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ORIGINAL REPORT

Epidermal Growth Factor Receptor Copy Number Alterations Correlate With Poor Clinical Outcome in Patients With Head and Neck Squamous Cancer

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ABSTRACT

Purpose
Overexpression of epidermal growth factor receptor (EGFR) is common in head and neck squamous cell carcinoma (HNSCC). Recent studies showed that EGFR inhibitors are effective for patients with HNSCC. This study analyzed the genetic nature of *EGFR* gene in HNSCC and its clinical correlations.

Patients and Methods
The *EGFR* gene copy numbers in 134 HNSCC tumors were determined using quantitative real-time polymerase chain reaction. The status of *EGFR* gene copy numbers was analyzed with clinical parameters including clinical outcome. Mutation status of *EGFR* exons 18, 19, and 21 was determined in the HNSCC tumors.

Results
Aberrant *EGFR* copy numbers were found in 32 (24%) of 134 tumors, including 22 (17%) with increased copy number and 10 (7%) with decreased copy number. Patients whose tumors had *EGFR* copy number alterations (particularly patients with increased copy numbers) had significantly poorer overall, cancer-specific, and disease-free survivals compared with patients with normal copy numbers ($P < .0001$). At 5 years after initial diagnosis, 20 (91%) of the 22 patients with increased copy numbers died of disease compared with 30 (29%) of the 102 patients with normal copy number. No mutations on *EGFR* exons 18, 19, and 21 were detected in any of the tumors.

Conclusion
A subset of HNSCC manifests *EGFR* copy number alterations, and this is associated with a poor clinical outcome, suggesting a biologic role of the alterations. The rare mutation or small deletion at *EGFR* exons 18 to 21 indicates a minimal role of these events in HNSCC.

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INTRODUCTION

Epidermal growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases, is overexpressed in a number of solid tumor types, including approximately 80% of head and neck squamous cell carcinomas (HNSCC). The overexpression occurs early in the head and neck tumorigenesis[1] and is associated with advanced stages of the disease and a poor survival.[2,3]

Clinical successes using EGFR inhibitors in treating refractory colorectal and lung cancers underscore the potential use of this drug class. However, the objective tumor response to the agents is limited to a small number of patients. In patients with recurrent or metastatic HNSCC, the EGFR inhibitors have shown encouraging clinical activity in phase II studies.[4,5] In a large, randomized, phase III study, the addition of cetuximab to high-dose radiation in patients with advanced HNSCC demonstrated a statistically significant prolongation in overall survival.[6]

Recently, somatic mutations in the kinase domain of *EGFR* have been identified in 10% to 15% of patients with non–small-cell lung cancer (NSCLC), particularly in females with adenocarcinoma histology, and have been correlated to response to gefitinib[7-10] or erlotinib.[11] In NSCLC, *EGFR* gene copy numbers have been associated with the protein expression levels, and *EGFR* gene amplification assessed by fluorescent in situ hybridization (FISH)[12-14] or by quantitative real-time polymerase chain reaction (Q-PCR)[10] was significantly associated with better clinical outcome in

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gefitinib-treated patients, suggesting that the copy number of the *EGFR* gene may be a useful predicting marker for patients treated with EGFR inhibitors.

To elucidate the nature of *EGFR* gene alterations in HNSCC, we examined *EGFR* gene copy numbers in 134 primary HNSCC tumors using Q-PCR and examined mutations of the kinase domain using a sensitive PCR-restriction fragment length polymorphism (RFLP) assay and correlated the results with clinical parameters and patient outcome.

PATIENTS AND METHODS

Study Population

The 134 HNSCC patients include 41 patients (31%) treated at Institut Gustave-Roussy (Villejuif, France) and 93 patients (69%) treated at The University of Texas M.D. Anderson Cancer Center (Houston, TX) between 1985 and 2003. The institutional review boards approved the study, and informed consent was obtained from all patients. For the French group, all fresh frozen tumor biopsy specimens had a proportion of tumor cells greater than 70%. Tumor DNA was extracted using the QIAamp Tissue kit (Qiagen, Courtaboeuf, France). For the US group, DNA was extracted from formalin-fixed, paraffin-embedded tissue blocks. Microdissection was performed in specimens with less than 70% tumor cells. All of the tumors from the US group were graded as well-, moderately, or poorly differentiated tumors. Clinicopathologic and follow-up information was obtained by reviewing patient pathology reports and hospital charts.

Quantification of EGFR Gene Copy Numbers

Q-PCR was used to quantify *EGFR* copy numbers in the tumor genomic DNA using TaqMan Universal PCR Master Mix and ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers (EGFRex2-205F and EGFRex2-266R) and TaqMan MGB probe (EGFRex2-228T) designed by using Primer Express software (Applied Biosystems) were in exon 2 of *EGFR*. The probe was labeled with the 6-carboxyfluorescein fluorophore and a nonfluorescent quencher. To normalize the *EGFR* copy number per cell, sequences from *β-globin* and *WNT9A* were used as endogenous references. Primer and probe sequences used in the experiments were as follows: EGFRex2-205F, 5′-CCAAGGCACGAGTAA-CAAGCT-3′; EGFRex2-266R, 5′-GGAGGCTGAGAAAATGATCTTCA-3′; EGFRex2-228T, 5′-CGCAGTTGGGCACTT-3′; hu-bglo-232F, 5′-TGAAGGCTCA TGGCAAGAAA-3′; hu-bglo-285R, 5′-GGTGAGCCAGGCCATCAC-3′; Wnt9a-int-F, 5′-CAAAGCGCAAGAAAAA-TAAATTCC-3′; Wnt9a-int-R, 5′-AGCCCCAAACGGAGAGAGA-3′; hu-bglo-253T, 5′-TGCTCGGTGC CTTT-3′; and Wnt9a-int-T, 5′-CCAGCTTGGAGGATT-3′.

We quantified each gene (*EGFR*, *β-globin*, and *WNT9A*) in separate reactions using a universal PCR protocol recommended by the manufacturer. Q-PCR reactions for each sample and each gene were performed in triplicate. The threshold amplification cycles (CT) at the normalized reporter signal minus the baseline signal level of 0.2 for each gene were determined, and their differences, ΔCT1 (*globin* − *EGFR*) and ΔCT2 (*WNT9A* − *EGFR*), were calculated. The cutoff for normal *EGFR* copy number was established as the 95% confidence minimum limit (mean ± 1.96 standard deviations) of ΔCT1 and ΔCT2 determined in DNA of normal WBCs from 22 healthy volunteers. The *EGFR* copy number was considered increased if the value was ≥ 2.1; normal if the value was more than 1.5 but less than 2.1, and decreased if the value was ≤ 1.5. Four NSCLC cell lines with known *EGFR* gene amplification[15] were used as positive controls.

EGFR Mutation Analysis

Mutations of *EGFR* exon 18 G719S (2155G>A) and exon 21 L858R (2573T>G) were screened using PCR-RFLP assay. These mutations create new restriction sites that can be cut by enzyme *Dde*I for G719S at exon 18 and *Sau*96I for L858R at exon 21. The restriction sites located upstream of the mutation sites were used as internal controls of enzyme efficiency. *EGFR* exon 19 deletions were screened by PCR analysis based on product size using GeneScan (Applied Biosystems). The sequences of the PCR primers were as follows: 21F, 5′-GGCATGAACTACTTGGAGGA-3′ and 21R, 5′-GGAAAA TGCTGGCTGACCTA-3′ for exon 21; 18F, 5′-CCATGTCTGGCACTGC TTT-3′ and 18R, 5′-CTGTGCCAGGGACCTTACC-3′ for exon 18; and 19F, 5′-TCTCTCTGTCATAGGGACTCTGG-3′ and 19R, 5′-AGCAGAAACTCA CATCGAGGA-3′ for exon 19.

For PCR-RFLP, PCR products were digested using respective restriction enzymes before determining sizes of the PCR products. For determining sizes of DNA fragments, fluorescently labeled DNA was separated on 4% polyacrylamide gel on an ABI 377 DNA Sequencer (Applied Biosystems). Fluorescent bands were analyzed using GENESCAN 2.1 software (Applied Biosystems) to determine fragment length. H2255 cells with exon 21 mutation, HCC827 cells with a 15–base pair deletion in exon 19, and H406 cells with a 9–base pair deletion were used as positive controls in each experiment. Serial dilutions of normal DNA (H292 cells) and mutated DNA were used to determine the detection sensitivity.

Analyses of EGFR Gene Copy Number and Expression by FISH and Immunohistochemistry

For *EGFR* gene copy number, FISH was performed based on protocols described previously.[12] Briefly, 4-μm paraffin-embedded tissue sections were deparaffinized and digested with proteinase followed by antigen retrieval. The sections were then hybridized with the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Abbott Laboratories, Downers Grove, IL). The signal analysis was performed on a BX51 bright-field and epifluorescence microscope (Olympus America, Lake Success, NY). The *EGFR* sequence was visualized with a Texas red filter, the chromosome 7 centromere sequence was visualized with a fluorescein isothiocyanate filter, and the nuclei were identified with a 6-diamidino-2-phenylindol filter. Tumors were considered FISH negative when there was no or low genomic gain (≤ four copies of the gene in > 40% of cells) and FISH positive when there was a high level of polysomy (≥ four copies of the gene in ≥ 40% of cells) or gene amplification (presence of tight gene clusters, a gene/chromosome per cell ratio of ≥ 2, or ≥ 15 copies of the genes per cell in ≥ 15% of analyzed cells).

For EGFR expression, 4-μm paraffin-embedded tissue sections were deparaffinized followed by antigen retrieval. The sections were incubated for 1 hour with mouse monoclonal antibody (31G7; Zymed Laboratories, Inc, South San Francisco, CA) diluted 1:50 in dilution buffer at room temperature and then incubated with Dako's Mouse EnVision+ Peroxidase System for 30 minutes (Dako, Carpinteria, CA). The peroxidase-catalyzed product was visualized with the BioGenex DAB Chromogen Kit (BioGenex, San Ramon, CA). Finally, the sections were lightly counterstained with Mayer's hematoxylin (Sigma Chemical Co, St Louis, MO) and mounted for analysis. The expression was scored as weak, moderate, or strong based on the staining strength of at least 1,000 tumor cells measured.

Statistical Analysis

The χ^2 and Fisher's exact tests were used to test equal proportion between groups in two-way contingency tables. Survival probability as a function of time was computed using the Kaplan-Meier method. The log-rank test was used to compare patient survival times between groups. Overall, cancer-specific survival (ie, patients who died of HNSCC-related causes) and disease-free survival (ie, patients who developed recurrence and/or metastasis) were analyzed. Multicovariate Cox regression was used to model the risks of abnormal *EGFR* gene copy numbers on survival time, with adjustment for clinical and histopathologic parameters. All statistical tests are two sided, and a $P \leq .05$ was considered statistically significant.

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RESULTS

Patient Characteristics

Forty-one patients were from France, and 93 were from the United States. The general characteristics of the patients are listed in Table 1. In the US group compared with the French group, there were significantly more female patients (47% *v* 17%, respectively; *P* = .0009), more oral cavity tumors (70% *v* 37%, respectively; *P* = .0008), and more T1-2 tumors (47% *v* 27%, respectively; *P* = .03; Table 1). The median follow-up time after surgery was 3.6 years for the overall population (3.9 years for the US group and 3.4 years for the French group). The 5-year overall survival rates were 48.6% for the US group, 19.1% for the French group, and 44.4% for the combined population.

EGFR *Gene Copy Numbers*

Thirty-two (24%) of the 134 tumors exhibited abnormal *EGFR* gene copy numbers, including 22 tumors (17%) with an increased *EGFR* copy number ranging from three copies to 12 copies (median, 3.9 copies) and 10 tumors (7%) with a decreased *EFGR* copy number (< two copies). *EGFR* copy numbers in the remaining 102 tumors were in the normal range. There was no significant difference in the frequencies of the overall abnormal copy numbers between the US group and the French group (22% *v* 29%, respectively; *P* = .33). However, a higher percentage of tumors exhibited increased gene copy numbers in the French group (27%) than in the US group (12%; *P* = .06).

The gene copy numbers were correlated with clinical parameters of the patients (Table 2). There was no significant association between the abnormal *EGFR* gene copies and age, sex, smoking status, tumor differentiation, tumor location, and tumor size. However, the aberrant gene copies were correlated significantly with increased lymph node metastasis (*P* = .045) and advanced pathologic stage (*P* = .03; Table 2).

Table 1. Characteristics of the Patient Populations

Characteristic	France (n = 41)	United States (n = 93)	*P*
	No. of Patients		
Age, years			.36*
Median	62	60	
Range	32-80	29-88	
Sex			.0009
Male	34	49	
Female	7	44	
Site			.0008
Oral cavity	15	65	
Pharynx	20	18	
Larynx	6	10	
T stage			.03
T1-2	11	44	
T3-4A	30	49	
N stage			.95
N0	24	55	
N1-2	17	38	
Disease stage			.18
I-III	16	48	
IVA	25	45	

*Wilcoxon rank sum test.

Table 2. Associations Between Abnormal *EGFR* Copy Numbers and Clinical Characteristics

Characteristic	Abnormal *EGFR* (n = 32)	Normal *EGFR* (n = 102)	*P*
	No. of Patients		
Age, years			.93*
Median	62	60	
Range	31-80	29-88	
Sex			.94
Female	12	39	
Male	20	63	
Country			.33
France	12	29	
United States	20	73	
Smoking status†			.99
Nonsmoker	4	17	
Smoker	16	56	
Tumor grade†			.80
Well	6	25	
Moderate	8	23	
Poor	6	24	
Site			.25
Oral cavity	16	64	
Pharynx	13	25	
Larynx	3	13	
T stage			.20
T1-2	10	45	
T3-4A	22	57	
N stage			.045
N0	14	65	
N1-2	18	37	
Disease stage			.03
I-III	10	54	
IVA	22	48	

Abbreviation: EGFR, epidermal growth factor receptor.
*Wilcoxon rank sum test.
†The smoking status and tumor differentiation grade were only available for the US population (n = 93).

EGFR *Copy Number Measured by FISH and EGFR Expression Measured by Immunohistochemistry*

We performed FISH and immunohistochemistry (IHC) analyses in 16 of the tumors from the US cohort, including five with reduced *EGFR* copy number, five with normal *EGFR* copy number, and six with increased *EGFR* copy number based on the Q-PCR analysis described earlier (Fig 1). All of the six tumors with increased *EGFR* copy number determined by Q-PCR showed *EGFR* amplification by FISH analysis, whereas none of the 10 tumors with normal or reduced *EGFR* copy number was FISH positive (*P* = .0001), supporting the role of Q-PCR in measuring *EGFR* copy numbers in clinical specimens. EGFR expression was also measured in the 16 tumors by IHC. Although five of the six tumors with increased *EGFR* copy number showed strong EGFR expression, three of the 10 tumors without *EGFR* amplification also showed strong EGFR expression (*P* = .12). Examples of *EGFR* copy numbers and protein expression measured by FISH and IHC are presented in Figure 1.

EGFR *Gene Copy Numbers and Survival*

Patients whose tumors had abnormal *EGFR* copy numbers experienced statistically significant poorer survival compared

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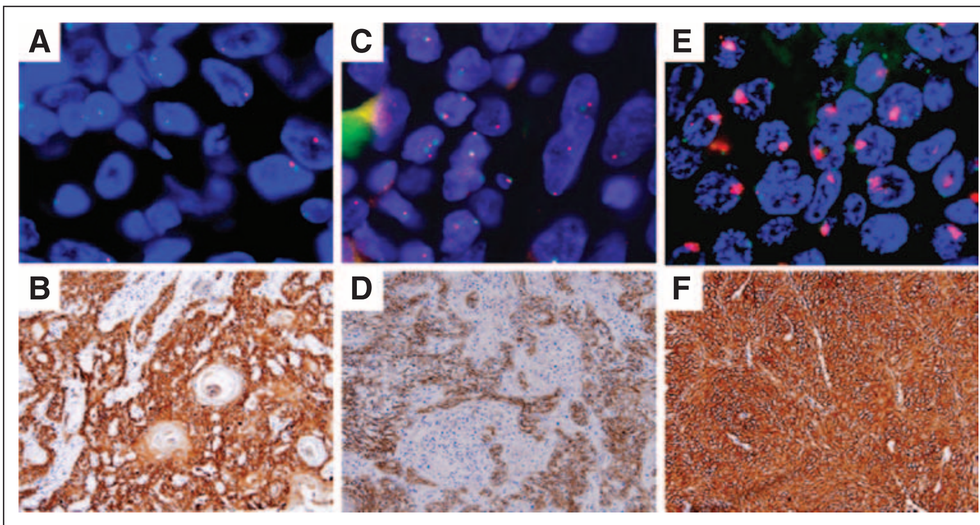

Fig 1. Fluorescent in situ hybridization (FISH) and immunohistochemistry (IHC) analyses of head and neck squamous cell carcinoma (HNSCC). Epidermal growth factor receptor (*EGFR*) copy numbers were measured by FISH analysis in three HNSCC tumors showing (A) reduced *EGFR* copy number, (C) normal *EGFR* copy number, and (E) *EGFR* amplification. (B, D, and F) EGFR protein expression of these tumors was measured by IHC. Table 3 lists the results of quantitative real-time polymerase chain reaction, IHC, and FISH in the 16 patients compared.

with patients whose tumors had normal *EGFR* copy numbers ($P < .001$ for overall, cancer-specific, and disease-free survivals; Fig 2). Because the biologic consequences are likely different for tumors with increased *EGFR* and decreased copy number, we analyzed the survival by considering this factor. Among the three groups (102 tumors with normal copy number, 22 with increased copy number, and 10 with decreased copy number), a striking difference was observed between the patients whose tumors exhibited normal *EGFR* copy number and patients whose tumors had increased *EGFR* copy numbers (Fig 3). At 5 years, 20 (91%) of the 22 patients with increased copy numbers died of the disease compared with 30 (29%) of the 102 patients with normal copy numbers (Fig 3B). Patients whose tumors had decreased *EGFR* copy numbers also had poorer cancer-specific and disease-free survivals (Figs 3B and 3C), although the differences were less striking compared with patients with increased copy numbers.

Multicovariate analysis was used to determine whether the gene copy number abnormality is an independent factor for patients' survival. Because differences in certain clinic parameters and patterns of *EGFR* abnormalities were observed between the US patients and the French patients, we stratified the data by patient country of origin. Table 3 lists the results of the multicovariate analysis. As expected, age, tumor size, and lymph node metastasis were independent prognostic factors for overall survival, whereas tumor size and lymph node metastasis were also independent prognostic factors for cancer-specific and disease-free survival. Interestingly, the aberrant *EGFR* copy number was a strong prognostic factor independent of all the other known factors in overall, cancer-specific, and disease-free survival.

EGFR *Gene Mutation*

Serial mixed dilutions of normal DNA and mutated DNA from control cell lines showed that the sensitivity of our mutation detection method for exon 18 and 21 mutations and exon 19 in-frame deletions was high (detected approximately 1% of mutated or deleted copies among the normal background; data not shown). We used positive

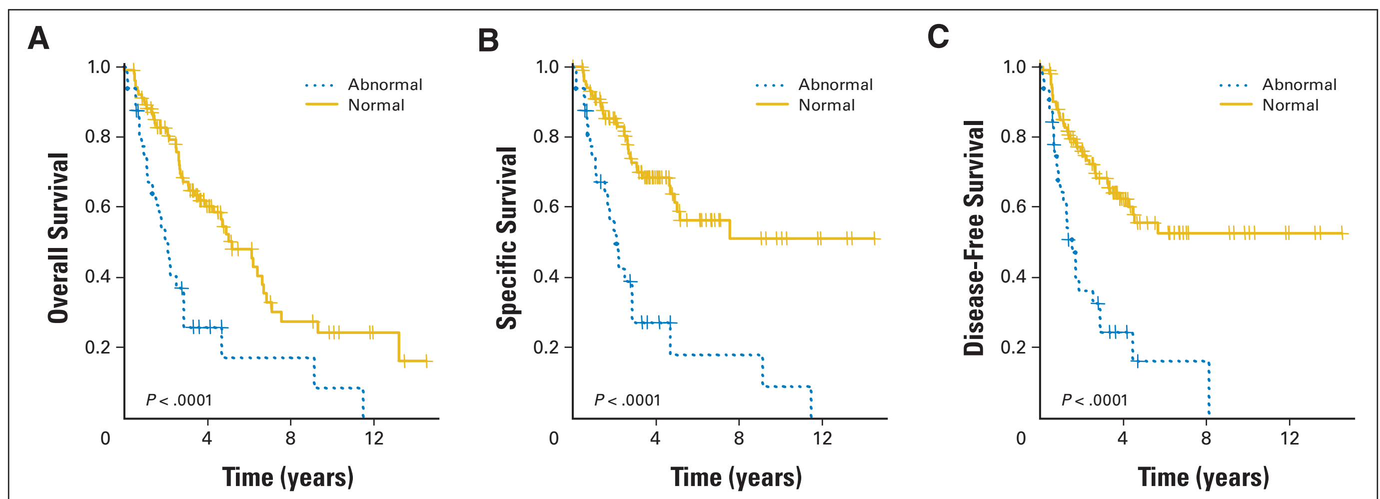

Fig 2. Kaplan-Meier survival curves for patients categorized by epidermal growth factor receptor (*EGFR*) gene copy number. (A) Overall survival. (B) Cancer-specific survival. (C) Disease-free survival. Normal copy number is displayed as solid lines, and abnormal (increased or decreased) copy number is shown by dotted lines. *P* values were estimated using the log-rank test.

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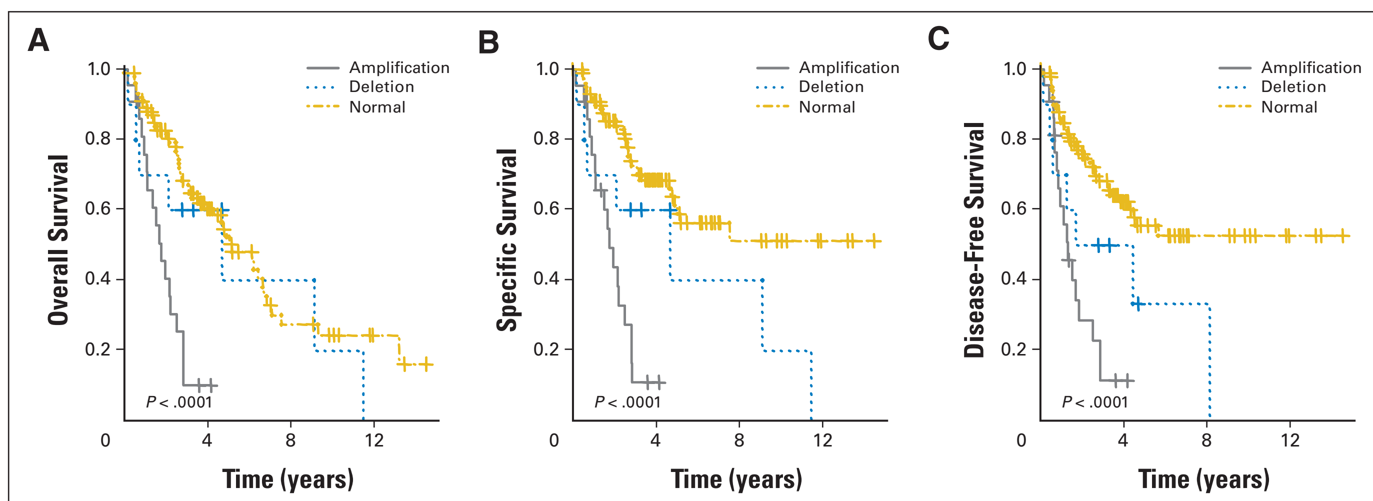

Fig 3. Kaplan-Meier survival curves for patients categorized by different epidermal growth factor receptor (*EGFR*) gene copy numbers. (A) Overall survival. (B) Cancer-specific survival. (C) Disease-free survival. *P* values were estimated using the log-rank test.

controls for mutations and deletions in each experiment. Among the 134 tumors, five samples showed PCR fragments suspicious for mutations or deletions. However, direct sequencing of these specimens failed to identify any mutation or deletion in the corresponding genomic regions.

DISCUSSION

The identification of mutations in EGFR kinase domain and the correlation between the mutations and tumor response to EGFR inhibitors have spiked extensive search for these mutations in various epithelial tumor types. In NSCLC, these *EGFR* mutations centralize in exons 18 to 21, with more than 90% located in the following three hotspots: in-frame deletions in exon 19, L858R (2573T>G) point mutation in exon 21, and G719S (2155G>A) point mutation in exon 18.[7-11] These mutations are more frequent in nonsmokers, women with adenocarcinoma, and patients from Asian nations.[10,16,17]

In this study, we failed to detect any mutation in these hotspots in 134 patients with primary HNSCC, suggesting that the mutations are rare in HNSCC patients in the US and French populations. It should be noted that only the three mutation hotspots were examined in this study. Although we failed to confirm mutation or deletion in the five tumors with suspicious alterations, it may be a result of the low sensitivity of direct sequencing analysis in the confirmative analysis, as suggested in a recent study.[18] Nevertheless, our finding is consistent with recent reports in which mutation or deletion was rarely identified in HNSCC specimens from whites.[19,20] In another study, Lee et al[21] detected three *EGFR*-mutated tumors (exon 19 deletion) among 41 HNSCC specimens from a Korean population, suggesting that mutations may be more frequent in Asian populations with HNSCC.

EGFR copy number in tumors is another factor important for EGFR expression level and response to EGFR inhibitors. Using FISH, increased copy numbers of *EGFR* have been observed in 10% to 13% of the HNSCC samples.[22,23] In this study, we showed that patients whose tumors had aberrant copy numbers were more advanced and had poorer clinical outcomes independent of other clinical parameters. In a recent study, Morrison et al[24] analyzed 59 laryngeal cancers and found similar frequencies of *EGFR* amplification (n = 14, 24%) and deletion (n = 6, 10%). These authors found that patients whose tumors had either *EGFR* amplification or deletion had a poorer survival.[24] Considering that the majority of the patients had oral or pharyngeal cancers in our study, these data together suggest that the alteration rates of *EGFR* copy numbers are similar at the different anatomic locations of HNSCC. It is possible that tumor cells with increased *EGFR* copy numbers possess a greater capability to invade under stress conditions because they might produce a higher peak level of receptors than cells with normal copy number. It should be noted that the prognosis and the rate of abnormal *EGFR* copy numbers were different between the US and French patients in the study, probably as a result of the more oropharyngeal tumors, more advanced disease stage, and more males in the French population.

We postulate that both genetic background and environmental etiology might contribute to the development of *EGFR* gene deletion or amplification. It is possible that the deletion occurs as a cellular response to reduce overexpressed EGFR and that the cells survive through alternative pathways. We observed some tumor cell clusters with reduced EGFR signal in the tumors with reduced *EGFR* copy number measured by Q-PCR in our FISH analysis (Fig 1A), which suggests that a subpopulation of the tumors might have contributed to the Q-PCR result. Whether the tumor cells with reduced EGFR capability developed other surviving mechanisms remains to be determined.

Tsao et al[11] analyzed 197 tumors for *EGFR* mutations and 221 tumors for *EGFR* gene copy numbers from patients with NSCLC who participated in a phase III clinical trial to determine efficacy of erlotinib. Although increased gene copy number was associated with longer survival in patients treated with erlotinib in a univariate analysis, such association was not statistically significant in multivariate analysis,[11] suggesting that gene amplification might be associated with other confounding factors in the study population. A recent study showed a lack of *EGFR* amplification in

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Table 3. Multicovariate Cox Model for Survival Stratified by Country

Variable	*P*	Hazard Ratio	95% CI
Overall survival			
Age	.004	1.03	1.01 to 1.05
T stage: T3-4 *v* T1-2	.0008	2.49	1.46 to 4.26
Node status: node positive *v* node negative	< .0001	2.78	1.72 to 4.51
EGFR copy number: abnormal *v* normal	.0007	2.41	1.45 to 4.01
Cancer-specific survival			
T stage: T3-4 *v* T1-2	.002	2.77	1.45 to 5.32
Node status: node positive *v* node negative	< .0001	3.26	1.85 to 5.75
EGFR copy number: abnormal *v* normal	< .0001	2.98	1.72 to 5.14
Disease-free survival			
T stage: T3-4 *v* T1-2	.0061	2.30	1.27 to 4.17
Node status: node positive *v* node negative	< .0001	2.88	1.69 to 4.92
EGFR copy number: abnormal *v* normal	.0001	2.81	1.65 to 4.80

Patient No.	Q-PCR *EGFR/globin*	IHC	*EGFR* copy ≥ 4 (% cells)	*EGFR/CEP7* ≥ 2 (% cells)	*EGFR* copy ≥ 15 (% cells)	FISH
1	0.32	+++	0	0	0	–
2	0.36	+	0	0	0	–
4	0.38	++	0	0	0	–
6	0.30	+	10	0	0	–
7	0.40	++	0	0	0	–
9	0.47	+++	0	13	0	–
10	0.47	+	0	0	0	–
11	0.48	+++	11	0	0	–
12	0.48	++	0	0	0	–
13	0.48	+	2	2	0	–
19	0.86	+	8	30	0	+
20	0.96	+++	0	35	0	+
21	1.18	+++	1	30	0	+
22	1.36	+++	1	51	0	+
23	1.48	+++	2	60	0	+
25	4.1	+++	0	75	0	+

Abbreviations: EGFR, epidermal growth factor receptor; Q-PCR, quantitative real-time polymerase chain reaction; IHC, immunohistochemistry; FISH, fluorescent in situ hybridization.

HNSCC tumors that responded to gefitinib treatment.[20] Major differences between NSCLC and HNSCC include the heterogeneous histology and the higher *EGFR* mutation rate in lung cancers. Nevertheless, patients with HNSCC seem to benefit from treatment with EGFR inhibitors. A recent phase III clinical trial using cetuximab in combination with high-dose radiation for patients with locally advanced HNSCC demonstrated an improved locoregional disease control and survival.[6] Because approximately 10% improvement was achieved at 2 years using this treatment strategy compared with radiotherapy alone, it is reasonable to hypothesize that patients whose tumors contain increased *EGFR* copy number might be the ones who benefited from the addition of the EGFR inhibitor. Analyzing tumor samples from these clinical trials or incorporating the genetic analysis into future clinical trials may answer this important question.

Although FISH is commonly used to determine gene copy numbers, Q-PCR can obtain reliable gene copy number and is less expensive. In FISH analysis, only a small part of tumors is analyzed, typically using the mean copy number of 60 cells, whereas real-time Q-PCR may be used to analyze a large area of tumors to minimizing the issue of tumor heterogeneity. The determination of gene copy numbers based on real-time Q-PCR is objective and, therefore, makes data interpretation more reliable.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

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Data analysis and interpretation: Stephane Temam, Hidetoshi Kawaguchi, Adel K. El-Naggar, Jaroslav Jelinek, Diane D. Liu, J. Jack Lee, Li Mao
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