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Receptor tyrosine kinase amplification is predictive of distant metastasis in patients with oral squamous cell carcinoma

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Key words

Distant metastasis, gene amplification, next-generation sequencing, oral squamous cell carcinoma, receptor tyrosine kinase

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This study aimed to clarify the genomic factors associated with the diagnosis and prognosis of oral squamous cell carcinoma via next-generation sequencing. We evaluated data from 220 cases of oral squamous cell carcinoma. Genomic DNA was eluted using formalin-fixed, paraffin-embedded samples, and targeted resequencing of 50 cancer-related genes was performed. In total, 311 somatic mutations were detected in 220 patients, consisting of 68 synonymous mutations and 243 non-synonymous mutations. Genes carrying mutations included *TP53*, *CDKN2A*, and *PIK3CA* in 79 (35.9%), 35 (15.9%), and 19 patients (8.6%), respectively. Copy number analysis detected amplification of *PIK3CA* and *AKT1* in 38 (17.3%) and 11 patients (5.0%), respectively. Amplification of receptor tyrosine kinases was found in 37 patients (16.8%). Distant metastasis was noted in nine of 37 patients (24%) with receptor tyrosine kinase amplification, accounting for 43% of the 21 cases of distant metastasis. The cumulative 5-year survival rate was 64.6% in the receptor tyrosine kinase amplification group *vs* 85.2% in the no receptor tyrosine kinase amplification group. Moreover, we identified significantly poorer prognosis in the *TP53* mutation/receptor tyrosine kinase amplification group, for which the cumulative 5-year survival rate was 41.6%. In conclusion, the results of this study demonstrated that receptor tyrosine kinase amplification is a prognostic factor for distant metastasis of oral squamous cell carcinoma, indicating the necessity of using next-generation sequencing in clinical sequencing.

It has become possible in recent cancer genome research to analyze a large volume of genomic data from a human sample due to remarkable technological innovations including next-generation sequencing (NGS). Large-scale cancer genome projects, such as The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium, provided an overview of genomic alterations in many cancers. Such data accumulation in cancer genome analyses and development of molecular-targeted drugs are fueling a shift in cancer treatment from conventional therapies selected on the basis of the organ of origin, histologic type, and stage of cancers to therapies selected according to the presence of gene mutations.

Annually, approximately 300 000 people worldwide develop oral cancer.[1] The majority of oral malignancies arise from epithelial tissue, and squamous cell carcinoma is the predominant tumor type.[2] Despite advances in diagnostic technology and therapeutic techniques, the survival rate of oral squamous cell carcinoma (OSCC) has improved by only 5% in the past 20 years, and the 5-year survival rate of OSCC is 60%.[3] Comprehensive analyses of gene mutations in head and neck squamous cell carcinoma (HNSCC) including OSCC using NGS have also been conducted, revealing substantial information about genomic alterations in HNSCC.[4–8] However, there is little evidence for selecting stratified therapies based on genomic alterations. In addition, repositioning of drugs and agents used in other organs should be examined, as only one adaptive molecular-targeted drug, cetuximab, an anti-EGFR monoclonal antibody drug, is used in the treatment of HNSCC.

This study aimed to clarify the genomic factors associated with the diagnosis and prognosis of OSCC by integrating analyses of genetic alterations in OSCC and clinicopathological information via target resequencing using NGS.

Materials and Methods

Patients and genomic DNA samples. We investigated 220 patients who received a histological diagnosis of OSCC

between 2001 and 2015 at the Department of Oral and Maxillofacial Surgery, Tokyo Medical and Dental University. Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissue from patients diagnosed with OSCC. Clinicopathological information was obtained from medical charts. The median follow-up period was 44 months (range 0.5–165 months). This study was conducted in accordance with the Declaration of Helsinki, and approved by the ethics committee of Tokyo Medical and Dental University, Faculty of Dentistry (No. 1087).

DNA extraction. The tumor areas in FFPE tissues were marked and hand-dissected using macrodissection methods to ensure tumor tissue inclusion, and genomic DNA was extracted from FFPE tissues using a QIAamp DNA FFPE Tissue Kit (Qiagen, Venlo, The Netherlands). The purified DNA was quantified using a Qubit DNA high-sensitivity assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Library preparation and sequencing. Library preparation was performed using an Ion AmpliSeq Library Kit 2.0 and Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific). The panel target's hotspot regions included more than 2800 COSMIC mutations of 50 cancer-related genes (Table S1a and b). After library preparation, each amplicon library was quantified using an Agilent 2100 Bioanalyzer and Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced using an Ion Proton platform and Ion PI Chip (Thermo Fisher Scientific). The average read depths were approximately 1700.

Sequencing analysis. Data were analyzed using Torrent Suite Software v4.2.191 (Thermo Fisher Scientific) and Ion Reporter Software v4.6 (Thermo Fisher Scientific). The read alignments were performed using the human reference genome hg19. Detected variants with quality scores of <20 and allele frequencies of <4.0%[9] were eliminated. Further, we filtered out possible germline mutations using the databases of the 1000 genomes project (http://www.internationalgenome.org/) and 5000 exomes project (http://evs.gs.washington.edu/EVS/), as matched normal tissue samples were not analyzed in this study. Copy number analyses were performed using Biomedical Genomics Workbench 2.5 (Qiagen). The algorithm implemented is based on a CNA detection tool called COpy Number Targeted Resequencing Analysis (CONTRA), which includes a module for efficiently creating a pseudo-control from multiple samples.[10] Non-cancer samples derived from FFPE specimens of nine patients with oral leukoplakia were used to create pseudo-control data. Each target region copy number was calculated using a log2 ratio. We used $|2| \leq$ adjusted fold-change (log2) as the criterion for CNAs. More details about this method are given in the Supporting information (Doc. S1, Fig. S1a–b).

Statistical analysis. The associations between clinicopathological variables and gene aberrations were evaluated using Fisher's exact test or the chi-squared test. Overall survival (OS) was measured as the time interval between the first date of visiting our department and that of the last follow-up or death. Cox proportional hazard models were used to assess the univariate and multivariate prognostic significance of clinicopathological variables and tumor gene aberrations regarding OS. Survival curves were estimated according to the Kaplan–Meier method, and these differences were examined using the log-rank test. All analyses were performed using PASW Statistics, version 18 (SPSS Inc., Chicago, IL, USA). Statistical analyses related to somatic mutations were performed for non-synonymous mutations, excluding synonymous mutations.

Results

SMs and CNAs in patients with OSCC. The 220 subjects consisted of 135 males and 85 females with a mean age of 58.7 years. Their oral subsites of tumor development were the tongue in 123 patients, gum in 64 patients, buccal mucosa in 21 patients, floor of the mouth in 10 patients, and hard palate in two patients (Table S2). The cumulative 5-year survival rates of subsites were 81.8% in the tongue, 87.3% in the gum, and 68.8% in others (buccal mucosa, floor of the mouth, and hard palate) (Fig. S2a). Their tumor stages according to the UICC stage classification were stage I in 53 patients, stage II in 48 patients, stage III in 33 patients, and stage IV in 86 patients, with cumulative 5-year survival rates of 97.1, 93.3, 86.4, and 64.5%, respectively (Table S2, Fig. S2b).

Figure 1 presents the results for the detection of somatic mutations (SMs) and copy number alterations (CNAs). Regarding SMs, 311 mutations were detected in 220 patients, producing an average of 1.41 mutations (0–23 mutations) per patient. The 311 mutations consisted of 68 synonymous mutations and 243 non-synonymous mutations. The non-synonymous mutations consisted of 161 missense mutations (66.3%), 49 nonsense mutations (20.2%), nine frame shift deletions (3.7%), 16 frame shift insertions (6.6%), one in-frame shift deletion (0.4%), one in-frame shift insertion (0.4%), and six splice site mutations (2.5%). Genes detected to have a mutation included *TP53* in 79 patients (35.9%), *CDKN2A* in 35 patients (15.9%), *PIK3CA* in 19 patients (8.6%), *NOTCH1* in nine patients (4.1%), *HRAS* in four patients (1.8%), *RB1* in three patients (1.4%), *FBXW7* in one patient (0.5%), and *PTEN* in onde patient (0.5%). No SM was detected in *CSF1R*, *CTNNB1*, *EZH2*, *FGFR2*, *IDH1*, *JAK3*, *MPL*, *NPM1*, and *PTPN11*.

Copy number analysis uncovered deletions of tumor suppressor genes, namely *CDKN2A* in 25 patients (11.4%), *NOTCH1* in eight patients (3.6%), and *SMAD4* in three patients (1.4%). Amplification was observed for *PIK3CA* in 38 patients (17.3%), *AKT1* in 11 patients (5.0%), *HRAS* in eight patients (3.6%), and *BRAF* in six patients (2.7%). Amplification of receptor tyrosine kinases (RTKs) was found in 37 patients (16.8%). The amplified RTK genes included *EGFR* in 17 patients (7.7%), *ERBB2* in seven patients (3.2%), *FGFR1* in seven patients (3.2%), *FGFR3* in six patients (2.7%), *ERBB4* in three patients (1.4%), *MET* in three patients (1.4%), *FLT3* in two patients (0.9%), *KIT* in one patient (0.5%), and *PDGFRA* in one patient (0.5%). Amplification of *KIT* and *PDGFRA* occurred in the same patient, in whom amplification of *EGFR*, *ERBB2*, *FGFR3*, and *MET* was also detected. No amplification of *FGFR2* was detected.

The frequency of *PIK3CA* SMs was significantly higher in stages III/IV than in stages I/II ($P = 0.023$). Meanwhile, the frequency of *PIK3CA* amplification was significantly higher in stages I/II than in stages III/IV ($P = 0.007$). Examination of these frequencies separately for T- and N-factors used for stage classification revealed that the frequency of *PIK3CA* SMs was significantly higher for patients with T3/4 lesions ($P = 0.015$), whereas the frequency of *PIK3CA* amplification was significantly higher in patients with N0 lesions ($P = 0.028$) (Table 1). Regarding mutations of *TP53* and *CDKN2A*, no significant difference was detected between stages. Concerning *HRAS*, SMs were detected in four patients, and their stage was IV in all cases (Tables S3–S4). There was

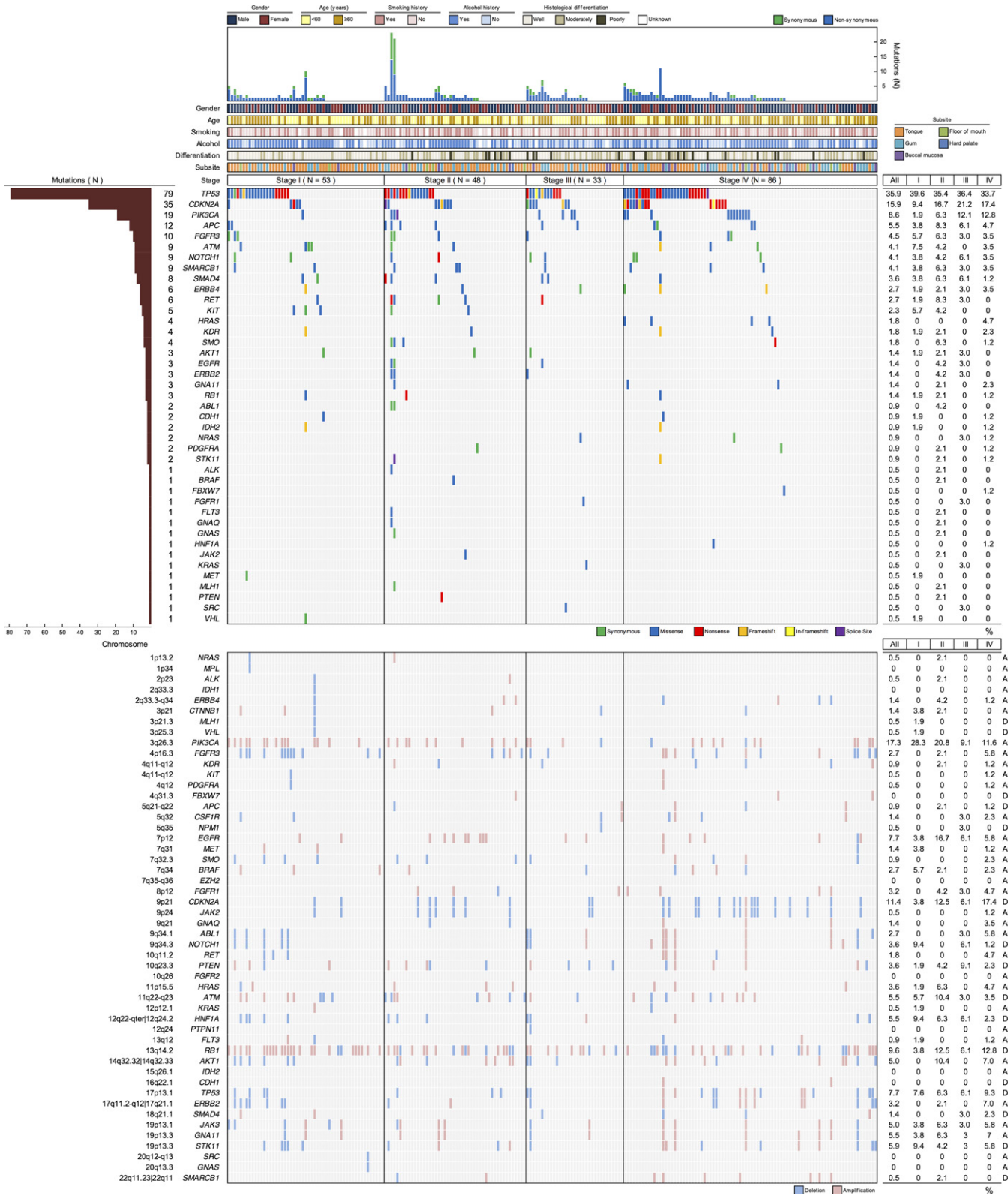

Fig. 1. Mutational landscape of oral squamous cell carcinoma in 220 patients. Patients were stratified into four subgroups according to stage. The figure shows mutation burdens, clinicopathological features, somatic mutations, and copy number alterations in order from the top panel. Concerning the percentage of copy number alterations, the figure shows the deletion rates for tumor suppressor genes and amplification rates for oncogenes. Amp., amplification; Del., deletion.

no relationship between the presence or absence of genetic alterations and age, gender, smoking history, alcohol history, subsite, or histological differentiation (Fig. S3 and Tables S3 and S4).

Somatic mutations and CNAs with higher frequencies among previously reported genetic alterations in OSCC were examined via gene classification (e.g., RTKs, PI3K pathway genes, tumor suppressor genes, and Ras/Raf pathway genes) (Fig. 2). Among RTKs, *CDKN2A*, and *PIK3CA*, SMs and CNAs exhibited a mutually exclusive trend. In addition, comparisons of each gene revealed that deletion of *CDKN2A* was exclusive with SMs of *TP53*, whereas amplification of *PIK3CA* was cooperative with SMs of *TP53*.

RTK amplification is predictive of distant metastasis in patients with OSCC. Distant metastasis was found in nine of 37 patients (24%) with RTK amplification. This accounted for 43% of the 21 cases of distant metastasis. Among the 220 patients, RTK amplification was detected in all three patients who were free of cervical lymph node metastasis (N0) but developed distant metastasis after therapy (Table 2). Moreover, in 21 patients who developed distant metastasis, the 10 patients who were clinically diagnosed with early-stage cancer and developed distant metastasis after primary therapy included four patients with RTK amplification and four patients with poorly differentiated histology (data not shown). In addition, no RTK amplification was detected in any of the five patients who developed distant metastasis and had poorly differentiated histology (Table 2).

Univariate and multivariate analyses according to clinicopathological factors and genes were conducted using the Cox proportional hazard model (Table 3). In univariate analysis for OS, a statistically significant difference was detected for RTK amplification (hazard ratio [HR] = 2.662, 95% confidence interval [CI] = 1.290–5.491, $P = 0.008$) and *CDKN2A* deletion (HR = 2.442, 95% CI = 1.059–5.634, $P = 0.036$). The cumulative 5-year survival rates were 64.6% (95% CI = 47.4–81.8) in the RTK amplification group (Fig. 3a) and 63.7% (95% CI = 41.6–85.8) in the *CDNK2A* deletion group (Fig. S4a). Regarding SMs of *TP53* with the highest frequency, no statistically significant difference was detected (Fig. S4b). In addition, no statistically significant difference was detected in CNAs and SMs of genes in the PI3K and/or Ras/Raf pathways. In multivariate analysis, we considered RTK and *CDKN2A* CNAs, and clinicopathological poor prognostic factors for OS, namely, age, subsite, histological differentiation, and clinical stage, which could be predictive before treatment. This analysis revealed that RTK amplification was an independent prognostic factor (HR = 2.410, 95% CI = 1.056–5.498, $P = 0.037$) (Table 3).

Although no statistically significant difference was detected in OS concerning the presence or absence of any SMs of *TP53*, comparing four groups according to the presence or absence of any *TP53* mutation or RTK amplification revealed significantly poorer prognosis in the *TP53* mutation/RTK amplification group (HR = 4.820, 95% CI = 1.869–12.43, $P = 0.001$) (Table 4). The cumulative 5-year survival rate of this group was 41.6% (95% CI = 10.9–72.2) (Fig. 3b). Additionally, similar comparisons among the four groups detected significantly poorer prognosis associated with the presence of RTK amplifications irrespective of *CDKN2A* deletion (no *CDKN2A* deletion/RTK amplification: HR = 2.626, 95% CI = 1.103–6.248, $P = 0.029$; *CDKN2A* deletion/RTK amplification: HR = 3.517, 95% CI = 1.196–10.34, $P = 0.022$) (Table 5). The cumulative 5-year survival rates of these two groups were 68.6 (95% CI = 48.6–88.6) and 56.3% (95% CI = 24.0–88.6), respectively (Fig. S4c). Examination of the cause of death in 25 cases in which *CDKN2A* deletion was detected revealed that two, one, two, and two patients died because of the primary lesion, cervical metastasis, distant metastasis, and another disease, respectively (data not shown).

Discussion

The clinical application of NGS, such as in the clinical trial NCI Molecular Analysis for Therapy Choice (NCI-MATCH), may provide insights into the genomic landscape of human cancers and identify therapeutic targets for molecular targeted agents.[11] In this trial, only the malignant tissue will be screened.[12] In our hospital, collecting sufficient matched normal oral tissue from patients with OSCC was challenging. This is a very common issue encountered in clinical contexts; therefore, we filtered out possible germline mutations using the databases of the 1000 Genomes Project and 5000 Exomes Project to identify SMs in the absence of matched normal controls. Recently, studies using similar approaches have been reported.[8,13] We expect that with increasing numbers of

Table 1. Association between stage and *PIK3CA* aberrations

Variable	*PIK3CA* soamtic mutation				*PIK3CA* copy number alteration			
	Wild type	Mutation	*P*-value	Direction	No amp.	Amp.	*P*-value	Direction
Stage								
I/II	97	4	0.023	Mutation worse	75	25	0.007	Amplification better
III/IV	104	15			107	13		
T status								
1/2	148	9	0.015	Mutation worse	126	31	0.126	
3/4	53	10			56	7		
N status								
0	123	10	0.466		104	29	0.028	Amplification better
1–3	78	9			78	9		

Amp., amplification.

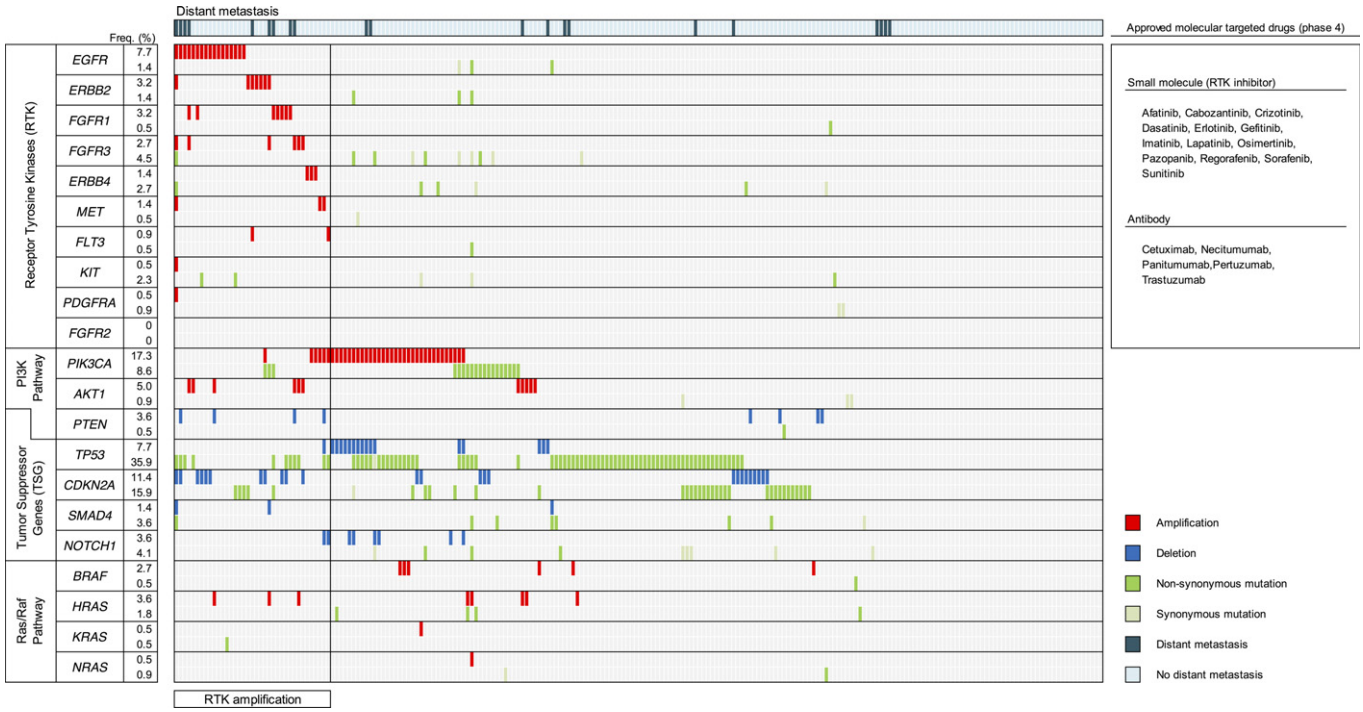

Fig. 2. Key genes and pathways in oral squamous cell carcinoma. Somatic mutations and copy number alterations indicated mutual exclusivity for receptor tyrosine kinases (RTKs), *CDKN2A*, and *PIK3CA*. *CDKN2A* deletions were exclusive with *TP53* somatic mutations, whereas *PIK3CA* amplifications were cooperative with *TP53* somatic mutations. The number of patients with RTK amplification and distant metastasis was nine. Meanwhile, 24 (37 patients) and 43% (21 patients) had RTK amplification and distant metastasis, respectively. The right panel of the figure shows approved molecular-targeted drugs against RTKs in malignant tumors. In head and neck squamous cell carcinoma (HNSCC), the only approved drug is cetuximab.

normal samples deposited in common databases, these methods can be further refined. Furthermore, the availability of these databases and methods allows future studies to decrease the cost of germline sequencing. This approach also obviates the issue of dealing with incidental or secondary findings that this generates.

The most prevalent genetic alteration detected in our cohort comprised a broad spectrum of *TP53* mutations (35.9%). Other frequently mutated genes include *CDKN2A* (15.9%) and *PIK3CA* (8.6%), and this pattern of Japanese OSCC genetic mutations was compared with other studies. The genetic mutations of oral tongue carcinoma in Singapore exhibited similar frequencies of mutations in *TP53* (38.3%) and *PIK3CA* (8.3%), whereas mutations in *CDKN2A* were less frequent.[8] In the HNSCC data of TCGA, mutation frequencies in *TP53*, *CDKN2A*, and *PIK3CA* were 72%, 22%, and 21%, respectively.[4] The *TP53* mutation frequency in gingivo-buccal OSCC in India was 62%.[7] On the other hand, the mutation frequencies of *TP53* in HNSCC and OSCC in COSMIC data (v78, released 05-SEP-16) were 32% and 42%, respectively (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Similarly, patterns attributable to etiology and ethnicity have been observed for other cancer types.[14]

The results of this study demonstrated that RTK amplification is a predictive factor for distant metastasis of OSCC. Regulation failure induced by RTK alteration is known to cause intercellular/intracellular signaling perturbation, and RTK alterations have been reported in many cancers.[15,16] In HNSCC, genetic alterations of not only *EGFR* but also *ERBB2*, *FGFR1*, *FGFR3*, and *MET* have been reported.[17–21] RTK gene alterations induce epithelial–mesenchymal transition,[22] allowing tumor cells to acquire migration capabilities. Overexpression of *EGFR* was demonstrated to be an adverse prognostic factor in HNSCC.[23] In addition, *HER2* overexpression in breast and gastric cancers and *FGFR1* overexpression in lung cancer have been identified as adverse prognostic factors.[24–26] *EGFR* amplification was detected most frequently in this study, similar to previous reports.[4,19] Meanwhile, amplification of other RTKs such as *ERBB2*, *FRFR1*, and *FGFR3* was also detected, albeit in a small number of patients, demonstrating that RTKs are adverse prognostic factors. Moreover, it was demonstrated that the group with both RTK amplification and *TP53* mutations had significantly poorer prognosis than the other groups, although no significant difference was detected in OS regarding the presence or absence of *TP53* mutations alone. This is likely because mutation-caused loss of function of *TP53* promoted tumor activation by RTK amplification, resulting in significant induction of distant metastasis. Meanwhile, *CDKN2A*, another tumor suppressor gene, has also been reported to undergo deletion in HNSCC.[27] However, it was not demonstrated that a synergistic effect of *CDKN2A* deletion and RTK amplification on OS.

Our examination of stages detected a statistically significant difference in the frequency of genetic alterations in *PIK3CA* between stages as well as their factors, namely local tumor progression (T status) and cervical lymph node metastasis (N status), separately. Regarding SMs of *PIK3CA* and stages, Kozaki *et al.*[28] reported that the frequency of SMs of *PIK3CA* was significantly higher in stage IV compared to stages I–III in OSCC, which is in line with our results. It has been found that precursor lesions of invasive cancers also exhibit *PIK3CA* amplification.[29,30] Given our result that the

Table 2. Summary of patients with receptor tyrosine kinase amplification and distant metastasis

Patient No.	Age	Gender	Smoking history	Alcohol history	Subsite	FFPE sample	Histological differentiation	T status	N status	Stage	Distant metastasis	Outcome	RTK amplification	Somatic mutation	Copy number alteration	
															Amplification	Deletion
1	56	M	Never	Yes	BM	B	M/D	1	0	I	-	NED	*EGFR*	*CDKN2A, KIT*	–	–
2	29	F	UNK	UNK	TON	B	W/D	1	0	I	–	LTF	*EGFR*	–	*GNA11, JAK3*	*CDKN2A, RB1*
3	67	M	Former	Yes	GUM	B	W/D	1	0	I	–	NED	*MET*	*ATM*	*PIK3CA*	–
4	41	F	Never	Yes	TON	B	W/D	1	0	I	–	NED	*FLT3*	–	*PIK3CA*	*HNF1A, NOTCH1, STK11*
5	76	F	Never	Yes	GUM	B	W/D	1	0	I	–	NED	*MET*	*TP53*	*PIK3CA*	*HNF1A, NOTCH1, PTEN, STK11, TP53*
6	36	M	UNK	UNK	TON	B	W/D	2	0	II	–	NED	*EGFR*	–	–	–
7	21	F	Never	No	TON	B	M/D	2	0	II	–	NED	*EGFR*	–	–	–
8	78	M	Former	Yes	GUM	P	M/D	2	0	II	–	NED	*EGFR*	*KIT*	–	*CDKN2A, RB1*
9	53	M	Current	Yes	TON	B	W/D	2	0	II	–	NED	*EGFR, FGFR1*	*SMARCB1*	–	*CDKN2A, RB1*
10	30	M	Current	No	TON	B	W/D	2	0	II	–	NED	*FGFR1*	*TP53*	–	*CDKN2A*
11	35	F	Never	No	TON	B	P/D	2	0	II	–	NED	*EGFR*	–	*AKT1, HRAS*	*PTEN, RB1*
12	35	F	Current	Yes	TON	B	M/D	2	0	II	–	NED	*EGFR*	*CDKN2A*	*GNA11, JAK3*	–
13	52	M	Former	Yes	GUM	B	M/D	2	0	II	–	NED	*ERBB4*	–	*PIK3CA*	–
14	30	M	Current	Yes	TON	B	M/D	2	0	II	–	NED	*EGFR*	*JAK2, RET*	–	–
15	65	M	UNK	UNK	BM	B	P/D	2	0	II	–	NED	*FGFR3*	–	*AKT1, ALK*	*ATM, CDKN2A, RB1*
16	35	F	Never	No	TON	B	W/D	2	0	II	–	NED	*ERBB4*	–	*PIK3CA*	–
17	65	F	Current	Yes	TON	B	P/D	2	0	II	–	NED	*ERBB2*	*CDKN2A*	–	–
18	74	F	Never	No	GUM	B	M/D	2	1	III	–	NED	*FGFR1*	–	–	–
19	36	M	Current	Yes	TON	B	M/D	2	1	III	–	LTF	*EGFR*	*CDKN2A*	–	–
20	62	F	Current	No	GUM	B	M/D	3	0	IVA	–	NED	*EGFR*	*KRAS*	*ABL1*	–
21	75	F	Never	UNK	GUM	B	M/D	4a	0	IVA	–	DID	*EGFR*	–	–	*CDKN2A*
22	54	F	Never	No	TON	P	M/D	2	2b	IVA	–	NED	*FGFR3*	*TP53*	*ABL1, AKT1, CSF1R, GNA11, HRAS, JAK3, RET, SMO*	*RB1*
23	63	M	Current	Yes	TON	B	W/D	3	2b	IVA	–	NED	*ERBB2*	–	–	–
24	50	M	Current	Yes	FOM	B	M/D	3	2b	IVA	–	NED	*FGFR1*	–	*ABL1, GNA11, GNAQ, JAK3*	*CDKN2A*
25	73	M	Current	Yes	FOM	B	M/D	4a	2c	IVA	–	NED	*ERBB2*	*PIK3CA*	*PIK3CA*	*CDKN2A*
26	82	F	Former	No	GUM	B	W/D	4a	2c	IVA	–	NED	*ERBB4*	–	–	–
27	62	M	UNK	UNK	HP	B	M/D	4a	2c	IVA	–	DOD(P,N)	*ERBB2*	–	–	*CDKN2A*
28	45	M	Never	Yes	GUM	B	W/D	4b	1	IVB	–	NED	*EGFR*	*TP53*	*AKT1*	–
29	58	M	UNK	UNK	FOM	B	M/D	2	0	II	Lung	DOD(M)	*EGFR*	*TP53*	–	*CDKN2A, PTEN*

Table 2 (Continued)

Patient No.	Age	Gender	Smoking history	Alcohol history	Subsite	FFPE sample	Histological differentiation	T status	N status	Stage	Distant metastasis	Outcome	RTK amplification	Somatic mutation	Copy number alteration	
															Amplification	Deletion
30	62	M	Current	Yes	BM	B	W/D	4a	0	IVA	Lung	DOD(M)	*FGFR1*	*CDKN2A, GNA11, PIK3CA, TP53*	–	–
31	57	F	Former	Yes	GUM	P	W/D	4a	0	IVA	Lung, bone	DOD(M)	*ERBB2, FGFR3*	*PIK3CA*	*ABL1, GNA11, GNAQ, HRAS, JAK2, JAK3, RET, SMO*	*APC, SMAD4*
32	24	M	Never	No	TON	B	M/D	1	2b	VIA	Lung	DOD(M)	*EGFR, ERBB2, FGFR3, KIT, MET, PDGFRA*	*ATM, ERBB4, FGFR3, IDH2, KDR, RB1, SMAD4, STK11, TP53*	*ABL1, GNA11, GNAQ, JAK3, RET*	*CDKN2A, RB1, SMAD4*
33	55	M	Never	No	BM	LN	W/D	2	2b	IVA	Lung	DOD(N,M)	*FGFR3*	*CDH1, TP53*	*ABL1, AKT1, RET*	*ATM, PTEN, RB1*
34	58	F	Never	Yes	TON	B	W/D	2	2c	IVA	Lung	DOD(P,N,M)	*ERBB2, FLT3*	–	–	–
35	63	F	Former	Yes	TON	B	M/D	3	2c	IVA	Lung	DOD(N,M)	*EGFR*	*TP53*	–	–
36	53	M	Never	Yes	TON	B	M/D	4a	2c	IVA	Lung, bone	DOD(M)	*EGFR, FGFR1, FGFR3*	–	*AKT1, GNA11, JAK3*	*RB1*
37	62	F	Current	Yes	TON	B	M/D	4a	2c	IVA	Lung, bone, liver	DOD(M)	*FGFR1*	*APC,TP53*	–	–
38	71	M	Current	Yes	BM	LN	P/D	2	1	III	Lung	DOD(M)	–	*CDKN2A, TP53*	–	–
39	74	F	Former	UNK	BM	P	M/D	2	1	III	Lung	DOD(M)	–	–	*CSF1R*	–
40	66	M	Current	Yes	GUM	B	W/D	4a	1	IVA	Lung, bone	AWD	–	*TP53*	–	*CDKN2A*
41	73	M	Never	Yes	TON	P	P/D	1	2b	IVA	Lung	DOD(M)	–	*SMO*	–	–
42	69	M	Current	Yes	TON	P	W/D	2	2b	IVA	Lung	DOD(M)	–	–	*AKT, CSF1R, HRAS*	*RB1*
43	65	M	UNK	Yes	TON	B	P/D	2	2b	IVA	Lung	DOD(M)	–	*TP53*	–	–
44	62	M	Never	Yes	BM	B	P/D	2	2b	IVA	Lung	DOD(M)	–	*TP53*	*PIK3CA*	*TP53*
45	74	M	Current	No	BM	B	P/D	2	2b	IVA	Lung	DOD(M)	–	–	–	*TP53*
46	32	M	Never	Yes	TON	B	M/D	2	2b	IVA	Lung, bone	DOD(M)	–	*SMARKB1*	–	–
47	65	M	Current	Yes	TON	B	M/D	3	2b	IVA	Lung, bone, liver	DOD(M)	–	*TP53*	–	–
48	67	M	Current	Yes	TON	B	W/D	2	2c	IVA	Lung	DOD(M)	–	*TP53*	*PIK3CA*	*STK11, TP53*
49	71	F	Never	No	GUM	B	M/D	4a	2c	IVA	Lung	DOD(N,M)	–	–	–	–

The upper part of the table shows patients with RTK amplification (Patients 1–28), the middle shows patients with both RTK amplification and distant metastasis (29–37), and the lower part shows patients with distant metastasis (38–49). AWD, alive with disease; B, biopsy sample; BM, buccal mucossa; DID, died of intercurrent disease; DOD, died of disease; F, female; FOM, floor of mouth; HP, hard palate; LN, lymph node sample; LTF, lost to follow up; M, male; M/D, moderately differentiated; NED, no evidence of disease; P, primary sample of surgical specimen; P/D, poorly differentiated; TON, tongue; UNK, unknown; W/D, well differentiated.

Table 3. Results of univariate and multivariate analysis for overall survival

Variable	Category	Hazard ratio for death (95.0% CI)	*P*-value
Univariate analysis			
Age (years)	< 60 *vs* ≥ 60	1.402 (0.690–2.850)	0.351
Gender	Male *vs* Female	0.754 (0.365–1.555)	0.444
Smoking	Non–smoker *vs* Smoker	0.992 (0.472–2.086)	0.984
Alcohol	No alcohol use *vs* Alcohol use	1.058 (0.463–2.418)	0.893
Subsite	Tongue/Gum *vs* Others (BM/FOM/HP)	2.711 (1.289–5.700)	0.009
Histological differentiation	Well/Moderately *vs* Poorly	2.014 (0.874–4.643)	0.100
cStage	cStage I–III *vs* cStage IV	1.901 (0.953–3.792)	0.068
Stage	Stage I–III *vs* Stage IV	6.505 (2.822–14.99)	<0.001
RTK	No amplification *vs* Amplification	2.662 (1.290–5.491)	0.008
PIK3CA	No amplification *vs* Amplification	0.543 (0.166–1.781)	0.314
	Wild type *vs* Mutation	1.197 (0.365–3.925)	0.767
RAS/RAF pathway	No amplification *vs* Amplification	0.731 (0.175–3.057)	0.668
	Wild type *vs* Mutation	0.901 (0.123–6.599)	0.918
CDKN2A	No deletion *vs* Deletion	2.442 (1.059–5.634)	0.036
	Wild type *vs* Mutation	0.874 (0.337–2.264)	0.781
TP53	No deletion *vs* Deletion	1.375 (0.419–4.510)	0.599
	Wild type *vs* Mutation	1.192 (0.593–2.397)	0.622
Multivariate analysis			
Age (years)	< 60 *vs* ≥ 60	1.256 (0.595–2.653)	0.550
Subsite	Tongue/Gum *vs* Others (BM/FOM/HP)	2.170 (0.958–4.918)	0.063
Histological differentiation	Well/Moderately *vs* Poorly	2.176 (0.917–5.162)	0.078
cStage	cStage I–III *vs* cStage IV	1.824 (0.879–3.788)	0.107
RTK	No amplification *vs* Amplification	2.410 (1.056–5.498)	0.037
CDKN2A	No deletion *vs* Deletion	1.104 (0.398–3.059)	0.849

RTK (*EGFR, ERBB2, ERBB4, FGFR1, FGFR2, FGFR3, FLT3, KIT, MET, PDGFRA*). RAS-RAF pathway (*BRAF, HRAS, KRAS, NRAS*). BM, buccal mucosa; cStage, clinical stage; FOM, floor of mouth; HP, hard palate.

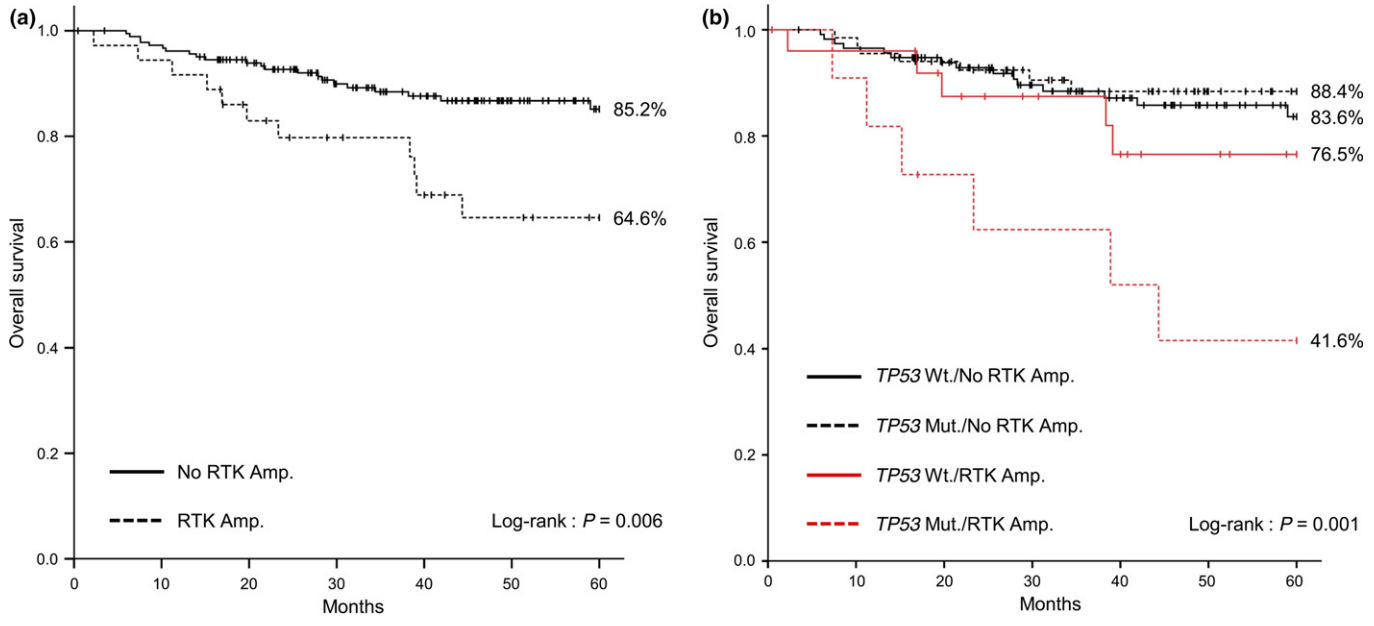

Fig. 3. Kaplan–Meier estimates of overall survival (OS) among patients according to genomic variables. (a) Patients were stratified into two subgroups according to receptor tyrosine kinase (RTK) amplification status. The cumulative 5-year survival rate was 85.2% (95% confidence interval [CI] = 79.1–91.3) in the no RTK Amp. group, *vs* 64.6% (95% CI = 47.4–81.8) in the RTK Amp. group. (b) Patients were stratified into four subgroups according to *TP53* mutation and RTK amplification status. The cumulative 5-year survival rates were 83.6% (95% CI = 75.6–91.7) in the *TP53* Wt./No RTK Amp. group, 88.4% (95% CI = 80.2–96.6) in the *TP53* Mut./No RTK Amp. group, 76.5% (95% CI = 58.2–94.9) in the *TP53* Wt./RTK Amp. Group, and 41.6% (95% CI = 10.9–72.2) in the *TP53* Mut./RTK Amp. group. Amp., amplification; Wt., wild type; Mut., mutation.

frequency of *PIK3CA* amplification was significantly higher in patients free of cervical lymph node metastasis, *PIK3CA* amplification is likely to reflect a tumor's early characteristics.

Since molecular-targeted drugs became available, it has become common in cancer treatment to choose drugs based on the presence or absence of a gene mutation. RTKs can be the

Table 4. Four-groups analysis according to the presence or absence of *TP53* and receptor tyrosine kinase genetic alterations

Variable	Category	No. patients	No. deaths	Hazard ratio for death (95.0% CI)	*P*-value
TP53/RTK	Wild type/No amplification	116	15	Reference	
	Mutation/No amplification	67	7	0.805 (0.328–1.976)	0.636
	Wild type/ Amplification	26	5	1.560 (0.567–4.294)	0.389
	Mutation/ Amplification	11	6	4.820 (1.869–12.43)	0.001

Table 5. Four-groups analysis according to the presence or absence of *CDKN2A* and receptor tyrosine kinase genetic alterations

Variable	Category	No. patients	No. deaths	Hazard ratio for death (95.0% CI)	*P*-value
CDKN2A/RTK	No deletion/No amplification	169	19	Reference	
	Deletion/No amplification	14	3	2.398 (0.708–8.120)	0.160
	No deletion/ Amplification	26	7	2.626 (1.103–6.248)	0.029
	Deletion/ Amplification	11	4	3.517 (1.196–10.34)	0.022

target molecules of various molecular-targeted drugs. Cetuximab, an anti-EGFR monoclonal antibody, has been demonstrated to provide clinical improvement in the treatment of locally advanced and recurrent/metastatic HNSCC.[31,32] Although clinical trials of various other RTK inhibitors and anti-RTK monoclonal antibody drugs have been conducted,[33–36] no efficient therapy equivalent to that of cetuximab has been identified. The reason may be that in most of these trials, the subjects had recurrent or metastatic lesions. Therefore, examination of the administration, methods, and timing of treatment may facilitate good outcomes. The use of a molecular-targeted drug as postoperative adjuvant therapy for the high-risk group as identified by RTK amplification through the analysis of genomic alterations using biopsy tissues is an example.

Cigarette smoking is the most cited risk factor for oral cancer. It raises the risk of developing oral cancer by threefold, and concomitant alcohol consumption, acting synergistically, increases the risk 10–15-fold.[37] The risk of cancer is higher in tissues which are in close contact with ingested alcohol, such as the oral cavity, pharynx, and esophagus.[38] However, it is not clear why alcohol use preferentially exerts a local carcinogenic effect. López-Lázaro discussed that the cytotoxic effect of ethanol activates the division of the stem cells that maintain the deeper layers of the mucosa in homeostasis.[38] Meanwhile, Hashibe *et al.*[37] reported that a substantial proportion of head and neck cancers cannot be attributed to tobacco or alcohol use, particularly for oral cavity cancer.[37] Therefore, we investigated the smoking status and alcohol consumption in our cohort; however, there were no significant correlations between the smoking status and/or alcohol consumption and SMs, CNAs, and clinicopathological features in our study. At the same time, smoking is known to affect the genome by causing certain types of mutation. The mutational events linked to smoking are traditionally reported as an increase in C>A mutations and a decrease in C>T mutations.[39] However, Pickering *et al.*[40] reported that smoking has only a minor impact on the types of mutations observed in oral tongue SCC and TCGA data also demonstrate that the genomic effects of smoking are tumor-site specific. Although data are sparse because of limited sequencing lesions, we have also investigated the events of somatic mutations detected in our study; no significant differentiations were revealed (data not shown) between 106 mutations in the non-smoking group (58 patients) and 159 in the smoking group (72 patients). These findings indicate that smoking and/or alcohol consumption only have a minor impact on carcinogenesis in oral cancer.

The panel that we used in this study does not include some important genes that have been detected in OSCC, such as *CASP8* and *FAT1*[4,5] (Table S1a). Moreover, we need additional consideration concerning the target region of each gene even though the panel is designed to target many hotspot regions (Table S1b). Oncogenes are recurrently mutated at the same amino acid positions, whereas tumor suppressor genes are mutated through truncating mutations throughout their length.[41] Regarding *NOTCH1*, most of the mutations in hematopoietic tumors have been identified in the heterodimerization and C-terminal polypeptide-enriched proline, glutamate, serine, and threonine domain,[42,43] and the panel we used only targeted these regions. Conversely, it has been demonstrated that in HNSCC, mutations, including truncating mutations, have also been detected in the N-terminal epidermal growth factor-like ligand-binding domain, and these mutations appeared to be loss-of-function mutations.[5,6] Therefore, a specific custom panel for OSCC with better target genes and regions may enable us to obtain more information and discover new treatments.

In conclusion, the results of this study using FFPE samples of cancer tissues and NGS demonstrate that RTK amplification is a prognostic prediction factor for distant metastasis of OSCC, indicating the necessity for using NGS in clinical sequencing. To achieve stratified therapies of OSCC based on genomic alterations, evidence must be accumulated.

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Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

CI	confidence interval
CNAs	copy number alterations

FFPE	formalin-fixed, paraffin-embedded
HNSCC	head and neck squamous cell carcinoma
HR	hazard ratio
NGS	next generation sequencing
OSCC	oral squamous cell carcinoma
OS	overall survival
RTK	receptor tyrosine kinase
SMs	somatic mutations
TCGA	The Cancer Genome Atlas

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Receiver operating characteristic curve to determine the cut-off point for (a) deletion and (b) amplification.

Fig. S2. Kaplan–Meier estimates of overall survival according to (a) subsite and (b) stage.

Fig. S3. The results for the detection of somatic mutations and copy number alterations according to subsite.

Fig. S4. Kaplan–Meier estimates of overall survival according to (a) *CDKN2A* deletion status, (b) *TP53* mutation status, and (c) *CDKN2A* deletion/receptor tyrosine kinase amplification status.

Table S1. Information for (a) target genes and (b) target regions.

Table S2. Summary of clinicopathological data.

Table S3. Associations of clinicopathological variables with somatic mutations and copy number alterations in 50 genes.

Table S4. Somatic mutations and copy number alterations according to stage and subsite.

Doc. S1. Supplementary Materials and Methods concerned with copy number analyses.