IDENTIFICATION OF THE RARE EGFR MUTATION p.G796S AS SOMATIC AND GERMLINE MUTATION IN WHITE PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Accepted 10 January 2008
Published online 4 June 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/hed.20831

Abstract: Background. Somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) are involved in tumorigenesis and response to targeted therapies in distinct cancer types. Squamous cell carcinomas of the head and neck (HNSCC) show an incidence of EGFR mutations varying from 7% in Asians to 0% to 4% in white patients. Mutational screening predominantly focuses on the analysis of hotspot regions of EGFR (exons 19 and 21).

Methods. In a follow-up study, we screened for mutations in exons 18 to 21 of the EGFR gene in 127 patients.

Results. In this cohort, a mutation frequency of 2.4% (3/127) was detected. In addition to the previously reported mutation p.K745R, the otherwise rare EGFR mutation p.G796S occurred in 2 patients with HNSCC (2/127).

Conclusion. EGFR kinase mutations are rare in white patients with HNSCC. Extension of mutational screening to exon 20 may clarify the frequency and impact of the mutation p.G796S.

Keywords: EGFR; mutation; HNSCC; tyrosine kinase inhibitor

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most frequent cancer worldwide, with estimated 900,000 cases diagnosed each year. Despite current therapeutic modalities, many tumors recur or develop metastases, highlighting the need for new therapeutic targets. HNSCC arises from a common premalignant progenitor followed by outgrowth of clonal populations associated with cumulative genetic alterations and phenotypic progression to invasive malignancy. Genetic aberrations result in inactivation of tumor suppressor genes and activation of proto-oncogenes by deletions, point mutations, promoter methylation, and gene amplification. Many epi-
Epithelial cancers, including those of the head and neck, overexpress epidermal growth factor receptor (EGFR), which has been correlated with a poor clinical prognosis.\textsuperscript{3–5} EGFR activation promotes cell proliferation and cell survival, and strategies that target EGFR are currently under investigation.\textsuperscript{6} These therapies include monoclonal antibodies blocking the ligand-binding domain and tyrosine kinase inhibitors (TKIs) inhibiting the activation of the cytoplasmic tyrosine kinase (TK) of EGFR.\textsuperscript{7,8} Anti-EGFR monoclonal antibodies have revealed activity in the therapy of advanced colorectal carcinoma and in a variety of epithelial tumor types, including HNSCC and non-small cell lung cancer (NSCLC).\textsuperscript{9–11} The development of anti-EGFR TKIs has primarily been focused on NSCLC, although responses have been reported for other cancer types. A phase II trial with oral TKI (gefitinib) in patients with recurrent or metastatic HNSCC showed significant response in 11\% of patients refractory to other therapies.\textsuperscript{12} The correlation between EGFR expression profile and sensitivity to EGFR blockers remains controversial. In several studies, no association was found between EGFR expression and response.\textsuperscript{13–17} Analysis in patients with NSCLC revealed a relation between somatic mutations in the EGFR TK domain and clinical response.\textsuperscript{18,19} All these EGFR mutations affect amino acids near the ATP-binding pocket that is targeted by TKIs. In NSCLC, 86\% of these mutations cluster in 2 hotspots, in exons 19 and 21. The remaining 14\% are rare and scatter throughout exons 18 to 21.\textsuperscript{20} The discovery of these somatic EGFR mutations might have important implications for tumor biology, clinical trial design, and treatment.\textsuperscript{21} Recently, a mutation frequency of 7\% was reported in a Korean patient cohort with HNSCC.\textsuperscript{22} In 5 further cohorts with white patients (including a study of our group) or patients of unspecified origin, 3 of 298 cancer samples carried mutations (1\%).\textsuperscript{23–27} To further explore the frequency of somatic EGFR TK mutations in white patients with HNSCC, we expanded mutation analysis in the 100 cancer samples already reported\textsuperscript{25} on exons 18 and 20, and, in addition, exons 18 to 21 were sequenced in 27 new samples. This representative patient group extends previously reported data on mutation frequency.

**PATIENTS AND METHODS**

**Patient Samples.** After obtaining informed consent, 100 formalin-fixed paraffin-embedded HNSCC samples\textsuperscript{25} and 27 fresh frozen samples were selected from the local tumor bank covering cases diagnosed between 1992 and 2005. All samples were obtained from white patients and analyzed independently by 2 pathologists (routine diagnostician and A.T.). The male-to-female ratio was 7.2:1, and patient age ranged from 30 to 97 years (median, 54 ± 2.1 years).

For subsequent DNA isolation and to exclude false-negative results, areas rich in tumor cells with a tumor cell proportion exceeding the stroma component by >90\% were selected on hematoxylin-eosin-stained slides. For DNA extraction, tissue cores (cylinders) with a diameter of 1.5 mm and a depth of 5 mm were punched from these areas using a TrapSystem biopsy needle (Medical Device Techn., Gainesville, FL). Nontumorous tissues or precancerous lesions were investigated only in patients carrying a mutation.

**DNA Extraction and EGFR Mutation Analysis.** DNA was extracted using the BioRobot M48 workstation with MagAttract technology (Qiagen, Germany). Polymerase chain reaction (PCR) of exons 18, 19, 20, and 21 of the EGFR gene was performed with primers described previously.\textsuperscript{19} Sequencing of PCR products was carried out using a Big Dye sequencing kit (Perkin-Elmer, Foster City, CA) on the ABI 3130xl Genetic Analyzer. All identified mutations have been confirmed with 3 independent PCRs of the original genomic DNA sample and were compared with nontumorous DNA samples to ensure that the finding was somatic.

**Immunohistochemistry.** EGFR expression was studied in a single patient (case 1), carrying the EGFR mutation p.G796S. No tissue samples from the second patient with this mutation (case 2) were available for immunohistochemical analysis. The paraffin-embedded tumor serial sections were cut and stained with hematoxylin-eosin stain. The immunohistochemical analysis of EGFR expression was done using Ventana nexus module (Ventana Medical Systems, Tucson, AZ) with monoclonal mouse anti-EGFR antibody (clone 3C6, Ventana detection kit-system) after pronase 1 pretreatment for 8 minutes. The intensity of EGFR-immunostained cells was scored according to the Dako scoring guidelines (http://pri.dako.com/08052_egfr_pharmdx_interpretation_manual.pdf; Dako, Denmark).

**RESULTS**

Tumor DNA for PCR analysis was extracted in 127 HNSCC samples. We analyzed 70 oral cavity
cancer samples (including 9 mouth floor, 2 tongue, and 6 tonsillar cancers), 45 laryngeal carcinomas, 10 hypopharyngeal carcinomas, and 2 nasopharyngeal carcinomas (squamous cell carcinomas). In addition to the already reported mutation in exon 19, p.K745R, direct sequencing of exon 20 of the EGFR gene revealed a missense mutation in the TK domain of 2 patients (1.57%). This mutation with a heterozygous G to A change at nucleotide c.2386 has not been shown before in HNSCC and results in a glycine to serine substitution at codon 796 (p.G796S) (Figure 1). The clinical features of the 2 unrelated patients with the mutation p.G796S are heterogenous. Case 1, a 53-year-old man, had oropharyngeal carcinoma (stage IVA) arising from the left tonsil and was treated by tumor resection and a protocol of combined radiation and chemotherapy with 5-fluorouracil and mitomycin C. Eighteen months after end of first-line therapy the patient had progressive disease with lymphangiosis carcinomatosa of both sides. He received palliative chemotherapy and died 26 months after diagnosis. Nontumorous samples from this patient displayed wild type, indicating that the mutation originated in the germline (Table 1). Immunohistochemical

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<td>Case 1</td>
<td>Tumor</td>
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analysis of EGFR expression in tumor samples of this patient revealed completely circumferential membrane staining of all cells, indicating strong EGFR expression (3+). Case 2, a 51-year-old woman, had the diagnosis of squamous cell carcinoma of the mouth floor (stage I) and was treated with local tumor resection. In a 5-year follow-up period, there was no recurrence of the tumor. The same mutation was seen in nontumorous samples, as well as in lymph nodes and peripheral blood, indicating that the mutation originated in the germline (Table 1). There was no evidence of neoplastic disease in family history.

DISCUSSION
EGFR kinase domain mutations seem to be a predictor of response to oral EGFR TKIs like erlotinib and gefitinib in patients with NSCLC. These somatic mutations are more frequent in females, Asians, nonsmokers, and adenocarcinomas. The 2 major types of mutations observed in NSCLC (deletions at codons 746–750 and the missense mutation p.L858R) cause increased and sustained phosphorylation of EGFR and consequently activated downstream antiapoptotic pathways (PI3K/AKT and STAT). EGFR and its ligands have also been studied extensively in HNSCC, and the efficacy of gefitinib has been elucidated. However, the biology of responsiveness to TKIs remains unclear. In contrast to NSCLC, responsive cases of HNSCC were indistinguishable from nonresponsive cases in terms of their histology or associated history of tobacco exposure. Similar to the results in lung cancer, colorectal, and prostate cancer, mutations in the EGFR in patients with HNSCC show ethnic differences in the frequency of their occurrence. In Korean HNSCC patients, a mutation frequency of 7.3% is described. In our previous study, we showed a frequency of 1% in white patients with HNSCC and 4 further HNSCC studies displayed a frequency of 0% to 4%, in total 3 mutations in 298 samples (1%). This study represents the largest cohort of white HNSCC patients and revealed a mutation frequency of 2.5% (3/127). Beside the already reported mutation p.K745R in exon 19, we found the mutation p.G796S in exon 20 of 2 patients with HNSCC. This mutation has not been described in HNSCC to date. Recently, Douglas et al showed the same mutation in a Korean patient with prostate cancer and overexpression of EGFR. This study group created a 3-dimensional structure of the TK domain revealing that p.G796S is located on the external surface within alpha helices of the C-terminal lobe. The missense substitution converts a nonphosphorylatable amino acid into a phosphorylatable serine. This change may be important in EGFR signaling and could induce a conformational change, but this assumption has to be proven in further studies.

Despite data on mutational analysis of 12,000 different cancer samples with 1750 EGFR mutations, p.G796S has only been found in 1 Korean patient with prostate cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic). In this study, this mutation is present in 2 patients with HNSCC. Whether this is a coincidental observation or is specific for HNSCC remains unclear. Exon 20 was only analyzed in 2 of 6 published mutation studies on HNSCC. Therefore, the incidence of p.G796S could be underestimated. Furthermore, only a few study groups performed direct sequencing and used mutation detection systems with lower detection rate like single-strand conformational polymorphism analysis. The extent of exon analysis as well as mutation detection methods hampers the comparability of available data. In this study, in 1 patient, the p.G796S mutation was somatic with strong EGFR expression; in another patient, with no family history of increased cancer incidence, it represented a germline event. Therefore, the impact of this mutation on tumorigenesis should be interpreted with caution. Further functional studies are required to characterize this mutation.

The mutational results of this large white patient cohort confirm that EGFR kinase mutations are rare in white patients with HNSCC. To date, EGFR mutational analysis in HNSCC is not used in clinical application, and the low frequency of this mutation raises questions about the usefulness of the analysis. However, in HNSCC, extension of mutational screening to exon 20 may be relevant to further elucidate the significance of the mutation p.G796S.

Acknowledgments. This work was supported by “MFF Tirol No. 108.” The excellent technical assistance of Ramona Berberich is gratefully acknowledged.

REFERENCES