cells (11). In a phase II study, Cohen et al. reported that 11% of the SCCHN patients with gefitinib monotherapy had an objective tumor response (10). This study suggested the possibility that like NSCLC, the SCCHN harbors the *EGFR* mutations which might be responsible for the clinical response of gefitinib in the SCCHN patients. To explore this possibility, we have analyzed 41 SCCHN tissues and found that *EGFR* gene is somatically mutated in the human SCCHN.

Materials and Methods

Tissue samples and microdissection. Twelve cases of methacarn-fixed and 29 cases of formalin-fixed SCCHN were randomly selected for the study. The tumors consisted of 34 larynx, five tongue, and two tonsil SCCHN. All of the tissues were reviewed independently by two pathologists. We analyzed the primary lesions in 18 cases, and both primary and nodal metastatic lesions in 23 cases. All of the patients were Korean. The male to female ratio was 37:4. Ages of the patients ranged from 43 to 79 years with an average of 59.8 years. The patients consisted of 27 current smokers, 8 former smokers, and 6 nonsmokers (Table 1).

Malignant cells and normal cells were selectively procured from H&E-stained slides using a 301/2 gauge hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator, as described previously (12). DNA extraction was done by a modified single-step DNA extraction method, as previously described (12).

PCR-single-strand conformational polymorphism analysis. All of the *EGFR* mutations in the NSCLC have been reported within exons 18, 19, and 21 (7–9). Thus, we analyzed the *EGFR* mutation in these three exons. Genomic DNAs from tumor cells and normal cells from the

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Table 1. Demographic data and *EGFR* mutations of the SCCHN

No.	Sex/age	Ethnicity	Smoking status	Primary tumor	EGFR mutation (predicted amino acid change)
1	M/60	Asian (Korean)	Previous smoker	Larynx	No
2	M/51	Asian (Korean)	Current smoker	Larynx	No
3	F/59	Asian (Korean)	Previous smoker	Larynx	No
4	M/58	Asian (Korean)	Current smoker	Larynx	No
5	M/68	Asian (Korean)	Previous smoker	Larynx	No
6	M/70	Asian (Korean)	Current smoker	Larynx	No
7	M/78	Asian (Korean)	Previous smoker	Larynx	No
8	M/67	Asian (Korean)	Current smoker	Larynx	No
9	M/64	Asian (Korean)	Current smoker	Larynx	No
10	M/50	Asian (Korean)	Current smoker	Larynx	No
11	M/68	Asian (Korean)	Current smoker	Larynx	Yes (E746_A750del)
12	M/61	Asian (Korean)	Nonsmoker	Larynx	No
13	M/51	Asian (Korean)	Current smoker	Larynx	No
14	M/64	Asian (Korean)	Current smoker	Larynx	No
15	M/75	Asian (Korean)	Previous smoker	Larynx	No
16	M/43	Asian (Korean)	Current smoker	Larynx	No
17	M/56	Asian (Korean)	Current smoker	Larynx	No
18	M/66	Asian (Korean)	Previous smoker	Larynx	No
19	M/62	Asian (Korean)	Previous smoker	Larynx	No
20	M/59	Asian (Korean)	Current smoker	Larynx	Yes (E746_A750del)
21	M/65	Asian (Korean)	Current smoker	Larynx	No
22	M/55	Asian (Korean)	Current smoker	Larynx	No
23	M/43	Asian (Korean)	Current smoker	Larynx	No
24	M/61	Asian (Korean)	Previous smoker	Larynx	No
25	M/47	Asian (Korean)	Current smoker	Larynx	No
26	M/50	Asian (Korean)	Current smoker	Larynx	No
27	M/59	Asian (Korean)	Current smoker	Larynx	No
28	M/45	Asian (Korean)	Current smoker	Larynx	No
29	M/64	Asian (Korean)	Current smoker	Larynx	No
30	M/50	Asian (Korean)	Current smoker	Larynx	No
31	M/65	Asian (Korean)	Current smoker	Larynx	No
32	M/60	Asian (Korean)	Current smoker	Larynx	No
33	M/64	Asian (Korean)	Current smoker	Larynx	Yes (E746_A750del)
34	F/48	Asian (Korean)	Nonsmoker	Larynx	No
35	F/66	Asian (Korean)	Nonsmoker	Tongue	No
36	F/78	Asian (Korean)	Nonsmoker	Tongue	No
37	M/57	Asian (Korean)	Current smoker	Tongue	No
38	M/63	Asian (Korean)	Current smoker	Tongue	No
39	M/65	Asian (Korean)	Current smoker	Tongue	No
40	M/57	Asian (Korean)	Current smoker	Tonsil	No
41	F/69	Asian (Korean)	Current smoker	Tonsil	No

same patients were amplified with three primer pairs covering exons 18, 19, and 21 of human *EGFR* gene. Numbering of cDNA of *EGFR* was done with respect to the ATG start codon. Radioisotope ([^{32}P]dCTP) was incorporated into the PCR products for detection by autoradiogram. The procedures of PCR and single-strand conformational polymorphism (SSCP) analysis were done as described previously (13). After SSCP, bands showing mobility shifts were cut out from the dried gel and reamplified for 30 cycles using the same primer sets. Sequencing of the PCR products was carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA). We repeated the experiments twice, including PCR, SSCP, and sequencing analysis to ensure the specificity of the results.

Results

Genomic DNAs of the SCCHN isolated through the microdissection were analyzed for the detection of mutations in exons 18, 19, and 21 of *EGFR* gene by PCR-SSCP analysis. Enrichment and DNA sequencing analysis of aberrantly migrating bands on the SSCP led to the identification of 3 *EGFR* mutations (cases 11, 20, and 33) of the 41 SCCHN (7.3%; Fig. 1 and Table 1). The mutations were detected in exon 19, but not in exons 18 and 21. Also, all of the mutations were the same type mutation (2236_2250delGAATTAAGAGAAGCA) which

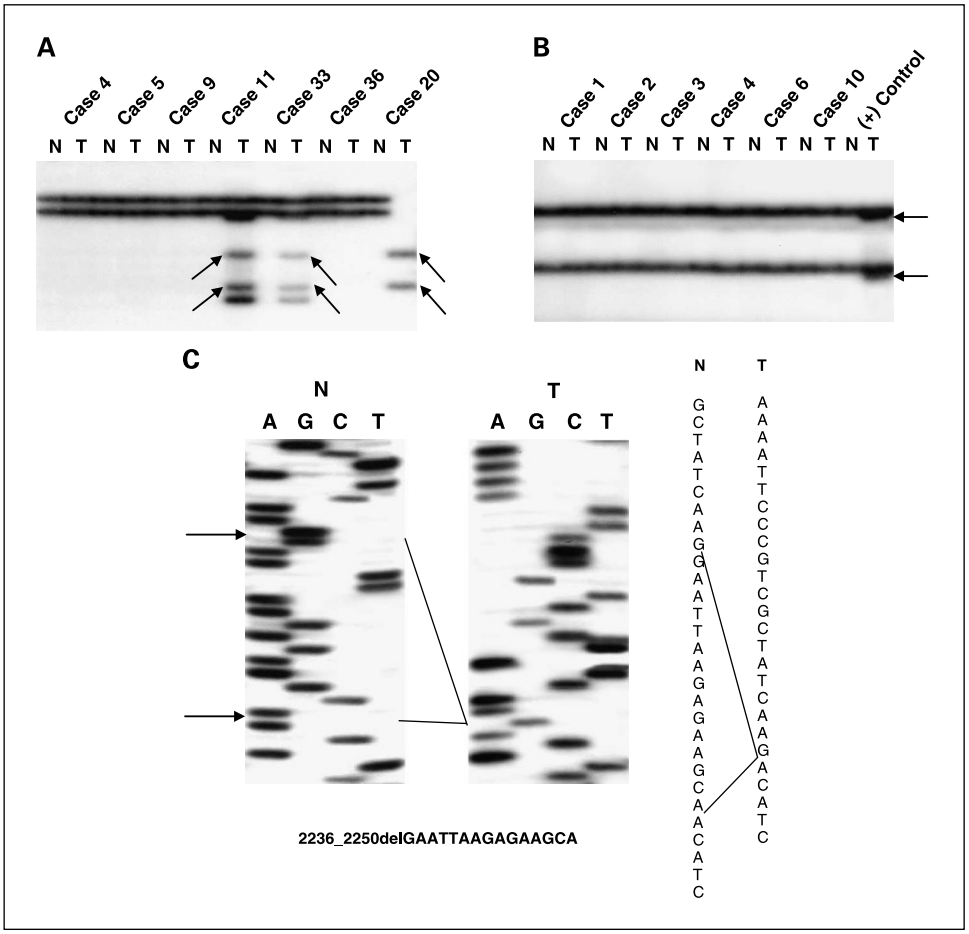

Fig. 1. Mutations of *EGFR* gene in the SCCHN. SSCP (*A* and *B*) and sequencing analysis (*C*) of the *EGFR* from tumors (*lane T*) and normal tissues (*lane N*). *A*, the SSCP (exon 19) of the tumors of cases 11, 33, and 20 shows aberrant bands as compared with SSCP from normal tissue (*N*). SSCP of the DNA from tumor of the case 20 shows only aberrant bands without any wild-type bands. Aberrant bands not indicated by the arrows were sequenced and proven to be heteroduplex bands. *B*, the SSCP (exon 21) of the SCCHNs showed no aberrant bands (*arrows*) compared with the SSCP of a lung adenocarcinoma with the L858R mutation [(+) *control*]. *C*, sequencing analysis from the aberrant band indicated by the arrows from the case 20 in the SSCP (*A*) shows a 15-bp deletion.

would result in a five-amino-acid deletion (E746_A750del). None of the normal samples from the same patients showed evidence of mutations by SSCP (Fig. 1A), indicating the mutations had increased somatically. We repeated the experiments twice, including PCR, SSCP and sequencing analysis, to ensure the specificity of the results, and found that the data were consistent (data not shown). As a positive control for the SSCP of exon 21, we included a lung adenocarcinoma tissue with the known hotspot *EGFR* mutation in exon 21 (L858R) by the same method (Fig. 1B). Also, to confirm the SSCP data on exon 18, we analyzed the PCR products by direct sequencing and found that there was no *EGFR* mutation in exon 18 (data not shown).

Whereas the SSCP patterns of cases 11 and 33 at the mutation sites showed both wild-type and aberrant bands, the SSCP pattern of the case 20 showed only aberrant bands of mutant allele without those of the wild-type allele (Fig. 1), and direct sequencing analysis also revealed only mutant sequence without wild-type one, indicating either homozygous mutations or hemizygous mutation with allelic loss.

All of the three *EGFR* mutations were identified in both primary and metastatic lesions. Also, all of the three mutations were detected in laryngeal squamous cell carcinomas, but the correlation was not statistically significant (Fisher's exact test, $P > 0.05$). All of the patients with the mutation were current smokers, but the association of smoking status and the *EGFR* mutation was not significant (Fisher's exact test, $P > 0.05$).

Discussion

Because gefitinib had an anticancer activity in the SCCHN and the *EGFR* kinase domain mutation is known to mediate the gefitinib sensitivity, we expected that human SCCHN tissues would carry the *EGFR* mutations. In this study, we found that 7.3% of the SCCHN harbored *EGFR* mutations in the DNA sequences encoding the kinase domain. These data could account for the responses seen in the phase II trials of gefitinib, in which 11% of the SCCHN patients had an overall tumor response (10). It is known that somatic mutations in the tyrosine kinase domain of *EGFR* are more common in adenocarcinomas and never smokers in NSCLC. However, the data in this study showed that in the head and neck cancers the *EGFR* mutation occurred in squamous cell carcinomas and smokers. These observations suggest that the etiology of *EGFR* mutations in SSCHN might be different from that of lung cancer.

In addition to NSCLC, Lynch et al. (7) sought to find *EGFR* mutations in exons 19 and 21 in a panel of 108 cancer-derived cell lines from various origins, including seven head and neck cancer cell lines, but no *EGFR* mutations were detected. Explanations for the discrepancy between the presence of *EGFR* mutations in the SCCHN tissues in this study and the absence of the mutations in the SCCHN cell lines include: (*a*) the SCCHN cell lines might not be representative for the original tumor tissues; (*b*) the number of cell lines analyzed was too small to detect the *EGFR* mutations; and (*c*) there might be an ethnic

difference in the distribution of the *EGFR* mutation in SSCHN as in the case of *EGFR* mutations of lung cancers. In regards to the second point, there is no statistical difference in the *EGFR* mutation frequency between the cell line study (0 of 7 samples) and our study (3 of 41 samples; Fisher's exact test, $P > 0.05$).

In the present study, all of the *EGFR* mutations in exon 19 were the E746_A750del. This is similar to the data of NSCLC, where the most common mutation in exon 19 has been the E746_A750del mutation among the 10 types of *EGFR* mutations reported (7–9). These data could raise the possibility that the contribution of *EGFR* mutations in the development of SCCHN might be similar to that of NSCLC. A central aim of cancer research has been to identify the mutated genes that are causally implicated in tumorigenesis. Mutations in cancer could be categorized either as functional alterations affecting key genes underlying the neoplastic process or nonfunctional "passenger" changes. The E746_A750del mutation seems to be a gain-of-function mutation that activates antiapoptosis pathways (14). Activation of the EGFR function and the relatively common incidence (7.3%) suggested that the *EGFR* mutations detected in this study may be functional alterations, but not passenger alterations in the SCCHN tumorigenesis.

The detection of only one type of *EGFR* mutations in exon 19 could raise the possibility that the PCR-SSCP method we used could not detect other types of mutations in these exons besides E746_A750del. However, this possibility is unlikely because we could detect nine types of *EGFR* deletion mutations in exon 19 from the genomic DNA of 80 lung adenocarcinoma and bronchioloalveolar cancer tissues by the same PCR-SSCP method.[5]

Mutant alleles of proto-oncogenes are considered dominant if they transform cells despite the presence of their normal alleles. *EGFR* gene, a proto-oncogene, has usually been mutated heterozygously in NSCLCs. However, Paez et al. and Pao et al. reported one and two nonheterozygous *EGFR* mutation(s) in exon 19 in NSCLC, respectively. In the current study, we also detected one nonheterozygous E746_A750del mutation in a SCCHN (case 20; Fig. 1). However, the functional difference between monoallelic and biallelic alterations of the *EGFR* gene in the tumorigenesis remains unknown at this stage.

Currently the *EGFR* kinase domain mutation seems to be the best predictor of sensitivity to gefitinib (15). However, it remains unknown as to whether the *EGFR* kinase domain mutation could be a predictor for the gefitinib therapy in SCCHN, too, although we found somatic mutations of the *EGFR* kinase domain mutation in the SCCHN. Clearly, therefore, studies are now needed that attempt to find the correlation between the *EGFR* kinase domain mutation and the gefitinib sensitivity in SCCHN.

[5] Unpublished data.

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