Common and Complex Notch1 Mutations in Chinese Oral Squamous Cell Carcinoma

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Abstract

Purpose: To determine Notch1 mutation status in oral squamous cell carcinoma (OSCC) from Chinese population and its potential clinical implications.

Experimental Design: Surgically resected OSCC tissues from 51 Chinese patients and 13 head and neck squamous cell carcinoma (HNSCC) cell lines were sequenced for mutations in the entire coding regions of Notch1 and TP53 using a next-generation sequencing platform. Sequences of the genes were also determined in corresponding normal tissues from 46 of the 51 patients. Mutations and their association with clinical parameters were analyzed.

Results: Six mutations in Notch1 and 11 mutations in TP53 coding regions were detected in 4 (31%) and 10 (77%) of the 13 HNSCC cell lines, respectively. Forty-two somatic Notch1 mutations, including 7 nonsense mutations and 11 mutations within the domain commonly harboring potential activating mutations in acute lymphoblastic leukemia, were detected in 22 (43%) of the 51 Chinese OSCC tumors. In comparison, 25 somatic TP53 mutations were observed in 21 (41%) of the 51 tumors. Patients whose tumors carried Notch1 mutation had significantly shorter overall and disease-free survivals (P = 0.004 and P = 0.001, respectively, by log-rank test) compared with those whose tumors carried no Notch1 mutation. Multivariate analysis showed that both Notch1 mutation and lymph node metastasis are independent prognostic factors in the patient population (P = 0.001). All 15 patients with both Notch1 mutation and nodal metastasis recurred or metastasized within 2 years after surgery.

Conclusions: Notch1 mutation is common in Chinese OSCC and associates with clinical outcomes. The complexity of the mutation spectrum warrants further investigation of Notch1 in Chinese patients with OSCC.

Clin Cancer Res; 20(3); 701–10. ©2013 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is among the most common cancers with 650,000 new cases and 350,000 cancer deaths worldwide each year (1). Oral squamous cell carcinoma (OSCC) accounts for more than 50% of all HNSCC. Higher OSCC incidences have been reported in Melanesia, South-Central Asia, and Central/Eastern Europe, whereas the incidences in Africa, Central America, and Eastern Asia are lower. The differences in OSCC incidence at different geographic locations and ethnic backgrounds may reflect to diverse etiologic factors and inherited genetic background (2).

Notch signaling is an evolutionally conserved pathway determining the cell fate and controlling cell proliferation, differentiation, and apoptosis (3). However, the role of Notch1 in cancer is complex and cell-context dependent. Although Notch1 has been suggested to play an oncogenic role in breast, colorectal, and pancreatic cancers, potential activating mutations have only been reported in lung cancers (4–11). Two research teams recently performed whole-exome sequencing of HNSCC and revealed an unexpectedly high rates of somatic Notch1 mutations (11%–15%) in the Caucasian populations (12, 13). Because the mutations reported in these studies are either leading to potential protein inactivation or located at domains impacting ligand binding, Notch1 is considered as a tumor suppressor in HNSCC (12, 13).

In this study, we sequenced the entire coding regions of Notch1 and TP53 genes in OSCC tissues from a Chinese patient cohort. We compared mutations with those reported in the Caucasian patients with OSCC and analyzed...
Translational Relevance

Notch signaling plays an important role in tumorigenesis. Recently, high frequencies of potentially loss-of-function mutations in Notch1 were observed in head and neck squamous cell carcinoma (HNSCC) of Caucasian populations. However, the Notch1 mutation status in HNSCC of Asian population is unknown. The relationship between the mutation status and clinical parameters in the patient populations is also unknown. In this study, we reported a high frequency (43%) of Notch1 mutation, including mutations within the domain commonly harboring activating mutations in acute lymphoblastic leukemia, in oral squamous cell carcinoma (OSCC) of Chinese patients, which is significantly higher than the frequencies (<20%) reported in Caucasian populations. We further observed a significant association between Notch1 mutation and shorter survival times in patients with OSCC. The results suggest an important role of Notch1 mutations in oral tumorigenesis and potential implications of these mutations as biomarkers and interventional targets.

Patients and Materials

Patients and cell lines

OSCC tissues were derived from surgically resected samples of 51 patients treated in the Department of Oral and Maxillofacial Surgery, Shanghai jiao Tong University School of Medicine (Shanghai, China) in compliance with and approved by the Institutional Review Board. All the patients received only surgical treatment before the samples were collected and followed-up from 3 months to 10.2 years with a median of 33 months. Tissues were formalin-fixed and paraffin-embedded (FFPE). Matched noncancerous tissues were available for the genetic analysis in 46 of the 51 patients from neck dissection samples during the same surgical procedure and were processed in the same way as the OSCC tissues. Thirteen HNSCC cell lines (WSU-HN4, WSU-HN6, WSU-HN12, WSU-HN13, SCC4, SCC9, UMSCC14B, UMSCC22A, UMSCC22B, UMSCC30, UMSCC38, MDA1186, and SqCC/Y1) derived from Caucasian were also used. WSU-HN4, WSU-HN6, WSU-HN12, and WSU-HN13 cells were obtained from Dr. Silvio Gutkind of NIH (Bethesda, MD) as described previously (14, 15). SCC4 and SCC9 were from the American Type Culture Collection. UMSCC14B, UMSCC22A, UMSCC22B, UMSCC30, UMSCC38, MDA1186, and SqCC/Y1 were obtained from Dr. Tom Carey of University of Michigan (Ann Arbor, MI) as described previously (16). All these cell lines were authenticated at October 7, 2013 by short tandem repeat testing done in the Fragment Analysis Facility, Johns Hopkins University School of Medicine (Baltimore, MD). Nine of the 13 cell lines were 100% matched with the current database (17). The four remaining cell lines (WSU-HN4, WSU-HN6, WSU-HN12, and WSU-HN13) showed distinct genotypes not matching with any in the databases. All the lines were recovered and maintained in Dulbecco’s Modified Eagle Medium containing 10% FBS.

DNA extraction

Five 10 μm FFPE tissue sections from each tissue block were deparaffinized and DNA was extracted using Biostic FFPE Tissue DNA Isolation Kit according to the manufacturer’s instructions (MO-BIO Laboratories). Sections immediately adjacent to the sections used for DNA extraction were stained with hematoxylin and eosin for histology examination. The tumor sections containing more than 50% cancer cells and noncancerous tissue sections without cancer cells were used for sequencing analysis (12). Genomic DNA from 14 HNSCC cell lines was extracted using QiAamp DNA Mini kit (Qiagen) according to the manufacturer’s instruction. Extracted DNA was quantified using Qubitds DNA Assay Kit according to the manufacturer’s instruction (Life Technologies).

PCR amplification

Primers were designed to cover the entire coding regions and intron–exon boundaries of TP53 and Notch1 based on the human reference genome (Human genome build hg19) from NCBI (Supplementary Table S1). The preparation and PCR setup were performed in a laminar flow biosafety hood inside a clean room. The PCR plate with DNA samples was sealed before taking out of the clean room. PCR was carried out in a volume of 25 μL containing 50 ng DNA, 5 pmol each of sense and antisense primers, and AmpliTaq Gold master mix (Life Technologies). PCR amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems) in a separate room dedicated to PCR. DNA was amplified in the following conditions: 95°C for 10 minutes followed by 20 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, followed by 7 minutes extension at 72°C.

Library preparation and single-molecule DNA sequencing

PCR products from each sample were pooled into one tube and purified using 0.4 × volume of Agencourt magnetic beads (Beckman) and a magnetic rack (Invitrogen) to remove genomic DNA. The supernatant containing the amplified DNA products were further purified by adding 1.4 × of Agencourt and recovering the DNA on the magnetic beads using the magnetic rack. The Agencourt beads were washed twice with 0.5 mL of 70% ethanol, air dried for 5 minutes, and resuspended with 20 to 30 μL of Tris-EDTA buffer followed by incubation at room temperature for 5 minutes. DNA was recovered in the supernatant using the magnetic rack, fragmented with ShearEnzyme (Ion Torrent) for 30 minutes. The sheared DNA was purified and recovered using 1.8 × volume of Agencourt as described previously in this section. DNA products from the PCR were treated with end-repairing enzyme (Ion Torrent) according...
to the manufacturer’s instruction. The PCR products were further purified and recovered with 1.8 × volume of Agencourt beads as previously described. The PCR products from each tissue were then ligated with a specific barcode adapter sequence using ligase and buffer (Ion Torrent) according to the manufacturer’s instruction to form libraries. Different libraries were then combined in one tube and underwent Nick translation followed by two cycles of PCR amplification using the Platinum High Fidelity DNA polymerase (Life Technologies). Approximately 70 million DNA molecules in 1 mL of PCR solution were used to mix with a fixed ratio (0.5–1.0) of Ion sphere particles (ISP; Ion Torrent) in the presence of PCR reaction mix and oil (Ion Torrent) to form tens of millions of droplets of emulsion particles. These droplets passed through an enclosed capillary PCR plate in OneTouch (Ion Torrent), which performed emulsion PCR amplification as the liquid and particles pass through the plate continuously. The ISPs were recovered by centrifugation for 3 minutes in OneTouch in a pair of collection tubes to remove most supernatant from the top but 50 µL at the bottom. The ISPs were washed once in 1 mL wash buffer and centrifuged for 3 minutes at 15,500 × g to remove most supernatant and leave 100 µL volume solution with ISPs at the bottom. ISPs containing amplified DNA were further enriched from ISPs without DNA by incubating with Dynabeads MyOne Streptavidin C1 magnetic beads at room temperature for 10 minutes in a rotating rack. The enriched ISPs were recovered by placing the tube on magnetic rack for 2 minutes and washed twice with 0.2 mL of wash buffer by pipetting and placing on magnetic rack for 2 minutes and discarding the supernatant. The ISPs were eluted from the Dynabeads MyOne Streptavidin C1 magnetic beads by incubation with 0.4 mL 0.125N NaOH and 0.1% Tween 20 for 7 minutes at room temperature in a rotating rack. The eluted ISPs were washed in 0.4 mL of wash buffer and centrifuged for 4 minutes at 15,500 × g to remove most supernatant from the top and leave 100 µL at the bottom. The ISPs were washed again in 1 mL of wash buffer and centrifuged for 4 minutes at 15,500 × g to remove most supernatant from the top and leave 100 µL at the bottom. The ISPs were resuspended by pipetting and placed on magnetic rack for 2 minutes to remove last traces of Dynabeads MyOne beads. The 100 µL of solution was transferred to a new tube as the final library of ISPs ready for quality control testing and sequencing. For sequencing, the ISPs were centrifuged for 3 minutes at 15,500 × g to remove most supernatant from the top and leave 20 µL at the bottom. The ISPs were resuspended by pipetting and transferred to a 0.2 mL PCR tube containing 150 µL annealing buffer. Five microliter Control Ion Spheres (Ion Torrent) was added to the ISPs mix and centrifuged for 3 minutes at 15,500 × g to remove most supernatant from the top to leave 15 µL at the bottom followed by adding 12 µL sequencing primer, denatured, and annealed at 95°C for 2 minutes, and 2 minutes at 37°C. After adding 3 µL DNA polymerase (Ion Torrent) before loading the sample into a semiconductor sequencing chip 316 (Ion Torrent). DNA sequencing was performed on a Personal Genome Machine (Ion Torrent; ref. 18).

Analysis of sequences and mutation calling

DNA sequences in the exon regions and intron–exon boundaries of Notch1 and TP53 were analyzed against hg19 reference sequences using VariantCaller 2.2 software from Ion Torrent on the Ion Server. A nucleotide variant was called if the variant occurred >14 times in a tumor tissue or >8 times in a normal tissue with a P < 10−7 as previously described (19). The raw read data were then manually verified. The mean coverage achieved was 968 times in the tumor tissues and 483 times in the normal tissues.

Statistical analysis

Time to death (overall survival; OS) and time to recurrence (disease-free survival; DFS) were defined as time from surgery to event of interest. All patients alive or free of disease at last follow-up date were considered right censored. Overall or DFS was estimated by the Kaplan–Meier method. The χ2 or Fisher exact tests were used to analyze the association between Notch1 or TP53 mutation with categorical clinical variables. A Cox proportional hazards model was used to identify independent predictors of survival. Statistical analyses were performed with SPSS version 12.0 (SPSS, Inc.). All tests were two sided, and P < 0.05 was considered to indicate statistical significance.

Results

Notch1 and TP53 mutations in HNSCC cell lines and Chinese patients with OSCC

We first examined the entire coding regions and intron–exon boundaries of Notch1 and TP53 genes in 13 HNSCC cell lines from 11 Caucasian patients, including 9 (70%) patients with OSCC. Six nucleotide substitutions in Notch1 gene in 4 (31%) and 11 substitutions in TP53 gene in 10 (77%) of the cell lines were detected (Table 1). Four (66%) of the 6 nucleotide substitutions in Notch1 gene were located at EGF repeat domain (amino acids 20 to 1426) including a nonsense mutation, C931 ×, at the Abruptex region (amino acids 907 to 1143). One mutation was at heterodimerization domain (amino acids 1570 to 1734) and the other at transcriptional activation domain (TAD, amino acids 2155 to 2374; Table 2). All the substitutions were likely nonsynonymous mutations because they would either result in amino acid changes or create stop codons.

In 51 OSCC tumors from the Chinese patients, 156 Notch1 nucleotide substitutions were identified. By analyzing matched normal tissues and reference sequences, 12 (8%) of the substitutions were considered insertion or deletion (INDEL) single-nucleotide polymorphism (SNP) in noncoding areas and 102 (65%) were synonymous variants. The remaining 42 (27%) were nonsynonymous somatic mutations in 22 (43%) of the tumors. The locations of the nonsynonymous mutations in Notch1 functional domains were shown in Fig. 1A and Table 2. More detailed information, such as the allele coverage and the numbers of mutant alleles detected for each mutation, were provided in Supplementary Table S2. The most common substitutions were C > T (40%) followed by G > A (24%) and A > G (21%) transitions with only three (7%) G > T transversions.
Seven (17%) of the mutations were nonsense mutations and 35 (83%) were missense mutations. EGF repeats domain (excluding Abruptex region) was one of the most common regions with mutations (12 or 29%), which resembled previously reported Notch1 mutations in HNSCC (Fig. 1B), whereas 12 (29%) of the mutations from 8 (36%) of the 22 tumors locate at Notch1 heterodimerization domain (Table 2) where most of the activating mutations were
reported in hematologic malignancies (Fig. 1C). Abruptex region, which was also located at the EGF repeat domain, contains the most mutations (13 or 31%). There were several mutation “hotspots,” including seven mutations at codon 1641 (P > L/S) in five (10%) of the 51 tumors, three mutations at codon 1713 (N > S) in three tumors, three mutations at codon 1133 (C > Y) in three tumors, and two mutations at codons 1107 and 1474 both resulted in a stop

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Abbreviations: LNR, Lin/NOTCH repeats (amino acids 1459 to 1574); HD, heterodimerization domain; RAM, RBP-JK–associated molecule region (amino acids 1757 to 1865).
Only two of these nonsynonymous mutations were previously reported (Supplementary Table S3). Corresponding normal tissues were not available for five tumors, including the tumor (SCC9) carried 10 nucleotide variations. For TP53, a total of 67 nucleotide substitutions were identified, including 41 (61%) SNPs (15 INDEL SNPs in the noncoding areas and 26 at codon 72 leading to P>R substitution). There were 26 somatic mutations including 25 nonsynonymous mutations from 21 (41%) of the tumors and 1 synonymous mutation. Among the 25 nonsynonymous mutations, 21 were missense and four were nonsense (Table 1 and Supplementary Table S4). The most common somatic mutations were G>A (28%) and C>T (28%) transitions followed by C>G (20%) transversions (Supplementary Table S4). Nineteen (83%) of the 23 distinctive nonsynonymous mutations were reported previously (Supplementary Table S5).

**Associations between Notch1 mutation status and clinical/pathologic parameters as well as treatment outcomes**

We analyzed associations between the mutation status of the genes and clinical/pathologic parameters. No significant association was observed between Notch1 mutation status and any of the clinical/pathologic parameters except a marginal significant difference between TP53 mutation and older age (Table 3). There was also no association between Notch1 and TP53 mutations in these tumors (Table 3).

Among the 51 patients with OSCC patients, 22 died during the follow-up period, including 18 died of OSCC and the remaining 4 patients died of second primary cancer, apnea, gastrointestinal hemorrhage, or cerebral hemorrhage, respectively. In univariate analysis, Notch1 mutation and lymph node metastasis were found to associate with the DFS of the patient population (P = 0.002 and P < 0.001, respectively; Table 4). Patients whose tumors carried Notch1 mutation had significantly shorter OS and DFS than those whose tumors carried no Notch1 mutation (Fig. 2A and B; P = 0.0042 and P = 0.001 by the log-rank test, respectively).

To determine whether the association between Notch1 mutation status and survivals was independent of other parameters, we used multivariate analysis to include lymph node metastasis as a cofactor. Both Notch1 mutation and lymph node metastasis were independently associated with DFS (P < 0.001 for both factors; Table 3). Patients who had
lymph node metastasis with tumors carried Notch1 mutation showed worst OS and DFS, whereas those who had no lymph node metastasis and no Notch1 mutation in the tumors showed the best survivals (Fig. 2C and 2D; \( P = 0.0001 \) and \( P < 0.0001 \), respectively, by log-rank test). All 15 (100%) patients with lymph node metastasis and Notch1 mutation in the tumors had tumor relapse or metastasis after curative treatment and 14 (93%) of the patients died.
during the follow-up compared with only 2 (15%) of the 13 patients with no lymph node metastasis and no Notch1 mutation did so (Fig. 2C and 2D).

Discussion

In this study, we examined Notch1 mutation status in OSCC from Chinese patients and observed a mutation rate (43%) as high as the mutation rate of TP53 (41%) tumor suppressor gene in the patient population. This result suggested that Notch1 plays a critical role in oral tumorigenesis of both Chinese and Caucasian populations (12, 13). Although the causes for the high mutation rate in the Chinese population remain to be determined, differences in the genetic background of ethnicity and etiologic factors of OSCC should be considered. It has been well documented that the mutation rates of EGFR receptor (EGFR) are highly variable in non–small cell lung cancer (NSCLC) among different ethnic populations. In Asians, 30% to 60% NSCLC tumors carry EGFR mutation compared with only 10% to 15% in Caucasian population (20, 21), which is clinically important because tumors with EGFR mutations are particularly sensitive to EGFR tyrosine kinase inhibitors. The mechanisms behind the differences of EGFR mutation rates among different ethnicities remain to be determined.

For Notch1, the exposure to different carcinogens and the inherited genomic background may contribute to the difference between Asian and Caucasian. Because exposure to tobacco and alcohol was associated with a high frequency of TP53 mutations in HNSCC (22), it will be interesting to determine its potential impact on Notch1 mutations. Notably, high-concentration liquor (>50% alcohol) is traditionally consumed in Chinese population compared with predominantly wine or beer in Caucasian. It remains unclear whether this cultural difference plays a role in Notch1 mutation spectrums. However, we cannot rule out the possibility that the high cytosine and guanine (CG) content in human Notch1 coding regions (64%) may impact the probability to detect certain mutations when different sequencing methods are used (18, 23). We noted that there were more C>T transitions in Notch1 (40%) than in TP53 (28%). Because C>T transition was associated with tobacco and alcohol consumption and mainly located in CpG islands (24), the higher incidence of C>T transition in Notch1 might be a result of the exposure to these risk factors and the high CG contents in Notch1 (65% in coding region). It should be noted that normal tissues were not available in five cases, making it difficult to rule out potential germline polymorphisms in these cases. One of these tumors (SCC9) exhibited 10 nucleotide variations including a nonsense

![Figure 2. Kaplan–Meier curves for Notch1 mutation status and for cervical lymph node metastasis (CM) status and patients’ survivals. A, patients whose tumors were with or without Notch1 mutation and OS. B, patients whose tumors were with or without Notch1 mutation and DFS. C, patients whose tumors were with or without Notch1 mutation as well as with or without CM and OS. D, patients whose tumors were with or without Notch1 mutation as well as with or without CM and DFS.](image-url)
What interesting is the significantly enhanced value by using an independent prognostic factor in the patient population. It is not surprising that cervical lymph node metastasis is another independent of other clinical and pathologic factors. It is likely negatively impact the ligand–receptor interaction (25–27). Another common domain with mutations is heterodimerization domain (12 or 29%) including two "hotspots" (P1641S in four tumors and N1713S in three tumors; Supplementary Table S5). Heterodimerization domain is one of the two regions where mutations are commonly observed in leukemia patients, including P1641S, with a predicted ligand-independent gain-of-function phenotype (28, 29). 

Notch1 domain contains the most mutations (13 or 31%) including three nonsense mutations and a hotspot mutation (C1133Y in three tumors; Supplementary Table S5). Because Abruptex domain plays a role in suppressing cis-inhibition of Notch1 signaling (30, 31), mutations in this region are thought as gain-of-function mutations. Our results, therefore, suggest a more complex Notch1 mutation spectrum, including the existence of mutations within the domain commonly harboring potential activating mutations in acute lymphoblastic leukemia, in OSCC of the Chinese population than their Caucasian counterparts. In solid tumors, activating Notch1 mutations have been reported, mostly in NSCLC (11). It, however, should be noted that most of the "activating" mutations were insufficient to induce malignancies in the experimental model systems (32). Further studies are necessary to determine the roles of various mutations in different cancers.

Importantly, our study is the first to reveal a relationship between Notch1 mutation status and clinical outcomes in patients with OSCC. Our results indicate that Notch1 mutation is a poor prognostic factor for both OS and DFS in the Chinese patients with OSCC. Furthermore, the associations are independent of other clinical and pathologic factors. It is not surprising that cervical lymph node metastasis is another independent prognostic factor in the patient population. What interesting is the significantly enhanced value by using the two factors together to predict clinical outcome. If the results can be validated in larger and prospective studies, these factors may serve as powerful markers for Chinese patients with OSCC.

We recognize the complexity of Notch1 mutations identified in our study and acknowledge several limitations, which will require further investigations. First, the sample size is relatively small and therefore mutation frequencies reported here may be biased. This factor may also impact the analyses with the TP53 mutation status. Second, only one high-throughput sequencing platform was used for the mutation analysis. Given the low cancer cell contents in OSCC, another next-generation sequencing platform and independent cohorts should be used to validate the findings. In addition, the study cohort had incomplete social/behavior information, such as tobacco and alcohol consumption, which prevented us from analyzing certain associations among key factors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: X. Song, H. Ren, L. Mao
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Song, Z. Long, L. Mao
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Acknowledgments
The authors thank Yuping Mei of the University of Maryland School of Dentistry for primer design, and Yunqiong Wu, Zhiu Wang, and Weiming Chu of the Institute of Stomatology, Nanjing Medical University (Jiangsu, China), for sample processing.

Grant Support
This work was supported in part by R01 CA126818 and P30 CA134274 from National Cancer Institute, the National Natural Science Foundation of China (31271341) and the Shanghai Science and Technology Committee (11DJ2291800). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 16, 2013; revised November 7, 2013; accepted November 12, 2013; published OnlineFirst November 25, 2013.

References
7. Meng RD, Sheltton CC, Li YM, Qin LX, Notterman D, Paty PB, et al. gamma-Secretase inhibitors abrogate oxiplatin-induced activation


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