Research Article

Functional Genomic Analysis Identified Epidermal Growth Factor Receptor Activation as the Most Common Genetic Event in Oral Squamous Cell Carcinoma

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Abstract

A 250K single-nucleotide polymorphism array was used to study subchromosomal alterations in oral squamous cell carcinoma (OSCC). The most frequent amplification was found at 7p11.2 in 9 of 29 (31%) oral cancer patients. Minimal genomic mapping verified a unique amplicon spanning from 54.6 to 55.3 Mb on chromosome 7, which contains *SEC61G* and *epidermal growth factor receptor* (*EGFR*). Results from fluorescence *in situ* hybridization, transcriptome, and immunohistochemistry analyses indicated that the expression level of *EGFR*, but not of *SEC61G*, was up-regulated and tightly correlated with DNA copy number in 7p11.2 amplified tumors. Among the members of the erbB family, *EGFR* (*HER1*) was found to be the most frequently amplified and highly expressed gene in both human and mouse oral tumors (*P* < 0.01). Genes for downstream effectors of EGFR, including *KRAS, mitogen-activated protein kinase 1*, and *CCND1,* were also found amplified or mutated, which resulted in activation of EGFR signaling in 55% of OSCC patients. Head and neck squamous cancer cells with different *EGFR* expression levels showed differential sensitivity to antitumor effects of AG1478, a potent EGFR inhibitor. AG1478-induced EGFR inactivation significantly suppressed tumor development and progression in a mouse oral cancer model. Our data suggest that EGFR signaling is important in oral cancer development and that anti-EGFR therapy would benefit patients who carry the 7p11.2 amplicon in their tumors. [Cancer Res 2009;69(6):2568–76]

Introduction

Cancer is a complex genetic disease frequently associated with genomic instability. Gene amplification is a common mechanism for activating oncogenes that control cell growth, development, differentiation, and apoptosis in cancer (1). Target-based therapy against oncogenes has been clinically useful in treating cancers, such as anti-HER2/neu for breast cancer and BCR/Abl inactivation for leukemia or gastrointestinal stromal tumor (2–4). Therefore, identification and characterization of oncogenes in cancers will help to advance drug development and clinical management.

Oral cancer is the third most common cancer in developing nations and the sixth most common cancer worldwide (5). Although oral cancer can be cured with proper treatment when detected early, patients who have had oral cancer have a high risk of developing secondary and/or recurrent tumors in the surrounding area, a phenomena called field effect. Once tumor cells spread to the lymph nodes, the overall mortality rate is high and the 5-year overall survival rate does not exceed 50%, which is among the lowest for all major cancers (6, 7). The lack of success in effectively treating oral cancer is primarily a consequence of a lack of understanding on the etiology of oral cancer and a lack of drugable targets.

To identify and characterize potential targets for treating oral cancer, a genome-wide approach was taken to quantitatively measure genomic alterations in oral squamous cell carcinoma (OSCC), which is the most common type of oral cancer (6, 8). To facilitate the discovery of key cancer-driving gene(s) in the observed 7p11.2 amplicon, we used a combination of assays to compare genomic copy number changes, mRNA expression profiles, and protein immunohistochemical staining. Epidemiologic studies indicate that betel quid chewing is one of the most important risk factors for oral cancer patients in Taiwan and other Southeast Asian countries (9). A strong casual association between betel quid chewing and oral cancer development has also been found (9). We, therefore, induced oral cancer in mice by treating them with arecoline and 4-nitroquinoline 1-oxide (4-NQO). The efficacy of anti–epidermal growth factor receptor (EGFR) therapies was also evaluated in this mouse OSCC model.

Materials and Methods

Sample preparation and DNA/RNA isolation. Fresh tumor specimens were collected from oral cancer patients and stored in liquid nitrogen. Tissue slides were prepared from the frozen samples and stained with hematoxylin. Cancer tissues were selected for further DNA and RNA extractions only when the tissues contained >85% tumor cells based on the staining results. Genomic DNA was purified by DNeasy kit (Qiagen) according to the procedure provided by the manufacturer. In addition, we also purified RNA from the same tumor samples using TRIzol reagent (Invitrogen) for gene expression profiling. Acquisition of tissue specimens was approved for this study by the institutional review board of the China Medical University Hospital. DNA or RNA from normal oral epithelia was used as reference samples in this study.

Single-nucleotide polymorphism array analyses. Genomic alterations in tumor samples were genotyped using 250K single-nucleotide polymorphism (SNP) arrays (Affymetrix). A detailed protocol is available on the Affymetrix Web page.[7] Briefly, genomic DNA was cleaved with the restriction enzyme Sty1, ligated with linkers, and amplified by PCR. The PCR products were further purified and digested with DNase I to sizes ranging from 250 to 1,000 bp. Later, fragmented products were labeled with biotin and hybridized to the array. Arrays were then washed on Affymetrix fluidics stations. The bound DNA was fluorescently labeled using streptavidin-phycoerythrin conjugates and scanned using Gene Chip Scanner 3000.

The dChip program was used to analyze SNP array data (10). Chip data were first normalized to a baseline array with median signal intensity at the probe intensity level using invariant set normalization method. A model-based (PM/MM) method was used to obtain signal values for each SNP in each array. Signal values for SNP probes were compared with the average intensities from eight normal samples. To infer DNA copy number from raw signal data, we used Hidden-Markov model (11) based on the assumption of diploidy for normal samples. A cutoff of >2.6 copies in more than three consecutive SNPs, with a copy number of >3.5 by quantitative real-time PCR, was defined as an amplification.

Prevalence of amplified regions by quantitative real-time PCR. We designed PCR primers for *EGFR* and *SEC61G*, which are within the defined 7p11.2 amplicon, using the Primer3 program[8] (see Supplementary Table S1). Quantitative real-time PCR was performed on 48 independent OSCCs. DNA content of each sample was then normalized to that of *LINE-1*, a repetitive element for which copy numbers per haploid genome are similar among all human cells (12). PCR reactions were performed in triplicate, and threshold cycle numbers were calculated using the iCycler software v2.3 (Bio-Rad Laboratories).

Gene overexpression was validated by quantitative real-time reverse transcription–PCR on cDNAs from experimental tumor samples and 10 normal oral epithelia. Expression levels of candidate genes within each sample were determined by normalization to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Tissue microarray for fluorescence *in situ* hybridization and immunohistochemistry. We arranged 128 formalin-fixed, paraffin-embedded oral cancer tissues and 20 normal oral epithelia onto tissue microarrays to facilitate fluorescence *in situ* hybridization (FISH) and immunohistochemical analyses. Three representative cores from each tumor were placed on the tissue microarrays. The histologic grading of OSCC was based on the WHO classification. Bacterial artificial chromosome clones, CTD-2199A14 and RP11-81B20, containing the genomic sequences of the 7p11.2 amplicon, were purchased from Invitrogen. BAC clone RP11-91E1 was used as the control probe for chromosome 7, because no copy number change was found within this region based on our SNP array data. The method used for FISH has been described in a previous study (13). Two individual scientists who are not aware of the tumor grade and clinical information evaluated FISH signals. Approximately 100 tumor cells were examined from each specimen. Gene amplification was defined as a ratio of gene probe signal to control probe signal exceeding 2.5.

Monoclonal antibodies against EGFR (clone 31G7) were purchased from Zymed Laboratories for immunohistochemical studies, with an antibody dilution of 1:10. Immunohistochemistry was performed using standard protocols with an EnVision+ System peroxidase kit (DAKO). Immunointensity of EGFR staining was independently scored by two pathologists using the four-tier system: weak positive (+1), moderately positive (+2), strongly positive (+3), or intensely positive (+4) membrane staining. We noted discrete nuclear immunoreactivity of EGFR in cases with a staining score of +3 or +4, and the functional roles of nuclear EGFR were extensively studied in recent reports (14, 15). For discordant cases, a third investigator was brought in to score, and the final intensity score was determined by the majority scores.

Statistical analysis and clinical correlation. The clinical information of OSCC samples studied in this study was collected from clinical note, including tumor site, clinical stage, treatment history, recurrent status, and survivorship. All patients who were treated at China Medical University Hospital were also interviewed uniformly for detail information on the history of cigarette smoking, alcohol drinking, betel quid chewing, and dietary preferences (Supplementary Table S2). Overall survival time was defined as the number of month between diagnosis and death or between diagnosis and the most recent follow-up. All calculations and statistical analysis were performed using the SAS/STAT software package (SAS Institute) and plotted as survival curves using Kaplan-Meier method. With this method, *P* values were evaluated by log-rank test.

Cell proliferation assay, apoptosis detection, and cell cycle analysis. Squamous cancer cells, BIC, SEG, KON, and SAT cells, were seeded in 96-well plates at a density of 4,000 per well and treated with or without the EGFR inhibitor AG1478 (Merck Biosciences). Cell number was determined indirectly by fluorescence intensity of SYBR Green I nucleic acid gel stain (Molecular Probes), as detected with a microplate reader (Fluostar, BMG). Data were expressed as mean ± SD from five replicates in each experimental group.

For apoptosis assay, apoptotic cells were detected by staining with Annexin V–FITC (BioVision). The percentage of Annexin V–positive cells was determined by counting at least 400 cells from five different fields under a microscope for each experiment. Furthermore, the treated cells were subjected for cell cycle analysis by the propidium iodide staining method, as previously described (16). The percentage of cells at each cycle stage was analyzed by ModFit LT software (Verity Software House).

Western blot analysis. Protein lysates were prepared from head and neck squamous cancer cell lines, including BIC, SEG, KON, and SAT cells. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Western blots were performed by hybridizing the membranes with anti-EGFR antibodies and anti-GAPDH (as loading control) for 2 h at room temperature, with a dilution of 1:1,000. After three washes with TBST (0.1% Tween 20 in TBS), the membranes were blotted with horseradish peroxidase–conjugated anti-mouse antibodies (Pierce) at a dilution of 1:1,000 for 1 h at room temperature. EGFR bands were revealed by chemiluminescence (Amersham).

Development of a mouse model for oral cancer. To mimic the etiology of oral cancer patients in Asia, we tried to generate an OSCC model in C57BL/6JNarl male mice by treating with different doses of arecoline (Sigma), as well as in combination with 4-NQO (Sigma). Although arecoline alone did not induce OSCC, it accelerated tumor growth when combined with 4-NQO (data not shown). The optimal conditions for OSCC formation are 0.5 mg/mL arecoline and 0.2 mg/mL 4-NQO in the drinking water of mice for 8 wk. The drinking water was changed every week, and mice were allowed access to the drinking water at all time during treatment. Cancer development was assessed every 2 wk. To evaluate the efficacy of anti-EGFR therapy, mice in tumor group were treated with AG1478 by i.p. (10 μg/mouse/treatment/wk for 4 wk) at 18 wk after the last treatment of arecoline plus 4-NQO. Mice were sacrificed at 28 wk, and tongues with tumors were excised, fixed, embedded, and sectioned for H&E staining. The lesions observed were classified into five types: epithelial hyperplasia, low-grade dysplasia, high-grade dysplasia, papilloma, and invasive carcinoma. The criteria to define tumor grades were described in a previous study (ref. 17; also shown in Fig. 6*C*). Differences of tumor progression were tested between AG1478-treated and untreated mice using Student's *t* test.

Results

Identification of 7p11.2 amplicon in oral squamous cell carcinoma. SNP array hybridization was performed on 29 OSCCs and 8 normal squamous epithelia using the 250K platform. Genetic alterations were revealed by detecting the signal density of each SNP probe along the chromosomes and normalizing gene copy number, taking the average signal intensity in normal tissues to be 2.0. As shown in Fig. 1*A*, 9 of 29 OSCC tissue samples (31%) showed

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[7] http://www.affymetrix.com/support/technical/manuals.affx
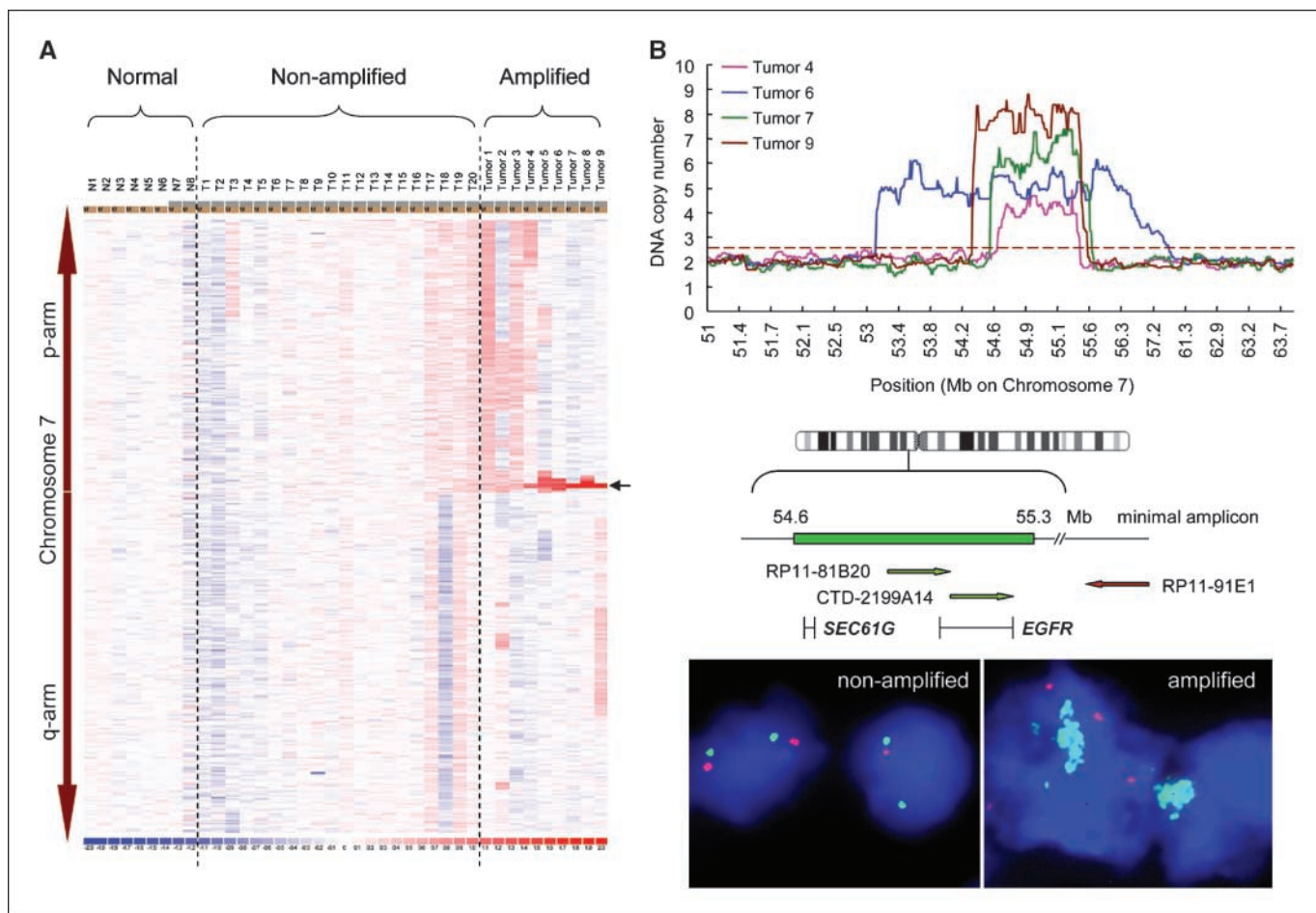[8] http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

Figure 1. Characterization of 7p11.2 amplicon in OSCC. *A,* in total, 29 OSCC tissues were genotyped by 250K SNP microarray, and DNA copy number was normalized with the average (as 2.0) of signal intensity in normal tissues adjacent to tumor sites. *B,* minimal amplified region was mapped by overlapping the array data of each amplified tissue (copy number > 2.6), spanning from 54.6 to 55.3 Mb on chromosome 7. *SEC61G* and *EGFR* were found in the minimal amplicon based on human genome databank alignment. Specific FISH probes were prepared to validate the amplicon in 7p11.2-amplified tissues (*right*) compared with nonamplified tissues (*left*).

detectable amplified gene copy numbers (>2.6; see the definition in Materials and Methods) at 7p11.2, whereas no evidence of gene amplification was found on chromosome 7 in any samples of normal oral epithelia. Results from the whole genome analysis indicated that the 7p11.2 amplicon was the most frequently amplified region (Supplementary Fig. S1 and Supplementary Table S3). Genomic quantitative PCR further confirmed the existence of the 7p11.2 amplicon (copy numbers, >3.5) in these nine OSCC tissue samples, which correlated strongly with the data generated by SNP array genotyping ($P < 0.01$; Supplementary Table S4). Alignment of these amplicons delineated a minimum over-lapped amplified region, spanning 54.6 to 55.3 Mb on chromosome 7 (Fig. 1*B*). Two genes, *EGFR* and *SEC61G*, were found within this minimal region, based on the University of California-Santa Cruz human genome databank.[9] To validate the genetic alterations, dual-color FISH (green signal for testing probes and red for the control probe) was performed to detect the amplicon on an independent panel of 128 clinical samples. A homogenous staining region (HSR) pattern was detected in 10 clinical samples (7.8%)

with a >6-fold increase in the FISH signal ratio (Fig. 1*B, right*). A high-level gain (ratio between 4.0-fold and 6.0-fold) was found in 10 clinical cases (7.8%) and a moderate gain (ratio between 2.5 and 4.0) was found in 12 clinical cases (9.4%). In normal tissues or in tumor tissues lacking the 7p11.2 amplicon, however, the signal ratios between the testing probes and the control probe were around 1 (Fig. 1*B, left*). Based on the genome-wide assay and cytogenetic analysis, our data indicate that 25% to 30% of oral squamous cancer patients carry the 7p11.2 amplicon.

EGFR is a key cancer-driving gene during OSCC development. According to the clonal selection/expansion theory, molecular genetic changes in cancer cells, such as gene amplifications or deletions, are heritable traits, and their accumulation is the result of Darwinian selection from a heterogeneous tumor population (18, 19). Therefore, a cancer-driving gene is predicted to exhibit gene copy number alterations consistent with mRNA expression levels and protein expression levels. To verify the biological relevance of the genes located in the 7p11.2 amplicon, we performed a genome-transcriptome comparison to select the cancer-driving gene(s). Real-time quantitative PCR was performed to detect the expression levels of *SEC61G* and *EGFR* using cDNA samples extracted from the same panel of tumor samples profiled by SNP

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[9] http://genome.ucsc.edu/cgi-bin/hgGateway

array method (Fig. 2*A*; see Supplementary Fig. S2 for the bar graph). Compared with the normal tissues, *SEC61G* expression levels in 7p11.2 amplicon-containing tumors did not show significant increases. In contrast, expression levels of *EGFR* in these tumors were much higher than in normal tissues (*P* = 0.006) and were significantly correlated with the DNA copy number increase (*P* < 0.01). Based on the above findings, we identified *EGFR* as the key cancer-driving gene within the 7p11.2 amplicon and selected it for further characterization.

Because EGFR belongs to the type I receptor tyrosine kinase (erbB) subfamily, we assessed whether other members of this family were involved in oral cancer development. DNA copy number and RNA expression levels of all erbB members, *EGFR (HER-1), HER-2, HER-3,* and *HER-4*, were measured by real-time quantitative PCR and normalized by the mean C_t of *LINE-1* and *GAPDH*, respectively. As shown in Fig. 2*B*, a 3-fold amplification of the *EGFR* locus was frequently found in OSCC (*P* = 0.004), whereas no significant genetic alterations were found for other erbB members. RNA expression levels of *EGFR, HER-2,* and *HER3* were all higher than normal in OSCC. However, only *EGFR* showed significant difference in both DNA (Fig. 2*B*) and RNA (Fig. 2*C*) levels. Interestingly, the expression levels of *HER-4* in OSCC were much lower than in normal tissues (*P* = 0.009). Taken together, our data suggest that *EGFR* is more important than other erbB members for oral cancer development.

Protein overexpression of EGFR correlates with gene amplification in OSCC and poor clinical outcome. To more comprehensively study the relevance of the *EGFR* amplicon in OSCC, the protein levels of EGFR were investigated by immuno-histochemistry on the same tissue slides used for FISH analysis

(Fig. 3*A*). Overall, tumor tissues with higher EGFR immunointensity tended to have higher FISH signal ratios in the immunohisto-chemistry staining assay (*P* < 0.001, Spearmen correlation; Fig. 3*B*). Notably, all of the tissues with HSR FISH patterns (*n* = 10; FISH ratios, > 6) had an intensely positive membrane staining with a strong nuclear staining (+4). Ninety percent (18 of 20) of the tumors with strong positive immunohistochemistry staining (+3) contained moderate to high levels of genetic gain (FISH ratios between 2.5 and 6). Ninety-six percent (94 of 98) of the tumors with weak positive (+1) or moderately positive (+2) immunohistochem-istry staining were verified as being *EGFR* nonamplified or low genetic gain (FISH ratios between 1 and 2.5; Fig. 3*A* and *B*). Normal squamous epithelium adjacent to the tumor site showed faint membranous staining (+1; Supplementary Fig. S3). Accordingly, our results indicated that 93.3% (28 of 30) of the tumors that showed intensely positive (+4) or strongly positive (+3) immunohistochem-istry staining carried the *EGFR* amplicon (FISH ratio, > 2.5). Thus, EGFR immunoreactivity might serve as a surrogate marker to predict *EGFR* amplification.

To determine if *EGFR* amplification/overexpression affects the overall survival, Kaplan-Meier survival analysis was performed to evaluate the clinical correlation. Among the 128 cases we tested, there were 115 patients whose long-term follow-up information was available and, thus, they were subjected to survival analysis (Supplementary Table S2). Consistent with previous studies (20–22), our immunohistochemistry data indicate that patients with immunostaining scores in the 0 to +2 range had a better overall survival (34.5 months) than did patients with scores in the +3 to +4 range (22.3 months; Fig. 3*C, left*). Consistent with our finding that EGFR expression correlates with gene copy number,
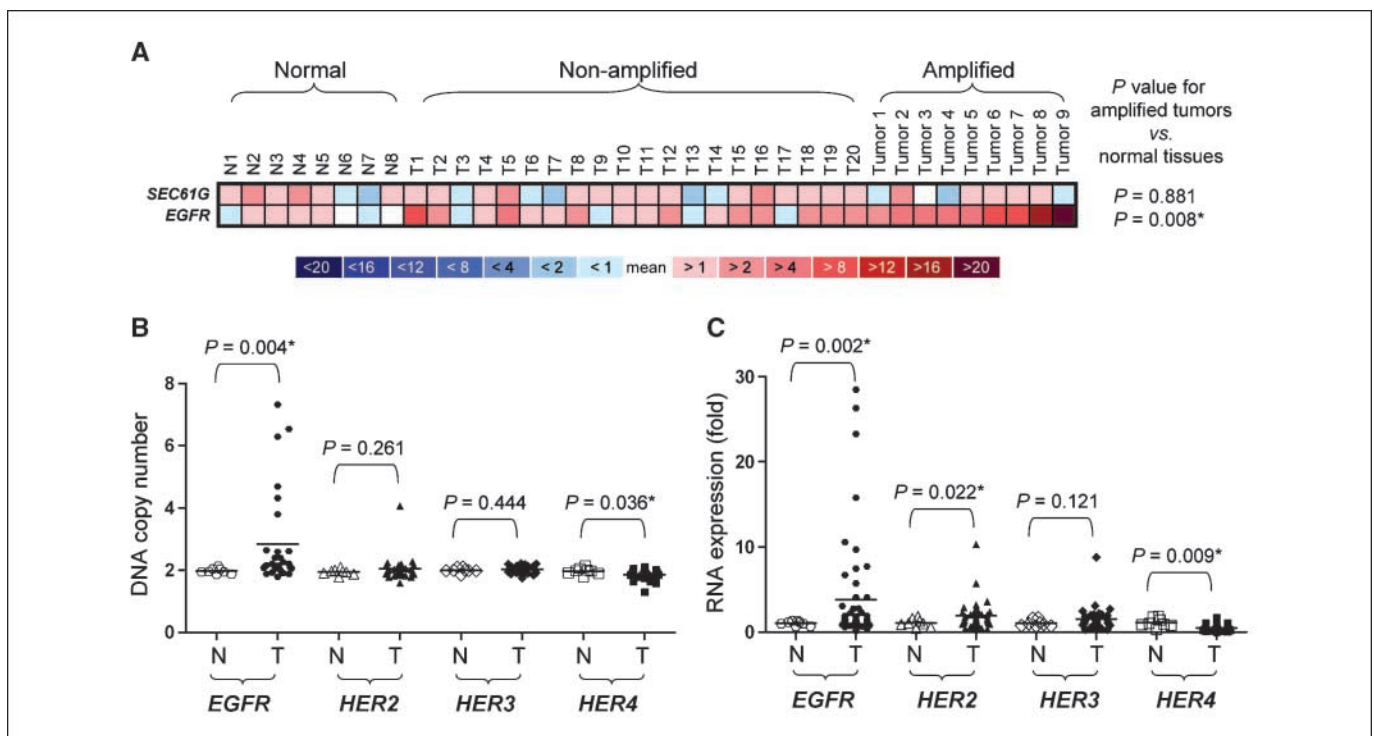

Figure 2. Gene expression analysis of the 7p11.2 minimal amplicon in OSCC. *A*, gene expression levels of *SEC61G* and *EGFR* were compared with DNA copy numbers using the same panel of tissues as shown in Fig. 1. To confirm the biological relevance of erbB members in OSCC, DNA copy number (*B*) and mRNA expression levels of erbB members (*C*), *EGFR, HER-1, HER-3,* and *HER-4*, in OSCC were analyzed by quantitative PCR and compared with the levels in normal oral epithelia.

patients with a high FISH signal (ratio, >2.5) had a shorter median survival time (21.8 months) than did patients with a low FISH signal (low genetic gain or nonamplified, 33.2 months; Fig. 3C, right). Our data suggest that *EGFR* amplification or protein overexpression is an indicator of poor prognosis for OSCC patients.

Activation of EGFR signaling in OSCC. To understand if genes involved in the EGFR signaling pathway can also be activated through gene amplification or mutation, we searched the same tumor samples analyzed by SNP array for other amplicons that contain downstream targets of EGFR. First, we noticed amplifications of *KRAS* and *mitogen-activated protein kinase 1* (*MAPK1*) in 1 (3.4%) and 4 (13.8%) of the 29 samples, respectively (Fig. 4A), suggesting activation of EGFR-KRAS-MAPK1 signaling in OSCC. In addition, *CCND1*, one of the most responsive downstream targets of activated MAPK1 (23), was also found amplified in five of the samples (17.2%) examined (Fig. 4A). Thus, the top three major amplicons in OSCC of Taiwanese patients all contribute to activation of the EGFR signaling (Supplementary Table S3). Gene expression profiling within the minimal amplicons further suggested that *KRAS, MAPK1,* and *CCND1* were potential key cancer-driving genes in their corresponding amplified regions (Supplementary Fig. S4A–C). Because activating mutations in the

RAS-RAF-MAPK pathway are frequent genetic events that promote tumor growth (24), DNA sequences in exons of the genes in the RAS, RAF, and MAPK gene families were analyzed. A mutational hotspot at codon 61 of *KRAS*, Q61H, was identified in 2 of the 29 samples (6.9%; Fig. 4B). The Q61H mutation was not identified in normal tissue counterparts or in other normal oral epithelia. No mutations were found in *NRAS, HRAS, ARAF, BRAF, CRAF, MEK1,* or *MEK2*. The genomic copy number of these genes also did not change in the 29 OSCC samples (Fig. 4C). Therefore, our data indicated that 55.2% (16 of 29) of oral cancer specimen carried genetic alterations that would be expected to promote activation of EGFR signaling. Notably, no mutations were found in the kinase domain (exons 18, 19, and 21) of EGFR in the OSCC samples tested.

Differential effects of EGFR inactivation on head and neck squamous cancer cells. It has been known that oncogenic signaling caused by gene amplification or activating mutation provides survival advantages for tumor growth. To investigate the possibility that squamous cancer cells in which EGFR was amplified or overexpressed depended on EGFR signals for survival, we analyzed squamous cell lines derived from head and neck cancers for expression levels of EGFR by Western blotting (Fig. 5A). Among the cell lines tested, SAT cells were found to carry the
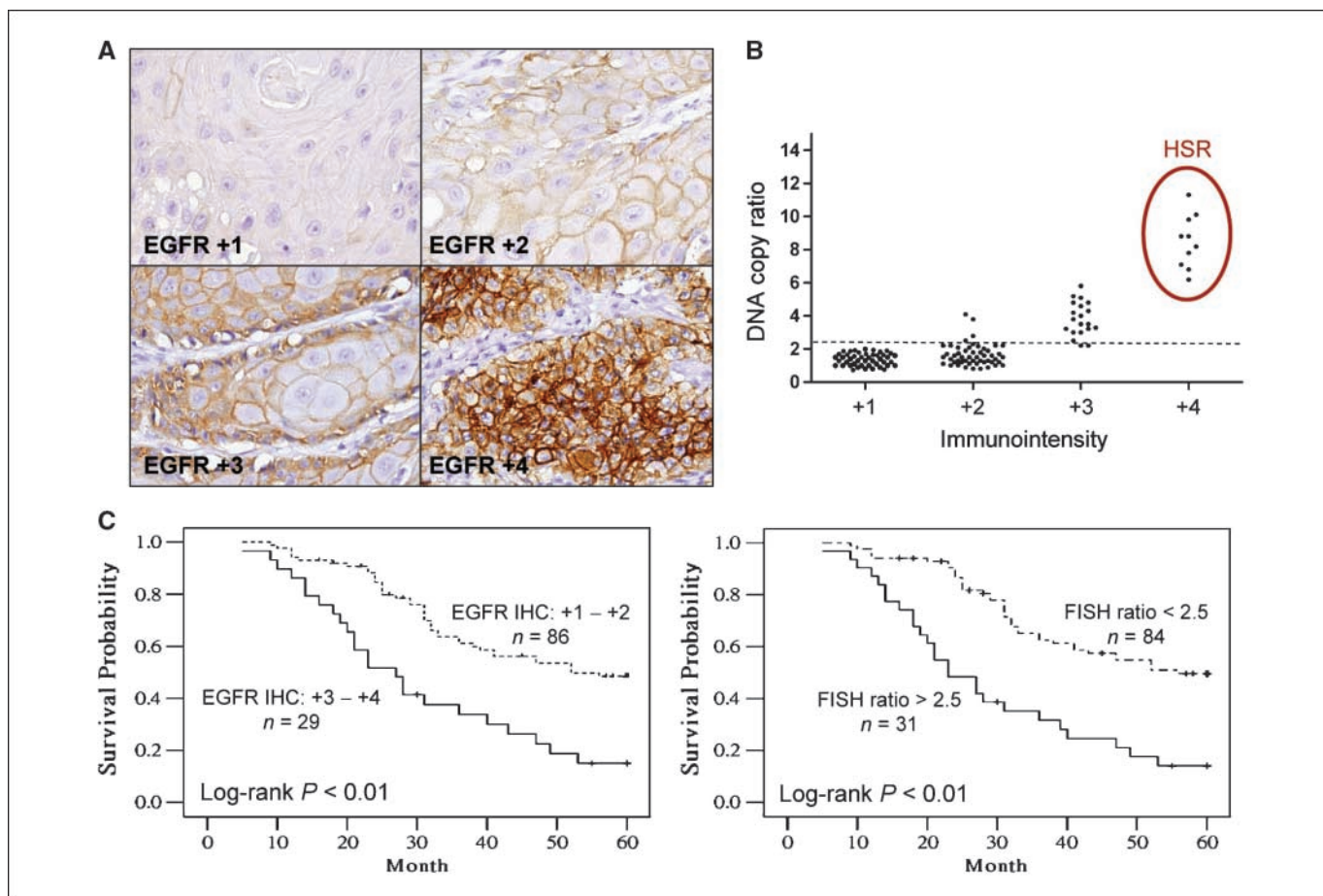

Figure 3. Expression level of *EGFR* correlates with DNA copy number and poor clinical outcome. *A,* immunohistochemistry (*IHC*) against EGFR was performed and scored (+1, +2, +3, +4) on the sample tissue arrays (*n* = 128) used for FISH analysis. The scoring criteria for immunointensity are described in Materials and Methods. *B,* OSCC tissues with higher DNA content in the 7p11.2 region were found to express higher protein levels of EGFR. Samples with HSR FISH patterns show the highest immunohistochemistry immunointensity (+4). Samples with DNA copy ratios of >2.5 were recognized as 7p11.2 amplified cancer tissues. *C,* Kaplan-Meier survival analysis for OSCC patients. The overall survival for patients was compared based on EGFR protein expression levels detected by immunohistochemistry (score, +1 to +2 versus +3 to +4; *left*) and the genetic content of *EGFR* detected by dual-color FISH (signal ratio < 2.5 versus ratio > 2.5; *right*).
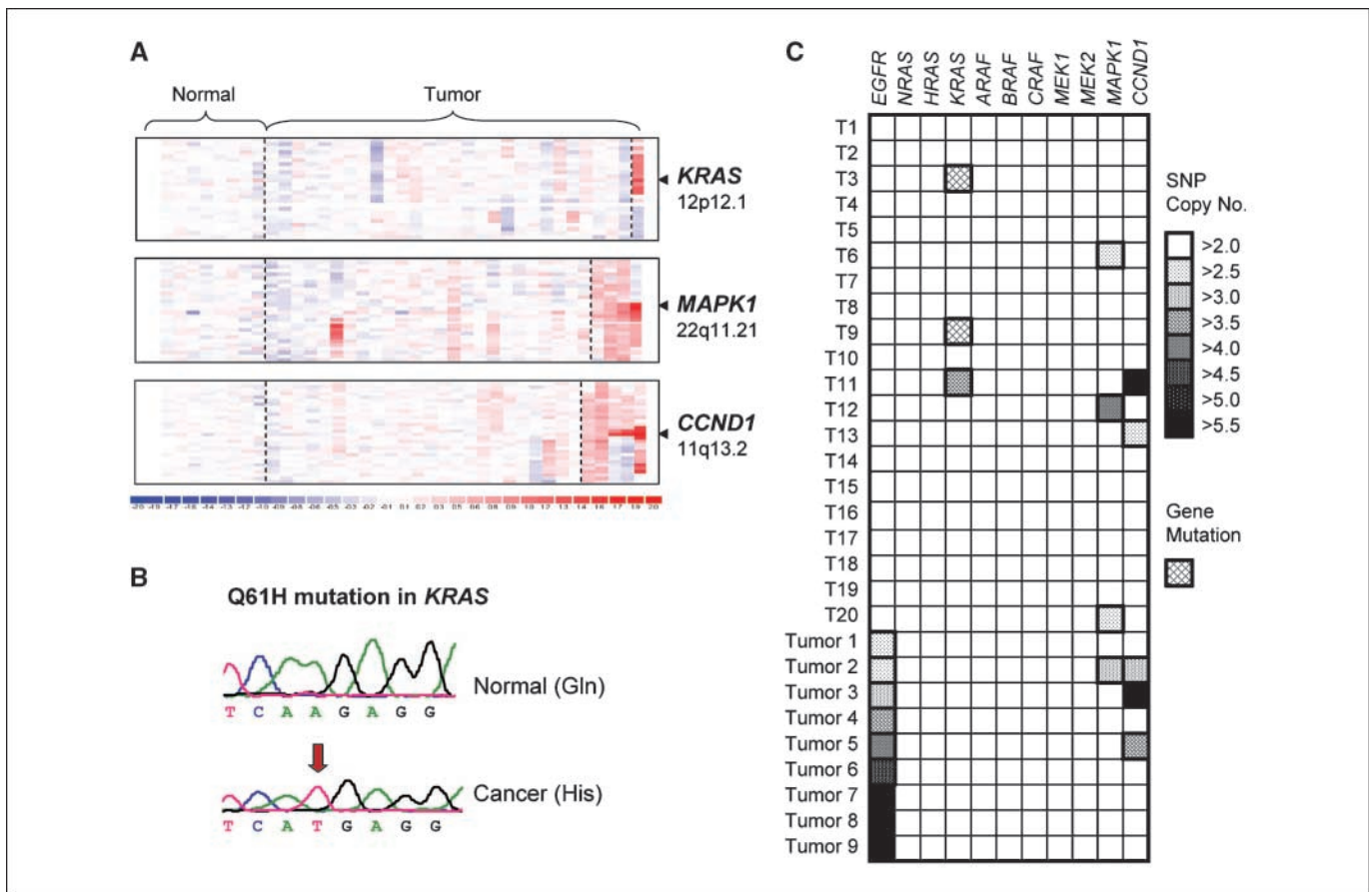
Figure 4. Activation of EGFR signaling in OSCC. *A,* the genetic contents of EGFR downstream targets were analyzed by 250K SNP array. Amplifications of *KRAS, MAPK1,* and *CCND1* were found in certain tumor samples. *B,* sequence analyses of EGFR downstream targets revealed the Q61H mutation in *KRAS* in 2 of the 29 OSCCs (6.9%) tested. *C,* summary of genetic alterations of genes involved in EGFR-RAS-RAF-MAPK-CCND pathway in OSCC samples. Exons analyzed for DNA sequencing were exons 1 and 2 of *NRAS*; exons 1 and 2 of *HRAS*; exons 1 to 3 of *KRAS*; exons 10, 11, and 13 of *ARAF*; exons 11, 12, and 15 of *BRAF*; exons 10, 11, and 14 of *CRAF*; exons 2 and 3 of *MEK1*; and exons 2 and 3 of *MEK2*.

7p11.2 amplicon and overexpress EGFR. KON cells expressed the EGFR protein but did not contain the amplicon. Low expression levels of EGFR were detected in BIC and SEG cells. Treatment with the EGFR inhibitor AG1478 had differential inhibitory effects on cell growth, depending on the *EGFR* status of treated cells, with the highest drug sensitivity in SAT cells and the lowest in BIC and SEG cells (Fig. 5*B*). When cells were treated with 10 μmol/L AG1478, a significant increase in cell death was found in SAT cells on day 3 and day 4, as measured by Annexin V staining (Fig. 5*C*), and DNA fragmentation (cells at sub-G_1 phase) could be detected on day 4 and day 5 (Supplementary Fig. S5). In contrast, treatment of BIC and SEG cells with the same condition did not result in cell death. Results from colony formation assays further indicated the effectiveness of EGFR inhibition on SAT cells in which *EGFR* was amplified or overexpressed, but there were minimal effects on BIC cells (Fig. 5*D*).

EGFR inactivation suggests a rational treatment for OSCC. Oncogenes serve as specific and unique targets for drug intervention, and target-based therapies against oncogenes have been proved clinically useful in treating cancer patients (2, 4). Because we have shown the requirement of EGFR signaling for cell survival in *EGFR*-amplified/overexpressing OSCC (Fig. 6), it is possible that anti-EGFR would be effective in treating OSCC *in vivo*. To mimic the etiology of OSCC among Asian patients who had

habits of chewing betel quid, we generated a mouse model for oral cancer induced by arecoline and 4-NQO. As shown in Fig. 6*A*, tumor lesions were found 28 weeks after the last treatment of arecoline plus 4-NQO. In addition, we showed significant differences in *EGFR* expression levels between oral tumors and normal tissues compared with other erbB members (Fig. 6*B*). Notably, *HER-2* has been suggested as an oncogene/tumor marker for OSCC in previous studies (25, 26). In our mouse tumor study, we also identified significant expression levels of *HER-2* in mouse oral tumors. Nevertheless, *EGFR* remains the most important target because of the greater changes in its expression, relative to other erbB members, in mouse oral tumors (Fig. 6*B*).

To address the efficacy of anti-EGFR therapy for OSCC, mice in the tumor group were treated with AG1748 (10 μg/mouse/ treatment/wk for 4 weeks) by i.p. injection when they developed leukoplakia on their tongues (around 18 weeks after the last treatment of arecoline plus 4-NQO). Mice treated with equal volumes of PBS were used as the control. At 28 weeks, all of the mice (*n* = 7) treated with PBS developed hyperplasia and high-grade dysplasia; 71.4% (five of seven) developed OSCC; 42.8% (three of seven), and 14.3% (one of seven) developed papillomas and low-grade dysplasia, respectively (Fig. 6*C*). Significantly, AG1478 treatment (*n* = 10) reduced the incidence of OSCC (from 71.4% to 10%, *P* < 0.001) and high-grade dysplasia (from 100% to 40%,

P = 0.008; Fig. 6*C*). In addition, a slower tumor progression rate for the AG1478-treated mice was observed, as more tumors remained at the low-grade dysplasia stage. These data suggest a clinical benefit of EGFR inhibitors in treating OSCC. Tumor volume was not compared in this study because tumor size did not correlate well with the cancer stage; for example, papillomas could be very large though they are only benign epithelial tumors. The histologic features of epithelial cells at a higher magnification for normal, hyperplasia, low-grade dysplasia and high-grade dysplasia lesions were shown in Supplementary Fig. S6.

Discussion

This study showed that the 7p11.2 amplification was the most common DNA copy number change in OSCC and provided cogent evidence that *EGFR* was the culprit gene within this amplicon, contributing to OSCC development. First, we identified the amplicon in around 30% of OSCC tissues in a genome-wide study. This amplicon was further validated by FISH analysis, a more sensitive and specific method of assessing gene copy number, on a larger scale using paraffin-embedded oral cancer tissues (*n* = 128). Second, we verified the presence of the key "cancer-driving" gene, *EGFR*, in the defined minimal amplified region, which showed consistent biological relevance from genomic copy alterations to

mRNA expression, protein expression levels, and clinical association. Third, we compared genetic contents and expression levels of all erbB members in the same OSCC samples and concluded that EGFR plays an important role in oral cancer development. Activation of EGFR downstream targets was also found to occur by genomic amplification or gene mutation. Fourth, cell-based studies confirmed the requirement of EGFR signaling for survival of cells with the EGFR amplification, but not for survival of cells that lacked the amplicon. Finally, using a mouse model, which mimics the etiology of OSCC in Asia, we showed the efficacy of anti-EGFR therapy for oral cancer.

Amplification of 7p11.2 is frequently found in many cancer types, including lung cancer, breast cancer, and glioblastomas (27–29). By overlapping the amplified regions among different samples, we further verified that the minimal amplicon contained only *EGFR* and *SEC61G*. EGFR overexpression in OSCC has been suggested as a prognostic marker for shortened survival and metastatic spread (20–22). Similar clinical patterns have also been found based on our clinical association studies (Fig. 3*C*). SEC61G is a subunit of the heteromeric SEC61 complex, which forms the core of the mammalian endoplasmic reticulum (ER) translocon, a transmembrane channel for translocation of proteins across ER membrane (30). However, the role SEC61G plays in cancer development is
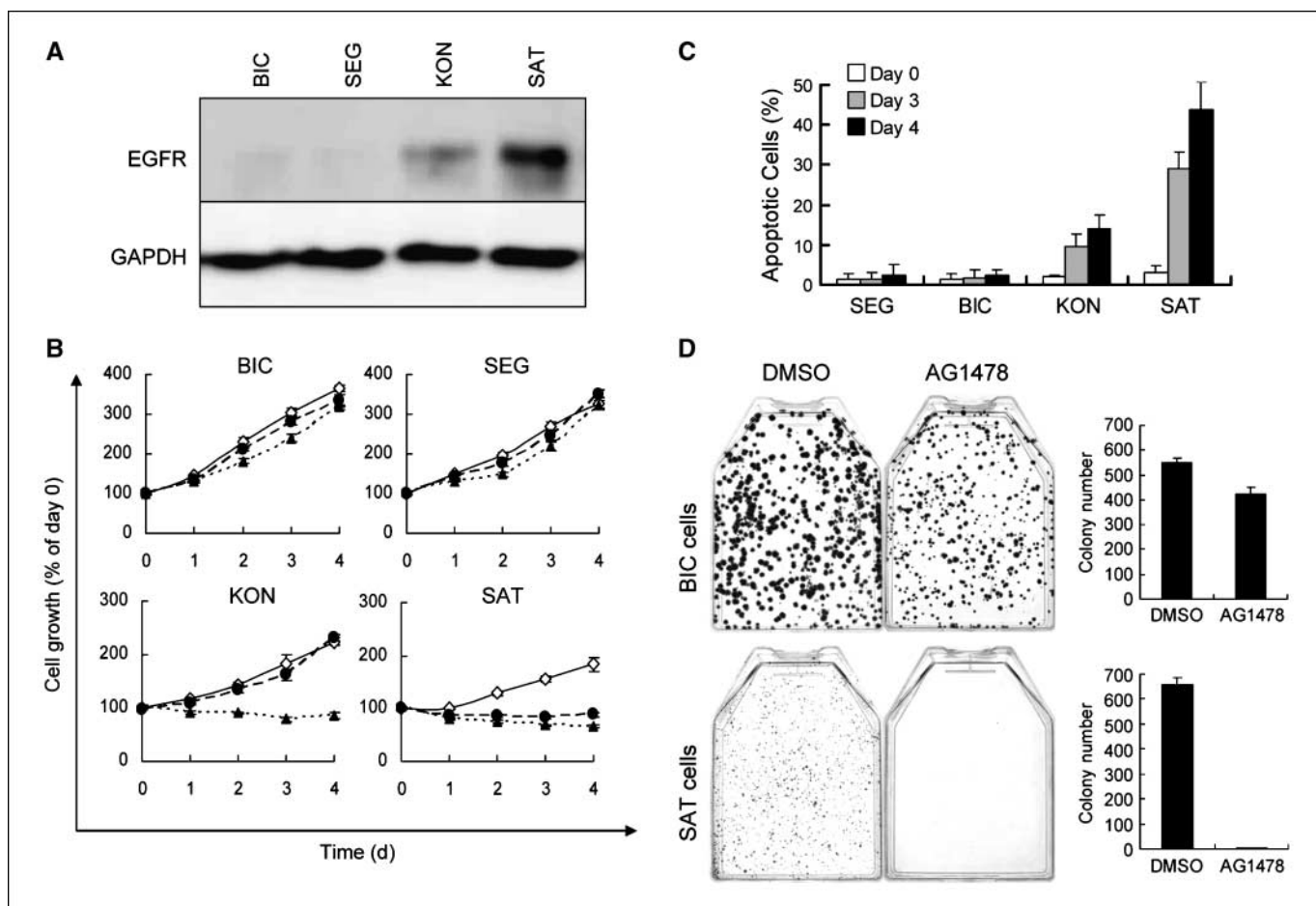

Figure 5. Biofunctional effects of EGFR inactivation on different head and neck cancer cell lines. *A*, Western blot indicates the protein level of EGFR in each cell line. *B*, cell lines were treated with 10 μmol/L (●) or 20 μmol/L (▲) AG1478 to inhibit EGFR function and cell growth was monitored for 4 d. Equal amount of DMSO was used as control (□). *C*, apoptosis was measured on AG1478-treated cells by Annexin V staining on days 3 and 4 using day 0 as controls. *D*, colony formation assays were performed to detect the inhibitory effects of AG1478 on BIC (low EGFR level) and SAT cells (high EGFR level).
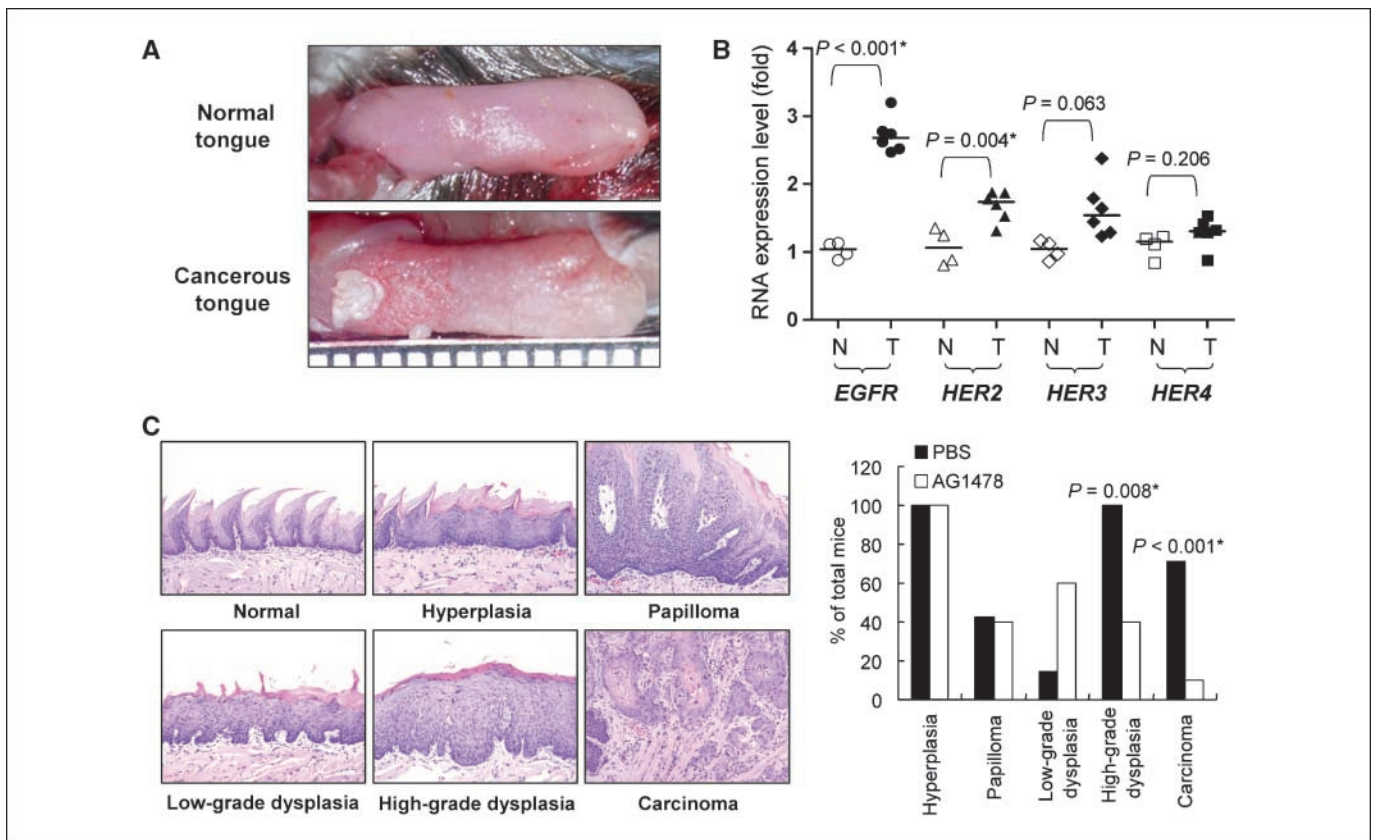
Figure 6. Validation of anti-EGFR therapy in a mouse oral cancer model. *A,* oral cancer lesions were induced on the tongues of C57BL/6JNarl mice by treatment with arecoline plus 4-NQO. *B,* gene expression levels of all erbB members in mouse oral cancer tissues were measured by quantitative real-time PCR. *C,* cancer development was observed in mice treated with arecoline plus 4-NQO. Tumor lesions were classified into epithelial hyperplasia, papilloma, low-grade dysplasia, high-grade dysplasia, and invasive carcinoma by H&E staining. Tumor staging of the tumor tissues from the PBS-treated group was compared with the AG1478-treated group.

poorly understood. Based on correlation studies between gene copy number changes and mRNA expression levels, our results suggest that SEC61G is merely a passenger gene for oral cancer and that its amplification is the by-product of the frequent genetic changes that occur during cancer development.

In addition to gene amplification, activating mutation of genes in kinase signaling pathways is another common genetic event during cancer development. For example, mutation of either *KRAS* or *BRAF* occurs in 65% to 88% of low-grade serous ovarian cancer (31, 32). EGFR and its downstream effectors have diverse cellular functions, including cell proliferation, differentiation, motility, survival, and tissue development (33). RAS-RAF-MAPK cascades are particularly active when cancer cells overexpress EGFR (34). Schulze and colleagues have further shown that the majority of RAS-RAF-MAPK–induced changes in gene expression are dependent on the status of *EGFR* (35), highlighting the critical roles of signal networking among different oncogenes in cancers (36). In our study, *EGFR, KRAS,* and *MAPK1* genes were found amplified in OSCC patients with frequencies of 31%, 3.4%, and 13.8%, respectively. Although *KRAS* amplification is a rare event in OSCC development, mutations at codon 61 were defined in 2 of the 29 OSCC samples (6.9%). Previous studies have shown that mutations in *KRAS, BRAF*, and *MAPK1* are usually mutually exclusive (31, 37). Our study on genetic alterations in the KRAS-BRAF-MAPK1 cascade also supports such conclusion (Fig. 4). Notably, coamplification of *EGFR* and *CCND1* was found in

three OSCC samples (10.3%; Fig. 4C). Because there are other downstream targets for EGFR and other upstream regulators for CCND1, these results may have important therapeutic implications for oral cancer patients.

Previous studies have shown that overexpression of other members in the erbB family, such as HER-2 and HER-3, is also prognostic of OSCC development (26, 38, 39), although the clinical significance of these molecules remains controversial (40, 41). In this study, we provide molecular evidence of EGFR activation in OSCC based on studies of both DNA copy number and mRNA expression levels in the same tumors. Our data reveal that the average mRNA level of *HER-2* in OSCC is higher than in normal tissues; however, few genomic alterations in *HER-2* could be identified. In support of our data, similar findings suggesting that there are no significant differences between the clinical associations with *HER-2*–amplified tumors and nonamplified tumors have also been reported (20, 42). This discrepancy in HER-2 associations might be due to different environmental factors that patients were exposed to. Recently, complications of chewing betel quid in oral cancer development have been found clinically meaningful and important. For example, *EGFR* amplification was reported to be associated with heavy betel quid users (43). Taken together, our data from human and mouse tumor tissues suggest that EGFR is the most important member of the erbB family for EGF signaling in OSCC, at least for oral cancer patients in Asia. Target-based therapies are widely accepted as the future of cancer treatment. Detection of

Cancer Research

EGFR status in oral cancer patients may identify patients who will benefit from anti-erb and/or anti-MAPK treatment(s).

The functions of *HER-4* in cancer development are still uncertain (44). Some previous studies have shown that overexpression of *HER-4* in breast cancer is associated with a well-differentiated phenotype and a better clinical outcome (45–47). A recent study reports that HER-4 functions as a proapoptotic protein, suppressing tumor growth and inducing cell death in breast carcinomas (48). In this current study, the gene copy number and expression levels of HER-4 in OSCC were found lower than in normal tissues. Therefore, it will be interesting to determine if HER-4 has similar tumor-suppressing roles in OSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Functional Genomic Analysis Identified Epidermal Growth Factor Receptor Activation as the Most Common Genetic Event in Oral Squamous Cell Carcinoma

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