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Discrepancy Between Clinical Diagnosis and Whole-exome Sequencing-based Clonality Analysis of Synchronous Multiple Oral Cancers

NAOTO NISHII[1,2], YOSUKE HIROTSU[3], NAMI KOIDA[1,2], YUKINOBU TAKAHASHI[1,2], YUKI TAKAGAWA[1,2], KENJI AMEMIYA[4], TOSHIO OYAMA[5], HITOSHI MOCHIZUKI[3,6], EMI FURUSAWA-NISHII[7], HIROYUKI HARADA[2] and MASAO OMATA[6,8]

[1]*Department of Oral Surgery, Yamanashi Central Hospital, Yamanashi, Japan;*
[2]*Department of Oral and Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan;*
[3]*Genome Analysis Center, Yamanashi Central Hospital, Yamanashi, Japan;*
[4]*Division of Genetics and Clinical Laboratory, Yamanashi Central Hospital, Yamanashi, Japan;*
[5]*Department of Pathology, Yamanashi Central Hospital, Yamanashi, Japan;*
[6]*Department of Gastroenterology, Yamanashi Central Hospital, Yamanashi, Japan;*
[7]*Department of Immunobiology, Institute of Development Aging and Cancer, Tohoku University, Sendai, Japan;*
[8]*The University of Tokyo, Tokyo, Japan*

Abstract. *Background/Aim: The definition of multiple oral cancers is based on the distances between the tumors. However, it is not possible to accurately predict tumor origins based only on clinical criteria. Patients and Methods: We performed whole-exome sequencing (WES) to analyze the genetic alterations in five tumors of two patients who underwent surgery in our hospital. Results: In case 1, the distances between tumors on the right mandibular gingiva and buccal mucosa were more than 15 mm, leading to a clinical diagnosis of multiple primary tumors. WES revealed common mutations between tumors, suggesting that the tumors were derived from the same clone. In contrast, in case 2, the distance between tumors on the right side of the tongue was only 10 mm, but the tumors were diagnosed as double primary tumors because their mutations were completely different. Conclusion: WES, rather than the available clinical criteria, can clarify the clonal origins of multiple oral cancers.*

In cases with synchronous multiple oral cancers, individual tumors may be derived from different clones, defined as multiple primary oral cancers, or from the same clone, reflecting intraoral metastasis. However, it is difficult to distinguish these conditions based on pathological features, because 90% of oral cancers are squamous cell carcinomas (SCCs). Clinical criteria are used to diagnose multiple oral cancers. Hong *et al.* have suggested that multiple primary oral cancers should meet the following criteria: (a) all tumors must be definitely malignant; (b) all tumors must be distinct; and (c) the distance between the tumors must exceed 20 mm (1). However, other clinicians use a distance of 15 mm (2, 3). Therefore, no consensus regarding the distance between tumors has yet been achieved.

In an effort to establish clear diagnostic criteria, Braakhuis *et al.* have proposed a new classification based on the mutational profile (4). If "recurrence or metastasis" is present, the mutations are almost the same; in "multiple primary cancers" the mutations are completely different. Furthermore, a "second field tumor (SFT)" category was proposed; some genetic markers are similar but others differ. SFTs are thought to arise from the same genetically altered mucosal field as the primary tumors, but additional independent oncogenic changes cause the tumors to share some, but not all, mutations.

Although 20 years have passed since this molecular-based classification was proposed, few studies on the mutational profiles of multiple primary oral cancers have been published, and they have focused only on some mutations (5). Synchronous multiple oral cancers have not been

Correspondence to: Naoto Nishii, Department of Oral Surgery, Yamanashi Central Hospital, 1-1-1 Fujimi, Kofu, Yamanashi 400-8506, Japan. Tel/Fax: +81 552537111, e-mail: nishii.osur@tmd.ac.jp

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subjected to whole-exome sequencing (WES), and are treated without clarifying the pathophysiology. Primary cancers and metastases may differ markedly in terms of prognosis and treatment requirements. In this study, we sought to distinguish multiple primary tumors, intraoral metastases, and SFTs *via* WES, and compared the result of clinical diagnosis with that of the mutational analysis.

Patients and Methods

Sample preparation. Serial sections of formalin-fixed paraffin-embedded (FFPE) tissue were stained with hematoxylin and eosin (H&E) and micro-dissected as described previously (6, 7). Tumor DNA was extracted using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany). FFPE DNA quality was evaluated as described previously (8). A peripheral blood sample was drawn from each patient, and DNA was extracted from the buffy coat using the QIAamp DNA Blood Mini QIAcube Kit (Qiagen). The DNA concentration was determined using a Nano Drop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) (9).

Whole-exome sequencing. WES was performed as previously described (10, 11). Sequencing libraries were prepared using the Ion AmpliSeq™ Exome RDY Kit (Thermo Fisher Scientific). Library purification was performed using Agencourt AMPure XP reagents (Beckman Coulter, Brea, CA, USA) and the KingFisher Duo Prime System (Thermo Fisher Scientific). The library concentration was determined using an Ion Library Quantitation Kit (Thermo Fisher Scientific). Emulsion PCR and chip loading were performed on the Ion Chef with the Ion PI Hi-Q Chef kit. Sequencing was performed using the Ion PI Hi-Q Sequencing Kit on the Ion Proton Sequencer (Thermo Fisher Scientific). Peripheral blood DNA was used as control to detect variants in tumors. Mutations with variant allele fractions (AFs) ≥10% were identified.

Ethics approval and consent to participate. This study was approved by the Institutional Review Board at Yamanashi Central Hospital. All patients signed the consent form, and all patient information will be kept confidential. Written informed consent for publication of their clinical details and clinical images was obtained from both patients.

Results

Case No. 1: Multiple oral tumors shared the same mutations, although the distances between the tumors exceed 15 mm

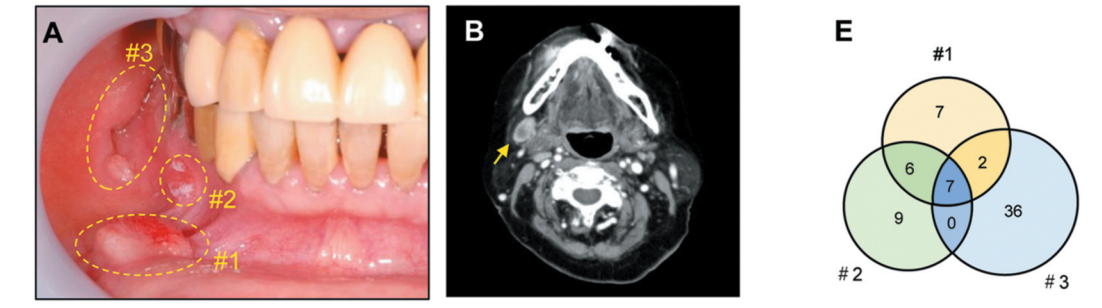
An 85-year-old woman visited our hospital for a consultation about a painless mass on the right buccal mucosa. Intraoral examination revealed three masses on the right mandibular gingiva (#1: 14×12 mm, #2: 5×5 mm) and buccal mucosa (#3: 25×8 mm) (Figure 1A). All tumors appeared to be independent, and the distances between them were 10 mm (#1 and #2), 16 mm (#2 and #3), and 17 mm (#1 and #3). A submandibular lymph node (LN) on the right side was metastatic. Magnetic resonance imaging revealed that tumors #1 and #2 were so close that they could not be distinguished, but tumors #1 and #3 were clearly independent. Computed tomography with contrast revealed that the right submandibular LN was markedly enlarged and suspected to be metastasis (Figure 1B). Pathological examination of biopsy samples taken from the three tumors revealed well-to-moderately differentiated SCCs (Figure 1C). As all cancers were SCCs, the macroscopic distance was the only way to distinguish primary from metastatic tumors using conventional diagnostic methods. However, in this case, the diagnosis differed by the diagnostic criteria used, because the distances between tumors #1, #2, and #3 were more than 15 mm but less than 20 mm.

To clarify the clonality of these independent but pathologically identical tumors, we performed WES on the three tumors (#1 to #3) and normal tissue near the tumors. The mean coverage depth was 164-fold. WES detected 67 somatic mutations with AFs ≥10% (Figure 1D). These somatic mutations were specific to the primary tumors. Sixty-six mutations (other than *TP53*) were passenger mutations. Strikingly, the three tumors shared seven mutations (*RNF17, SEPT10, CCDC60, SEMA6C, JUP, FGA*, and *SLC22A6*) (Figure 1D and E), indicating that the tumors were derived from the same clone. Tumors #1 and #2 shared six mutations and tumors #1 and #3 shared two mutations (Figure 1E). The oncogenic mutation TP53 was shared by tumors #1 and #2. Thus, genetic analysis suggested that the individual SCCs (#1 to #3) were metastatic tumors or SFTs rather than multiple primary tumors.

The patient underwent suprahyoid neck dissection on the right side, and tumor excision with marginal mandibulectomy after neoadjuvant chemoradiotherapy. One year has passed, and there is no evidence of tumor recurrence or metastasis.

Case No. 2: Mutational analysis revealed that two close tumors on the right side of the tongue were multiple primary tumors

A 57-year-old man scheduled for surgery to treat an esophagus carcinoma was referred to us because of pain on the right side of the tongue. Intraoral examination revealed a 10×10 mm white lesion with erosion on the right side (#1, Figure 2A), which was diagnosed as an SCC *in situ* by biopsy. Although the patient had not complained of it, 4×3 mm white patch with a smooth surface was found on the left side of the tongue (#2, Figure 2B), and was clinically diagnosed as leukoplakia. After neoadjuvant chemotherapy in the department of surgery, the patient underwent partial glossectomy of SCC #1, and resection of leukoplakia #2. Specimen #1 was diagnosed as an SCC *in situ* (Figure 2C), and the margin near the floor of the mouth was positive. We performed additional resection of #1, and then, found a microinvasive SCC at the margin near the floor of the mouth (#3, Figure 2C). Surprisingly, tumors #1 and #3 were not continuous, although the distance between them was only 10



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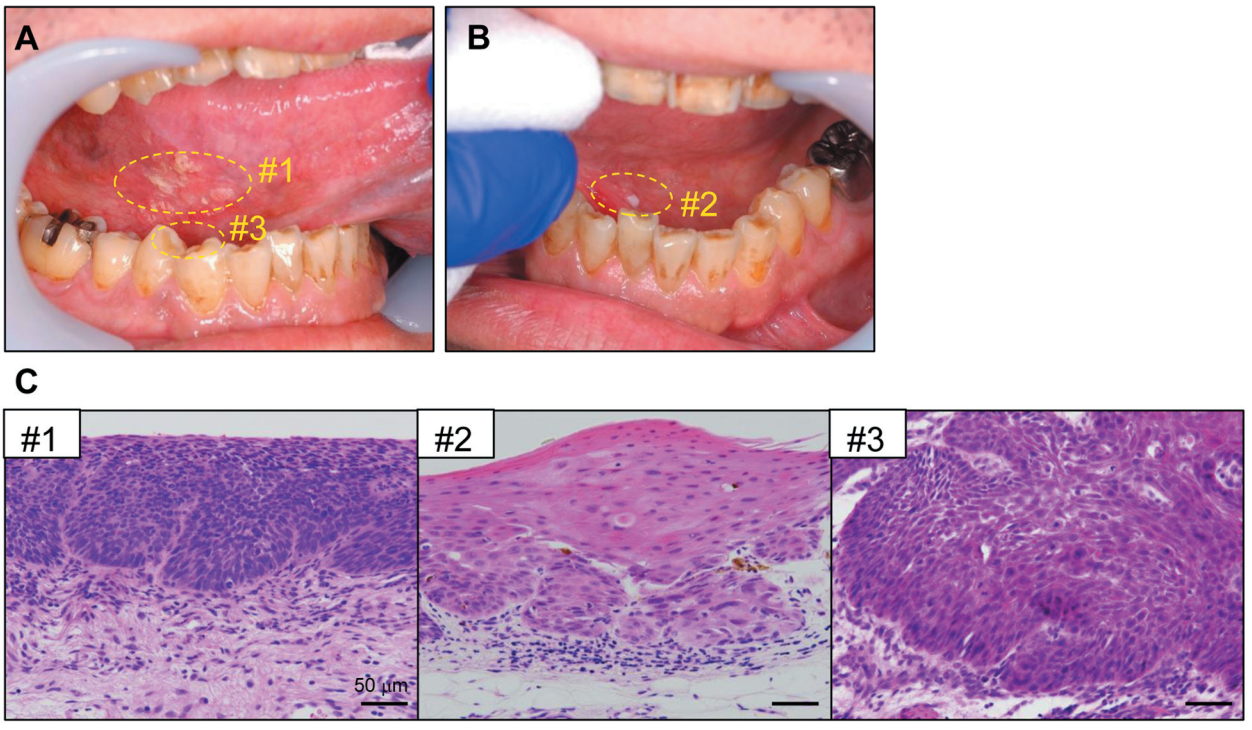
Gene	Mutation	OncoKB	Primary tumor #1	Primary tumor #2	Primary tumor #3	Non tumor
RNF17	p.T484A	NA	40	25	14	
SEPT10	p.K422R	NA	32	25	20	
CCDC60	p.V393I	NA	27	15	16	
SEMA6C	p.H435P	NA	27	16	21	
JUP	p.A538G	NA	24	19	28	
FGA	p.R790S	NA	22	12	21	
SLC22A6	p.A479T	NA	17	18	21	
TP53	p.R248Q	LO	31	20		
TXNDC16	p.L691F	NA	27	19		
GRIN2B	p.M508I	NA	26	15		
SERAC1	p.N255I	NA	26	14		
EMILIN1	p.R55H	NA	25	14		
SMOC2	p.R391P	NA	18	14		
TTN	p.I3439T	NA	33		19	
FAM98C	p.S293fs	NA	12		26	
GABRG2	p.R448G	NA	26			
CNTD2	p.F183del	NA	24			
KCNT1	p.R474C	NA	22			
HUWE1	p.E2654K	NA	17			
GPR158	splice site	NA	16			
SLC26A4	p.E643K	NA	14			
PCDH20	p.Q871K	NA	14			
PAPD4	p.L199Ter	NA		21		
FAT1	p.V3840M	U		17		
NDNF	p.K458N	NA		17		
EDEM3	p.D882N	NA		16		
GRID1	p.R683L	NA		16		
RBSN	p.R173W	NA		16		
LRRC4C	p.L269V	NA		14		
RNASET2	p.D243H	NA		13		
PRRC2C	p.R507S	NA		12		
TUBB8	p.R121K	NA			26	
JAKMIP1	p.R64W	NA			22	
FBL	p.R218Ter	NA			22	

Gene	Mutation	OncoKB	Primary tumor #1	Primary tumor #2	Primary tumor #3	Non tumor
NACAD	p.E1113K	NA			21	
OR52M1	p.S9Ter	NA			21	
GK	p.L463M	NA			21	
SUSD3	p.E83K	NA			20	
NUCB1	p.F248L	NA			18	
HDAC4	p.D1074H	U			18	
GRIK1	p.A699V	NA			18	
PKD1L1	splice site	NA			18	
SOHLH2	p.E341Q	NA			17	
CCDC169-SOHLH2	p.E418Q	NA			17	
TECPR2	p.S109L	NA			17	
TNIP1	p.E514L	NA			17	
MSH5	p.S596L	NA			17	
WDR72	p.I496M	NA			17	
KLHL13	p.E41Q	NA			17	
USH1C	p.E454Q	NA			16	
PUM3	p.P315S	NA			16	
CCNQ	p.D230H	NA			16	
SFSWAP	p.E101Ter	NA			16	
NQO1	p.E218Q	NA			16	
FCRL3	p.S658R	NA			16	
FREM2	p.E1302Ter	NA			15	
HRH2	p.Leu67F	NA			15	
PDZRN4	p.E743K	NA			15	
MYH4	p.E871Q	NA			15	
ADAT1	p.W480C	NA			14	
FBXW10	p.C32R	NA			13	
DCAF4L2	p.G170E	NA			13	
DNAJC14	p.S57C	NA			13	
MAP7D2	p.A70D	NA			13	
PRCP	p.R23W	NA			12	
ERC1	p.E356Q	NA			12	
AKAP17A	p.E498V	NA			11	

AF
1% 100%
No mutation

Figure 1. *Synchronous multiple tumors in case 1, which clinically suspected to be multiple primary tumors, were diagnosed as metastatic tumors or SFTs based on genomic analysis. (A) Intraoral photograph of three tumors on the right lower gingiva (#1 and #2) and buccal mucosa (#3), obtained at the first visit. (B) CT revealed an enlarged right submandibular LN. (C) H&E-stained images of biopsy samples taken from the three tumors. Tumors #1 and #3 were well-differentiated SCCs, and tumor #2 was a moderately-differentiated SCC. Scale bars=50 μm. (D) Genomic analyses by WES: Heat maps of the mutations in each sample. The left column lists the mutated genes with the corresponding amino acid changes. AF: Allele fraction; LO: likely oncogenic; U: unknown; NA: not available in the OncoKB database. (E) A Venn diagram illustrating the distributions of the validated mutations in the three lesions. Common mutations were defined as identical nucleotide changes at the same genomic co-ordinates.*



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Gene	Mutation	OncoKB	Primary tumor #1	Primary tumor #3	Non tumor
MAGEE1	p.P297A	NA	44		
C16orf89	p.S31R	NA	30		
IQGAP1	p.I1295V	NA	29		
PPFIA4	p.R514W	NA	25		
MAP4	p.P831H	NA	21		
TAB3	p.H521R	NA	19		
POTEC	p.C102_C103delinsFH	NA	18		
POTEC	p.R110K	NA	17		
POTEC	p.C105R	NA	17		
POTEC	p.V117M	NA	16		
CCSAP	p.R28H	NA	16		
LUZP1	p.H849R	NA	15		
NAV1	p.A909P	NA	15		
DNAJC3	p.I352K	NA	14		
AKT3	p.Y38C	U	13		
XIRP2	p.T1910A	NA	12		
FADS1	p.A65D	NA	12		
BAZ2A	p.V1657L	NA	12		
OR8J3	p.A98G	NA	12		
BRCA1	p.G1677A	U		16	
SSH2	p.T118N	NA		15	
NEB	p.F1481L	NA		11	

AF
1% 100%
No mutation

Figure 2. *Synchronous multiple tumors on the right side of the tongue of case 2, located close together, were diagnosed as multiple primary tumors based on genomic analysis. Intraoral photograph of tumors on the right (A) and left side (B) of the tongue at the first visit. Tumor #3 was found during the second surgery. (C) H&E-stained images of the surgical specimens. Tumor #1 (SCC in situ) and tumor #2 (a microinvasive SCC) were taken from the right and left sides of the tongue, respectively, during the first surgery. Tumor #3 (a microinvasive SCC) was taken from the right side of the tongue during the second surgery. Scale bars=50 mm. (D) Genomic analyses by WES: Heat maps of mutations detected in each sample. The left column lists the mutated genes with the corresponding amino acid changes. AF: Allele fraction; LO: likely oncogenic; U: unknown; NA: not available in the OncoKB database.*

mm. Tumor #2 was diagnosed as a microinvasive SCC (Figure 2C). Further additional resection of tumors #2 and #3 revealed that no cancer cells remained.

Although the pathological diagnoses of #1 and #3 were not consistent, the short distance between the tumors implied that they may have arisen from the same genetically altered field, and would thus share at least some mutations. We subjected tumors #1 and #3, and normal tissue near the tumors, to WES. We could not analyze #2; the amount of DNA extracted was too low. Twenty-two somatic mutations with AFs ≥10% were detected (Figure 2D). The mean coverage depth was 106-fold. Tumors #1 and #3 harbored 19 and 3 mutations, respectively. Of note, the mutational patterns differed completely, suggesting that they were not SFTs, but rather double primary oral tumors.

The patient underwent subtotal esophagectomy at the time of the first oral operation, and the specimen contained two lesions, SCC and SCC *in situ*. The patient has remained alive without recurrence of oral and esophagus disease for 1 year after the last surgery.

Discussion

WES analysis demonstrated that there was a discrepancy between clinical diagnosis and mutational profile-based diagnosis of synchronous multiple oral cancers. In case 1, the distance between independent SCCs ranged from 15 to 20 mm, leading to different clinical diagnoses depending on criteria used. Mutational analysis revealed that seven mutations were common, indicating that the tumors were metastases from one primary tumor, or SFTs, as opposed to triple primary cancers. On the other hand, the tumors of case 2, which were close together, were double primary tumors with completely different mutations.

Several laboratory techniques have been used to analyze the clonal relationships between multiple oral tumors. Ribeiro *et al*. have analyzed the *TP53* mutation profile of the tumors clinically diagnosed as multiple primary oral cancers, and found that tumor pairs had different *TP53* mutations; in turn, this indicated that they were in fact multiple primary tumors (12). Scholes *et al*. have analyzed the loss of heterozygosity (LOH) of tumors clinically diagnosed as multiple primary oral cancers, and revealed that the tumors originated from the same clone in three out of five patients. (2). These results suggested that analysis of the gene mutational profile facilitates accurate diagnosis of multiple primary cancers and intraoral metastases. However, earlier studies examined only a few genes or the LOH. We found that mutational profiles obtained by WES discriminated between multiple primary tumors and metastases/SFTs. This is the first report to use WES for clonality-based diagnosis of synchronous multiple oral cancers.

The prognosis of synchronous multiple oral cancers diagnosed *via* mutational analysis remains unclear. In patients with metachronous multiple oral cancers, mutational analysis of mitochondrial DNA revealed that the postoperative prognosis of patients with SFTs was better than that of patients with locoregional recurrences or multiple primary cancers (13). Thus, treatment responses may differ according to the molecular-based classification of multiple oral cancers.

Regarding multiple cancers in other organs, the prognoses of patients with intrapulmonary metastases are poorer than those of patients with multiple primary lung cancers; discrimination between multiple primary tumors and metastases is essential to guide appropriate treatment. Goto *et al*. have successfully identified the clonality of multiple lung cancers *via* genomic profiling, indicating that mutational analysis could help clinicians select the optimal treatments (14, 15).

Oral squamous cell carcinoma (OSCC) has been conventionally treated *via* surgery, radiation, and chemotherapy, but new modalities have been rapidly introduced in recent years. In Japan, an immune checkpoint inhibitor was approved for OSCC treatment in 2017. Genetic tests help clinicians to select the best drugs; the cost began to be covered by the Japanese public health insurance system in 2019. Surgery is the first treatment of choice for oral cancer, but if a poor postoperative prognosis can be predicted at diagnosis, treatment should be promptly switched to a non-surgical modality. We found that classification of multiple oral cancers using the mutational profiles, rather than the clinical criteria, clearly identified the clonality of individual tumors. Further studies on the relationship between mutation analysis-based classification and long-term prognosis are required to help clinicians predict the prognosis and the best treatments for multiple oral cancers.

Conflicts of Interest

All Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

N.N. and Y.H. contributed to conception and design of the research, performed the experiments, data acquisition, analysis, interpretation and wrote the paper. N.K. and Y.T. performed the surgeries. T.O. performed the pathological examinations. K.A., Y.T., and H.M. participated in the genomic analyses. E.F.-N., H.H., and M.O. contributed to conception and critical discussion of the research and manuscript preparation. All Authors have read and approved the final manuscript.

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