Published in final edited form as:

Clin Cancer Res. 2014 February 1; 20(3): 701–710. doi:10.1158/1078-0432.CCR-13-1050.

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 cancer (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 ALL, 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 a significantly shorter overall and disease-free survivals ($P=.004$ and $P=.001$, respectively, by log-rank test) compared to 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 ($P=.001$). 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.

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The authors indicated no potential conflicts of interest.

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. While 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 OSCC patients and analyzed potential association between the mutations and clinical outcomes in the patient population.

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 in compliance with and approved by the Institutional Review Board. All the patients received only surgical treatment before the samples being 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 non-cancerous 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, SqCC/Y1) derived from Caucasian were also used. WSU-HN4, WSU-HN6, WSU-HN12, WSU-HN13 cells were obtained from Dr. Silvio Gutkind of National Institute of Health as described previously (14, 15). SCC4 and SCC9 were from the American Type Culture Collection (ATCC). UMSCC14B, UMSCC22A, UMSCC22B, UMSCC30, UMSCC38, MDA1186, SqCC/Y1 were obtained from Dr. Tom Carey of University of Michigan as described previously (16). All these cell lines were authenticated at October 7, 2013 by short tandem repeats (STR) testing done in the Fragment Analysis Facility, Johns Hopkins University School of Medicine. Nine of the 13 cell lines were 100% matched with current database (17). The 4 remaining cell lines (WSU-HN4, WSU-HN6, WSU-HN12, WSU-HN13) showed distinct genotypes not matching with any in the databases. All the lines were recovered and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS.

DNA extraction

Five 10- μ m 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 (H&E) for histology examination. The tumor sections containing greater than 50% cancer cells and non-cancerous 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 (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 μ L 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 $\times$ 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 $\times$ 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 re-suspended with 20–30 μ L 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 $\times$ 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 $\times$ 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 2 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 μ L at the bottom. The ISPs were washed once in 1 mL wash buffer and centrifuged for 3 minutes at 15,500 $\times$ g to remove most supernatant and leave a 100 μ L 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 IPSs 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 0.125 N 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 100µL 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 100µL 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 100µL of solution was transferred to a new tube as the final library of ISPs ready for QC 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 20µL at the bottom.

The ISPs were resuspended by pipetting and transferred to a 0.2 mL PCR tube contain 150µL 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 153 L at the bottom followed by adding 12µL sequencing primer, denatured and annealed at 95°C for 2 minutes, and 2 minutes at 37°C. After adding 3µL 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 (PGM) (Ion Torrent) (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-value <10⁻⁷ as previously described (19). The raw read data were then manually verified. The mean coverage achieved was 968× in the tumor tissues and 483× in the normal tissues.

Statistical Analysis

Time to death (overall survival) and time to recurrence (disease-free survival) 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 disease-free survival was estimated by the Kaplan-Meier method. The χ^2 or Fisher's 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., Chicago, Illinois). All tests were two-sided, and a *P* value of less than 0.05 was considered to indicate statistical significance.

RESULTS

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

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 (HD, 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 non-synonymous 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 INDEL single nucleotide polymorphism (SNP) in non-coding areas and 102 (65%) were synonymous variants. The remaining 42 (27%) were non-synonymous somatic mutations in 22 (43%) of the tumors. The locations of the non-synonymous mutations in *Notch1* functional domains were shown in Figure 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 Table S2. The most common substitutions were C>T (40%) followed by G>A (24%) and A>G (21%) transitions with only 3 (7%) G>T transversions. Seven (17%) of the mutations were nonsense mutations and 35 (83%) were missense mutations. Epidermal growth factor (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* HD 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 “hot spots”, including 7 mutations at codon 1641 (P>L/S) in 5 (10%) of the 51 tumors, 3 mutations at codon 1713 (N>S) in 3 tumors, 3 mutations at codon 1133 (C>Y) in 3 tumors, and 2 mutations at codons 1107 and 1474 both resulted in a stop codon. Only 2 of these non-synonymous mutations were previously reported (Table S3). Corresponding normal tissues were not available for 5 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 non-coding areas and 26 at codon 72 leading to P>R substitution). There were 26 somatic mutations including 25 non-synonymous mutations from 21 (41%) of the tumors and 1 synonymous mutation. Among the 25 non-synonymous mutations, 21 were missense and 4 were nonsense (Table 1 and Table S4). The most common somatic mutations were G>A (28%) and C>T (28%) transitions followed by G>T (20%) transversions (Table S4). Nineteen (83%) of the 23 distinctive non-synonymous mutations were reported previously (Table S5).

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

We analyzed associations between the mutation status of the genes and clinical/pathological parameters. No significant association was observed between *Notch1* mutation status and any of the clinical/pathological 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 OSCC patients, 22 died during the follow-up period, including 18 died of OSCC and the rest 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 disease-free survival (DFS) of the patient population ($P=.002$ and $P<.001$, respectively; Table 4). Patients whose tumors carried *Notch1* mutation had significantly shorter overall survival (OS) and DFS than those whose tumors carried no *Notch1* mutation (Fig. 2A and 2B; $P=.0042$ and $P=.001$ by 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 co-factor. Both *Notch1* mutation and lymph node metastasis were independently associated with DFS ($P<.001$ 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; $P=.0001$ and $P<.0001$, 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 to 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 epidermal growth factor receptor (EGFR) are highly variable in non-small cell lung cancer (NSCLC) among different ethnic populations. In Asians, 30–60% NSCLC tumors carry *EGFR* mutation compared to only 10–15% 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. Since 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 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 there were more C>T transitions in *Notch1* (40%) than in *TP53* (28%). Since 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 GC contents in *Notch1* (65% in coding region). It should be noted that normal tissues were not available in 5 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 mutation (Q1733X) which is probably a somatic event. Five variants observed in the other 4 tumors are also possible somatic mutations based on the frequencies of the variant copies in the tumor tissues. Polyphen2 software predicted 4 of the 5 variants as “probably damaging”, a suggestion of functional involvement of the mutations.

Another key finding in our study is the spectrum of the *Notch1* mutations. One of the common domains with mutations is the epidermal growth factor (EGF) repeats (12 or 29%), which is consistent with previous reports (12, 13) for HNSCC although all but one (E424K) of the mutations at different codons (Tables S4). Nevertheless, these mutations are likely

negatively impact the ligand-receptor interaction (25–27). Another common domain with mutations is HD (12 or 29%) including 2 “hotspots” (P1641S in 4 tumors and N1713S in 3 tumors) (Table S5). HD 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). *Abruptex* domain contains the most mutations (13 or 31%) including 3 nonsense mutations and a hotspot mutation (C1133Y in 3 tumors) (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 OSCC patients. Furthermore, the associations are independent of other clinical and pathological 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. Additionally, the study cohort had incomplete social/behavior information, such as tobacco and alcohol consumption, which prevented us from analyzing certain associations among key factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This work is supported in part by P30 CA134274 from National Cancer Institute, the National Natural Science Foundation of China (31271341) and the Shanghai Science and Technology Committee (11DZ2291800).

We thank Yuping Mei from University of Maryland School of Dentistry for primer design, and Yunong Wu, Zilu Wang, Weiming Chu from Institute of Stomatology, Nanjing Medical University for sample processing.

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STATEMENT OF 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. It is also unknown the relationship between the mutation status and clinical parameters in the patient populations. 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 (ALL), 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 OSCC patients. The results suggest an important role of *Notch1* mutations in oral tumorigenesis and potential implications of these mutations as biomarkers and interventional targets.

Figure 1.

Distribution of *Notch1* mutations. **A**, *Notch1* mutations in primary OSCC of the Chinese population; **B**, Reported *Notch1* mutations in HNSCC of Caucasian populations; **C**, Reported “hotspot” gain-of-function *Notch1* mutations in hematopoietic malignancies. Arrows, missense mutations; ×, nonsense mutations; Red or purple marks represent newly identified mutations whereas blue marks represent mutations reported in literatures. EGF, epidermal growth factor; LNR, Lin/NOTCH repeats; HD, heterodimerization domain; RAM, RBP-JK-associated molecule region; ANK, ankyrin repeats; TAD, transactivation domain; PEST, sequence rich in proline, glutamic acid, serine, and threonine.

Figure 2. Kaplan-Meier curves for *Notch1* mutation status and or cervical lymph node metastasis (CM) status and patients' survivals. **A**, Patients whose tumors with or without *Notch1* mutation and overall survival (OS); **B**, Patients whose tumors with or without *Notch1* mutation and disease-free survival (DFS); **C**, Patients whose tumors with or without *Notch1* mutation as well as with or without CM and OS; **D**, Patients whose tumors with or without *Notch1* mutation as well as with or without CM and DFS.

Table 1

Notch1 and *TP53* mutation in cell lines and primary OSCC

Cell lines	<i>Notch1</i> mutation		<i>TP53</i> mutation
WSU-HN4	C478F	V2285I	
WSU-HN6			H179L
WSU-HN13	N623D		V173L
SCC4			P151S
SCC9	E848K		G279A
UMSCC14B			R280S
UMSCC22A	E1679×	C931×	Y220C
UMSCC22B			Y220C
UMSCC38			K132N
MDA1186			E258G
SqCC/Y1			C176S P142H
Primary	<i>Notch1</i> mutation		<i>TP53</i> mutation
SCC 1	C1397F	P1641L P1641S	G279E
SCC 2			E286K R175H
SCC 3			C242S
SCC 4	C1218Y		E294×
SCC 5	C1133Y		R282W
SCC 6			N235D
SCC 7			E2K
SCC 9	Q1733× R1762W N1347D M1737I	R203C T232I P1641L	R217W S1189F S1389I
SCC 10	P1641S		
SCC 11			Q317× V31I
SCC 13			E286G P152L
SCC 14	C1133Y	N1713S	R282W
SCC 15	C1133Y		P278S
SCC 19	E606×		C141Y
SCC 21	N1489D		V157F

Cell lines	<i>Notch1</i> mutation	<i>TP53</i> mutation
SCC 22	Q1134R	
SCC 23	Q1134R	
SCC 26	E1084D N1713S	
SCC 27		V274A Q104×
SCC 28	Q1134R	C135F
SCC 30		D281E
SCC 31	N994K P1641S	
SCC 32	C1074Y	Q331×
SCC 33	E424K	
SCC 35	Q1080× N1713S	
SCC 37		R248Q
SCC 38		C141G
SCC 42	R1107×	G266V
SCC 47	N548D P1641S	P1641L
SCC 48	R1107×	W1474×
SCC 50	E1084D	G266V
SCC 51	R1784Q	

Table 2

Notch1 mutation distribution in different domains

Cell line/Tumor	EGF	Abruptex	LNR	HD	TAD
WSU-HN4	C478F				V2285I
WSU-HN13	N623D				
SCC9	E848K				
UMSCC22A		C931×		E1679×	
SCC1	C1397F				
SCC1				P1641L	
SCC1				P1641S	
SCC4	C1218Y				
SCC5		C1133Y			
SCC9	R203C				
SCC9	R217W				
SCC9	T232I				
SCC9	S1189F				
SCC9	N1347D				
SCC9	S1389I				
SCC9				P1641L	
SCC9				Q1733×	
SCC9				M1737I	
SCC9					R1762W
SCC10				P1641S	
SCC14		C1133Y			
SCC14				N1713S	
SCC15		C1133Y			
SCC19	E606×				
SCC21			N1489D		
SCC22		Q1134R			
SCC23		Q1134R			
SCC26		E1084D			
SCC26				N1713S	

Cell line/Tumor	EGF	Abruptex	LNR	HD	TAD
SCC28		Q1134R			
SCC31		N994K			
SCC31				P1641S	
SCC32		C1074Y			
SCC33	E424K				
SCC35		Q1080×			
SCC35			W1474×		
SCC35				N1713S	
SCC42		R1107×			
SCC47	N548D				
SCC47	E606G				
SCC47				P1641L	
SCC47				P1641S	
SCC48		R1107×			
SCC48			W1474×		
SCC50		E1084D			
SCC51					R1784Q

EGF, epidermal growth factor; LNR, Lin/NOTCH repeats (amino acids 1459 to 1574); HD, heterodimerization domain; RAM, RBP-JK-associated molecule region (amino acids 1757 to 1865); TAD, transcriptional activation domain.

Table 3Correlation of *Notch1* or *TP53* mutation with clinical characteristics

Characteristic	Patients	<i>Notch1</i> mutation		<i>TP53</i> mutation		<i>P</i>
		+	-	+	-	
Age	51	22	29	21	30	
<55 yr	17	7	10	3	14	.051
55-64 yr	16	8	8	9	7	
>64 yr	18	7	11	9	9	
Sex						
Male	37	16	21	17	20	.261
Female	14	6	8	4	10	
Pathological TNM stage						
3	16	6	10	4	12	.172
4	34	15	19	17	17	
5	1	1	0	0	1	
T stage						
2	5	2	3	1	4	.19
3	12	4	8	3	9	
4	34	16	18	17	17	
Pathological grade						
I	42	18	24	16	26	.460
II	9	4	5	5	4	
Cervical node metastasis						
N+	31	15	16	12	19	.656
N-	20	7	13	9	11	
<i>TP53</i> mutation						
+	21	11	10			.265
-	30	11	19			

Table 4

Univariate and Multivariate Cox regression analysis of clinical characteristics or mutation with disease-free survival

Univariate	<i>P</i>	Risk Ratio	95% CI	
Age	0.316	0.798	0.514	1.240
Sex	0.892	1.058	0.473	2.366
T Stage	0.917	0.971	0.559	1.688
TNM stage	0.347	1.476	0.626	2.965
Grade	0.345	1.502	0.646	3.493
<i>TP53</i> mutation	0.941	0.973	0.476	1.990
<i>Notch1</i> mutation	0.002	3.132	1.508	6.504
Cervical Metastasis	0.001	4.552	1.824	11.361
Multivariate				
<i>Notch1</i> mutation	0.001	3.445	1.611	7.369
Cervical Metastasis	0.001	5.041	1.957	12.988