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Mutational analyses of the *BRAF*, *KRAS*, and *PIK3CA* genes in oral squamous cell carcinoma

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Abstract

OBJECTIVES—The development of oral squamous cell carcinoma (OSCC) is a complex, multistep process. To date, numerous oncogenes and tumor-suppressor genes have been implicated in oral carcinogenesis. Of particular interest in this regard are genes involved in cell cycling and apoptosis, such *BRAF*, *KRAS*, and *PIK3CA* genes.

STUDY DESIGN—Mutations of *BRAF, KRAS*, and *PIK3CA* were evaluated by direct genomic sequencing of exons 1 of *KRAS*, 11 and 15 of *BRAF*, and 9 and 20 of *PIK3CA* in OSCC specimens.

RESULTS—Both *BRAF* and *KRAS* mutations were detected with a mutation frequency of 2% (1/42). *PIK3CA* mutations were detected at 3% (1/35).

CONCLUSIONS—This is the first report implicating *BRAF* mutation in OSCC. Our study supports that mutations in the *BRAF*, *KRAS*, and *PIK3CA* genes make at least a minor contribution to OSCC tumorigenesis, and pathway-specific therapies targeting these two pathways should be considered for OSCC in a subset of patients with these mutations.

Keywords

BRAF; KRAS; PIK3CA; oncogene mutation; hot-spot mutation; oral squamous cell carcinoma; OSCC

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BACKGROUND

Oral squamous cell carcinoma (OSCC), a subset of head and neck squamous cell carcinoma (HNSCC), is one of the most common human malignancies worldwide, ranking sixth amongst all human cancers [1]. The 5-year survival rate for OSCC is a mere 50%, a figure that has remained relatively unchanged for decades [2]. Consequently, there has been an increasing focus on identifying key genetic players that may contribute to OSCC pathogenesis, with the overall goal of preventing onset and progression of disease. Furthermore, such knowledge may aid in refining early detection techniques and in developing novel therapeutic approaches. To date, numerous oncogenes and tumor suppressor genes have been implicated in the development of OSCC. Of interest in this regard are mutations in the oncogenes *BRAF*, *KRAS*, and *PIK3CA*.

BRAF is a serine/threonine kinase of the *RAF* family, which is an integral part of the *RAS-RAF-MEK-ERK-MAP* kinase pathway. This pathway plays a role in mediating cellular response to cell growth. Somatic point mutations of *BRAF*, such as those that occur at hot-spot V599E of its kinase domain, can result in elevated kinase function in *BRAF* [3]. Constitutive *ERK* activation ensues, which then influences the cell cycle at the G1/S transition via cyclins D and E, and also p21 [4]. *KRAS*, a protein within the *RAS* family, functions in the same pathway as *BRAF* and is located just upstream to it. *KRAS* appears to be involved in signal transduction and cell cycle regulation [5–8]. To date, there has been only limited investigation of both *BRAF* and *KRAS* mutations in HNSCCs. Of note, mutations in the *RAS* gene family (including *H-, K-*, and *N-RAS*) have been implicated in upwards of 30% of all human cancers; however, mutation frequencies within OSCCs are varied (5–50%) and appear to be dependent on the specific RAS gene and interestingly, geographic location of the study population [9–15].

Phosphatidylinositide-3-kinases (*PI3K*) are a family of enzymes that form inositol lipid products; inositol lipid products play key roles in mediating several intracellular pathways [16]. *PIK3CA*—a heterodimeric, Class 1A enzyme—encodes the p110α catalytic subunit of *PI3K*, which is located at the human chromosomal site 3q26.3 [17]. “Hot spot” mutations of this enzyme have been shown to be located at E542K, E545K, and H1047R [18, 19], and result in increased cell survival by inhibiting apoptosis [20]. Somatic mutations in *PIK3CA* have been documented in a number of human cancers, including hepatocellular, breast, gastric, lung, esophageal, ovarian, pancreatic, and head and neck cancers [21–25]. Three previous studies have shown the presence of *PIK3CA* amplification or overexpression in OSCCs [26–28].

There appears to be limited literature documenting *BRAF, KRAS*, and *PIK3CA* mutations in both HNSCCs and OSCCs. In this paper, we aimed to examine mutational frequencies of all three genes by polymerase chain reaction (PCR) amplification and direct genomic sequencing in a cohort of OSCC specimens.

PATIENTS AND METHODS

Patients and Tissue Samples

Forty-five formalin-fixed, paraffin-embedded OSCC specimens were retrieved from Columbia University’s Oral Diagnostic Biopsy Service. This is a non-overlapping set of specimens from our previous HNSCC studies [29, 30]. The histologic diagnosis and grading of each tumor was verified on hematoxylin-eosin stained slides using the criteria established by the World Health Organization [31]. A board-certified Oral Pathologist (Dr. Woo), who was blinded from all clinical data, performed this verification of the specimens. Demographic data, tumor location, and tumor differentiation for all samples analyzed are

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provided in Table 1. Detailed clinicopathologic information (e.g. TNM staging) was often difficult or impossible to analyze owing to the absence of such data in biopsy-obtained specimens. All procedures were performed with approval from the Institutional Review Board (IRB) of Columbia University Medical Center and in accordance with Health Insurance Portability and Accountability Act (HIPAA) regulations.

DNA Samples and Mutation Analysis

Each specimen was microdissected and the genomic DNA was extracted using the QIAmp DNA Mini Kit (California, USA), following the manufacturer's tissue protocol. All DNA concentrations were subsequently measured by spectrophotometer to ensure the presence of adequate amounts of DNA. PCR amplification of genomic DNA was performed and analyzed for mutations in the following genes: *BRAF* (exons 11 and 15), *KRAS* (exon 1), and *PIK3CA* (exons 9 and 20). These regions included the most common *KRAS*, *BRAF*, and *PIK3CA* mutations previously observed in human cancers [18, 32–34]. Direct sequencing of each individual PCR product was then carried out. Each sample, consisting of 40ng of genomic DNA, was amplified with primers that covered the entire coding region and the exon/intron boundaries of the exon to be analyzed (E11F/ E11R and E15F/E15R of *BRAF*; E1F/E1R of *KRAS*; E9F/E9R and E20F/E20R of *PIK3CA*) as per previous studies [29, 35, 36]. The genomic sequencing was performed with ABI's 3100 capillary automated sequencers at the DNA Core Facility of Columbia University Medical Center. Upon analysis of the sequencing results, all mutations were verified by independent PCR analysis and successive reverse-sequencing of the PCR product. PCR primers were also utilized as the sequencing primers. Corresponding normal tissues derived from surrounding nontumorous tissue or from a tumor-free block (as determined by Dr. Woo) served as the normal control for each patient.

RESULTS

A total of three mutations were found within the 45 cases of OSCC. Due to varying concentrations of DNA in the specimens and varying sensitivities of the primers, each gene that was analyzed had a different sample size. One of the 42 samples analyzed demonstrated a *BRAF* mutation (2.4%, exon 11, G412A). The specimen containing the mutation was a moderate-to-poorly differentiated OSCC obtained from the maxillary alveolar mucosa (Figure 1). One of the 42 samples analyzed demonstrated a *KRAS* mutation (2.4%, exon 1, G12D). This specimen was a moderately differentiated OSCC obtained from lateral tongue (Figure 2). Lastly, one of the 35 samples analyzed demonstrated a *PIK3CA* mutation (3%, exon 9, E545K). This specimen was a moderateto-poorly differentiated OSCC obtained from the ventral tongue (Figure 3).

Normal tissue from the specimens containing the *PIK3CA*, *BRAF* and *KRAS* mutations were examined by sequencing analysis. No mutations were detected in the corresponding normal tissues, suggesting that these mutations were somatic in nature.

DISCUSSION

The results of our study show a mutation frequency of 2.4% in *BRAF*; 2.4% in *KRAS*; and 2.9% in *PIK3CA*, respectively. It should be noted that the sample size was variable between the three genes (see Table 1). We believe that this was due to either the variability in tumor cellularity of each specimen or the sensitivity of the primers. Although all three of these genes have been previously implicated in HNSCCs, there is little data regarding their involvement in OSCCs. Shelly et al examined *BRAF* mutations occurring in exon region 15 in canine oral cancer specimens and found no mutations in their cohort of samples [37]. Weber et al investigated both *BRAF* and *KRAS* mutations via PCR analysis of genomic

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DNA in HNSCC of various sites 14. This group demonstrated a 3% mutation frequency of *BRAF*, also in the exon 15 region, in their pharynx and hypopharynx specimens but none in oral specimens. They also identified a 6% mutation frequency of *KRAS*, which were found in specimens deriving from the pharynx and floor of mouth. Hoa et al noted overexpression of the *KRAS* protein by reverse transcriptase-PCR (RT-PCR) in their HNSCC cell lines 38. Also, oncogenic activation of *KRAS* was previously shown to have a causal role in the development of oral cancer in mice and humans; this was demonstrated via mouse modeling and subsequent RT-PCR 39 and cell transfection assays 40. It is interesting to note that variability in the frequency of *KRAS* mutations has been linked to ethnicity and certain environmental factors, such as use of chewing tobacco 11, 13, 41–43.

The missense mutation detected in *PIK3CA* exon 9 in our study was a previously reported "hot spot" mutation for squamous cell carcinoma 44, 45. Our results are in agreement with Samuels et al who confirmed that an increasing number of mutations (>75%) were found to be located in the helical and kinase domains of *PIK3CA*, which includes exons 9 and 20 18. *PIK3CA* mutants were noted to have increased lipid kinase activity, seemingly due to alterations in the p110α catalytic subunit, with a subsequent downstream constitutive activation of *Akt* signaling 18, 19. Mutations—such the E545K mutation—were shown to promote aberrant cell growth *in vitro* and induce tumorigenesis at a rate of 50% in newly hatched chicks 19. The mutation frequency for *PIK3CA* for our study is slightly lower than those reported in other SCC studies: namely, 11% in a HNSCC series, and 7.4 and 21.4% in OSCC clinical specimens and cell lines, respectively 29, 44. The reason for this disparity remains unclear to us; although we propose that sample size and geographic differences in the study populations (Asian *vs*. North American) may play roles.

We do recognize that there are several limitations to this study, including the restricted sample size and lack of clincopathologic data. Investigating the mutational frequencies of these three genes in OSCC resection specimens will be desirable in the near future, where factors of prognostic significance, such as patient-related factors (e.g. ethnicity, tobacco history, etc.) and tumor-related factors (e.g. tumor thickness, perineural invasion, etc.), are more easily accessible. Of particular interest is exploring a possible correlation with histologic differentiation, as the three mutations identified in this study occurred in moderately and poorly differentiated tumors.

Although somatic mutations of *KRAS, BRAF*, and *PIK3CA* are not frequent events in OSCC, as suggested by our study, the detection of these mutations is important to support the notion that the *RAS-RAF-MEK-ERK-MAP* kinase and *PIK3CA-PTEN-AKT* pathways are involved in OSCC tumorigenesis. The oncogenic activations of these pathways may include additional mechanisms other than small mutations, such as amplification and overexpression. For example, it has been shown previously that *PIK3CA* is frequently amplified in OSCC 26–28, 44. Although *PIK3CA* amplification has not been shown to be a useful prognosis marker in OSCC, *PIK3CA* mutation and amplification have been associated with advanced stages of OSCC and metastasis 28, 44. Future studies should investigate possible roles of PIK3CA in metastasis. Our findings advocate that pathway-specific therapies targeting these two pathways should be pursued in OSCC.

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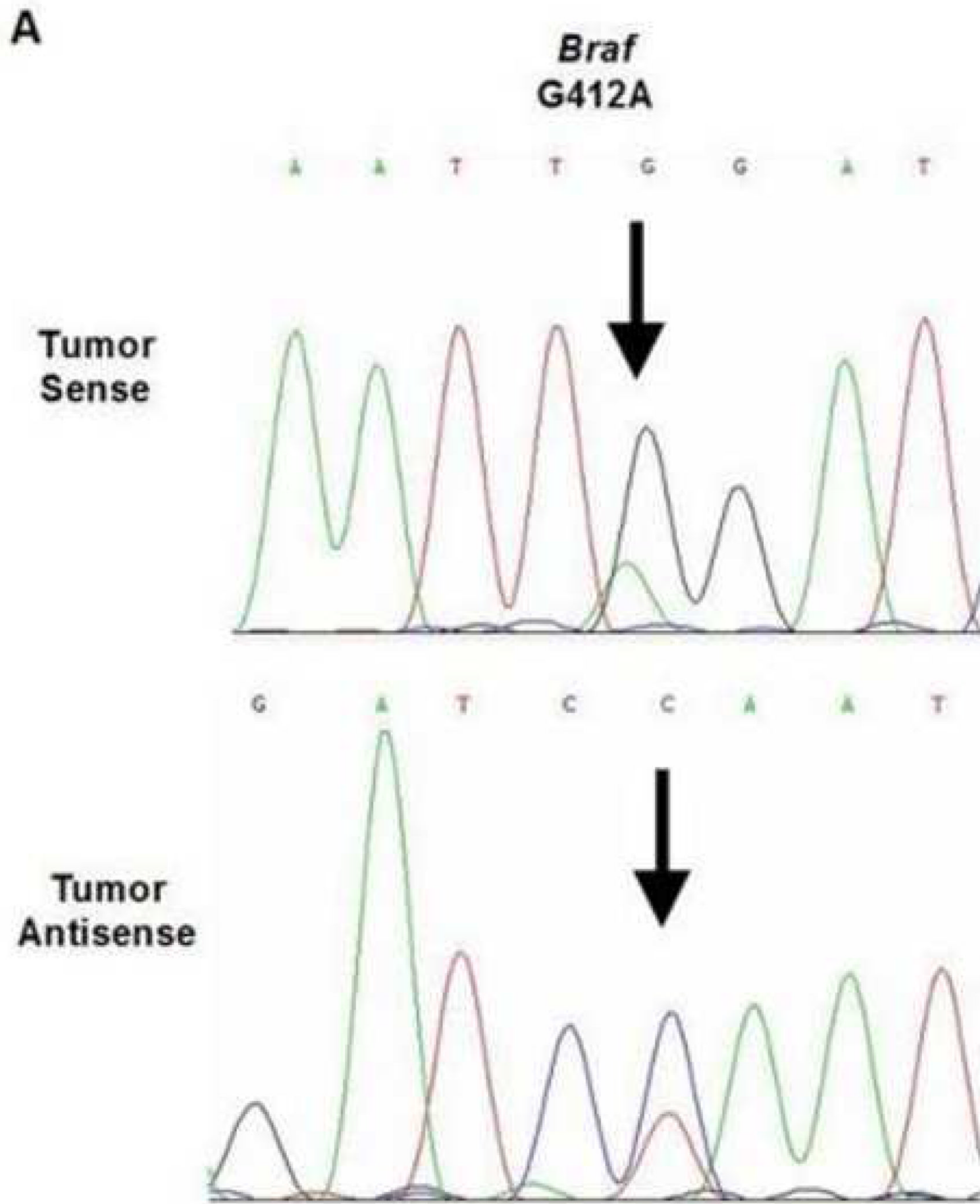

Figure 1.
BRAF mutation in a maxillary alveolus OSCC. A. Direct genomic sequencing result. All mutations within the nucleotide sequences are indicated by the black arrows. All mutations were verified by a second independent sequencing analysis and anti-sense sequencing.

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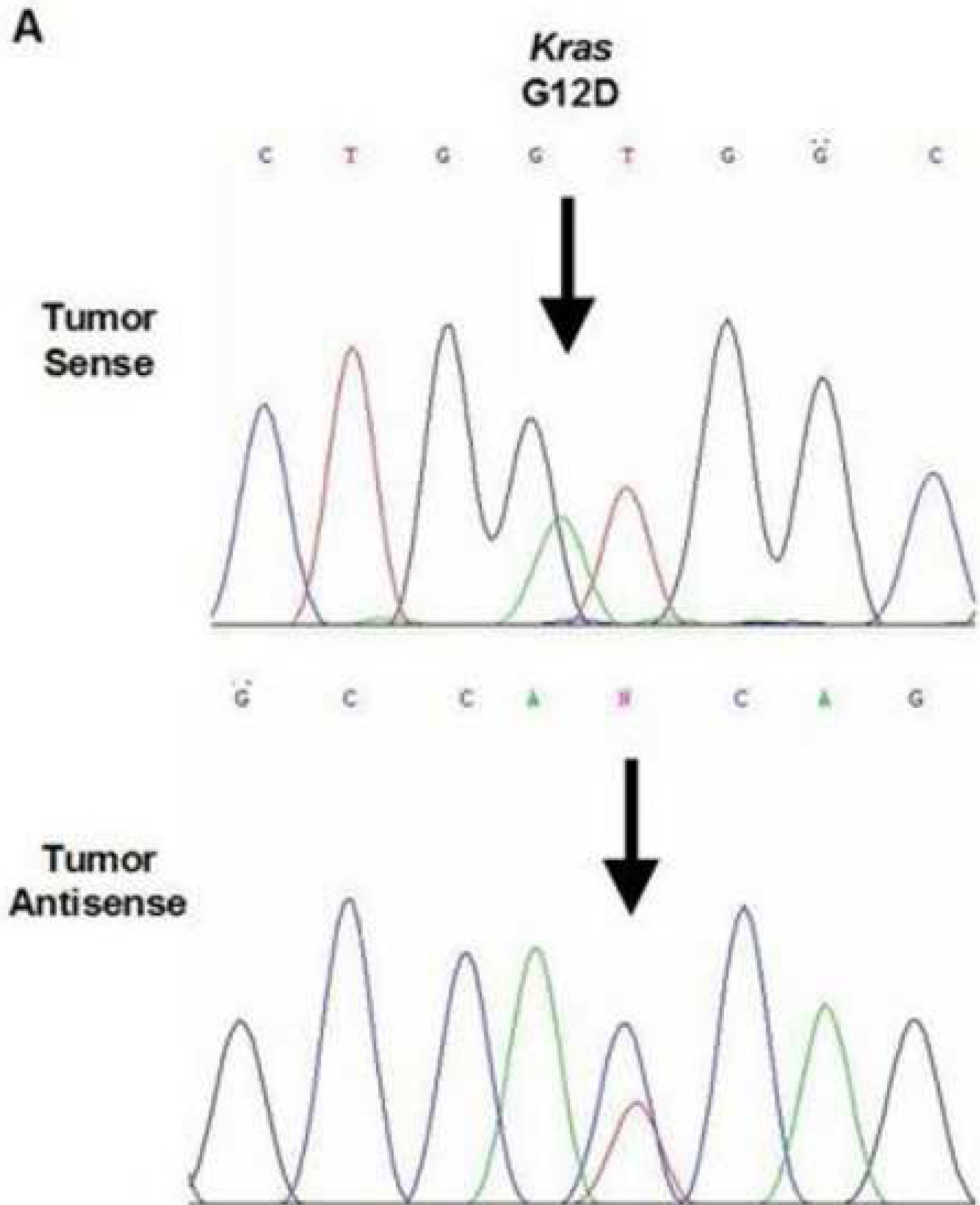

Figure 2.
KRAS mutation in a lateral tongue OSCC. A. Direct genomic sequencing result. All mutations were verified by a second independent sequencing analysis and anti-sense sequencing.

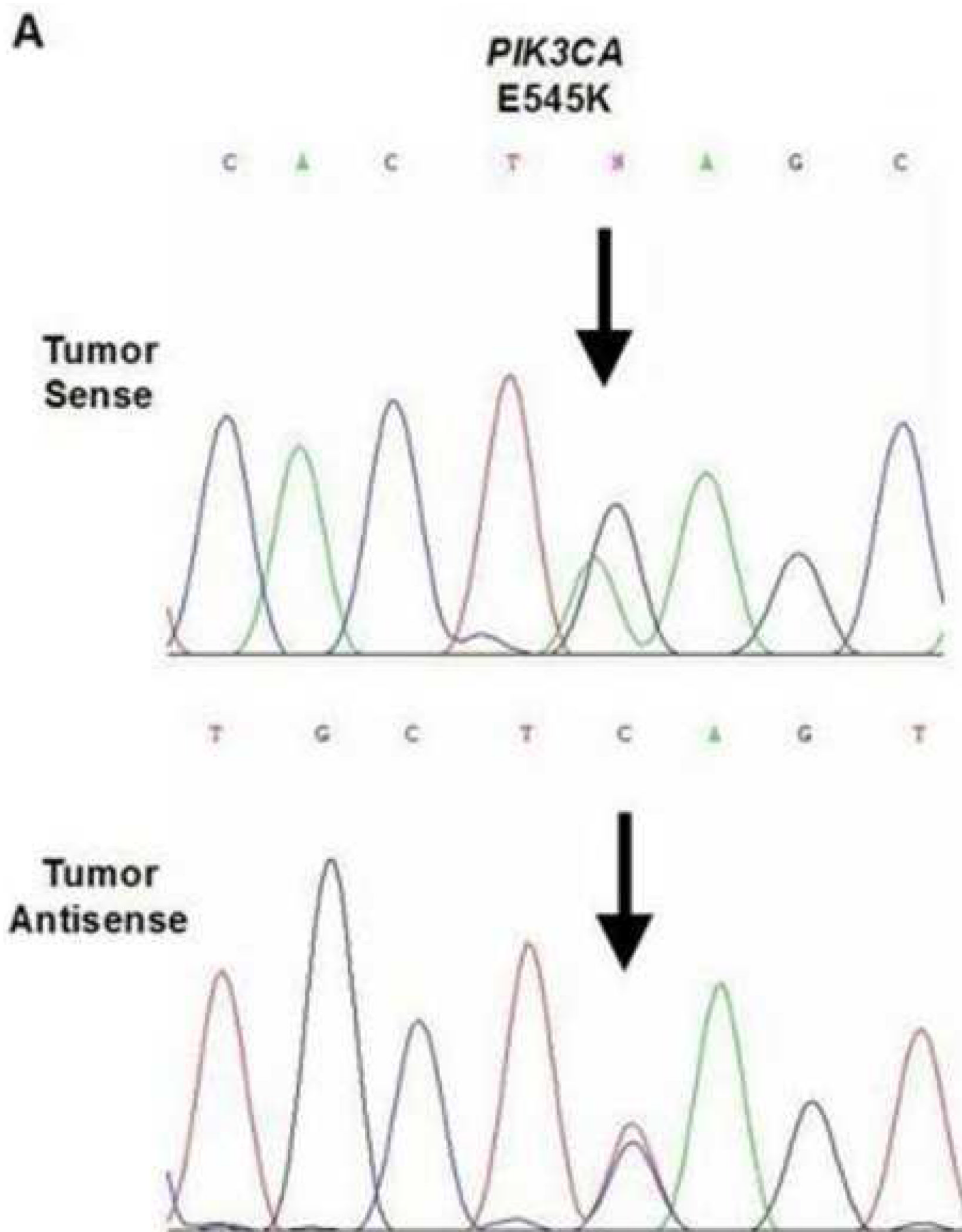

Figure 3.

PIK3CA mutation in a ventral tongue OSCC. A. Direct genomic sequencing result. All mutations were verified by a second independent sequencing analysis and anti-sense sequencing.

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Table 1

Summary of clinicopathologic data and identified mutations in OSCCs

Case	Gender	Age	Location	Tumor differentiation	Genes analyzed *BRAF*	*KRAS*	*PIK3CA*	Mutation
1	M	44	R buccal mucosa	well	Yes	Yes	Yes	No
2	F	82	Alveolar ridge	well	Yes	Yes	Yes	No
3	M	54	R soft tissue	well	Yes	Yes	Yes	No
4	F	73	Gingiva	moderate	Yes	Yes	Yes	No
5	F	57	R ventrolateral tongue	moderate-poor	Yes	Yes	Yes	No
6	F	78	L maxillary alveolus	moderate-poor	Yes	Yes	Yes	Yes *(BRAF)*
7	M	59	R lateral tongue	poor	Yes	Yes	Yes	No
8	M	71	R lateral tongue	poor	Yes	Yes	Yes	No
9	M	88	L mandibular mucosa	poor	Yes	Yes	Yes	No
10	M	28	L mandibular alveolar ridge	well	Yes	Yes	Yes	No
11	F	62	R retromolar area	well	No	Yes	No	No
12	M	68	Anterior floor of mouth	well	Yes	Yes	Yes	No
13	F	84	L alveolar ridge	well	Yes	Yes	Yes	No
14	F	84	L buccal mucosa	well-moderate	Yes	Yes	Yes	No
15	F	57	R ventrolateral tongue	well-moderate	Yes	Yes	No	No
16	F	62	R lateral tongue	moderate	Yes	No	No	No
17	M	59	buccal alveolar ridge	moderate	Yes	Yes	Yes	No
18	M	75	R buccal mucosa	moderate	Yes	Yes	No	No
19	F	73	R lateral tongue	moderate	Yes	Yes	No	No
20	M	88	Ventral tongue	moderate-poor	Yes	Yes	Yes	Yes *(PIK3CA)*
21	F	74	L floor of mouth	poor	Yes	Yes	No	No
22	M	60	L lateral tongue	poor	Yes	Yes	Yes	Yes *(KRAS)*
23	F	91	L posterior maxilla	well	Yes	Yes	Yes	No
24	F	86	R lateral tongue	moderate	No	Yes	No	No
25	M	73	L floor of mouth	poor	Yes	Yes	Yes	No
26	M	75	R maxillary alveolus	well	Yes	Yes	Yes	No

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Case	Gender	Age	Location	Tumor differentiation	Genes analyzed			Mutation
					BRAF	*KRAS*	*PIK3CA*	
27	F	79	R mandibular alveolar ridge	well	Yes	Yes	No	No
28	M	72	Floor of mouth	well-moderate	No	Yes	Yes	No
29	F	88	R mandibular alveolar ridge	well-moderate	Yes	Yes	Yes	No
30	M	63	R maxillary alveolus	well-moderate	Yes	Yes	Yes	No
31	F	89	R mandibular region	well, focal poor	Yes	Yes	Yes	No
32	M	34	R lateral tongue	well-moderate	Yes	Yes	Yes	No
33	M	83	R lateral tongue	well	Yes	Yes	Yes	No
34	M	80	Posterior hard palate	well	Yes	Yes	Yes	No
35	M	60	R mandibular retromolar pad	well	Yes	Yes	Yes	No
36	F	75	L lateral tongue	well-moderate	Yes	Yes	Yes	No
37	F	70	L posterolateral tongue	moderate	Yes	Yes	Yes	No
38	M	67	L lateral tongue	moderate-poor	Yes	Yes	Yes	No
39	F	38	R mandibular gingiva	well	Yes	Yes	Yes	No
40	M	78	L anterior floor of mouth	poor	Yes	No	No	No
41	F	78	L floor of mouth	well	Yes	Yes	Yes	No
42	F	41	L border (tongue?)	moderate-poor	Yes	No	No	No
43	F	68	L mandibular retromolar pad	poor	Yes	Yes	Yes	No
44	F	75	R maxillary coronal sulcus	well-moderate	Yes	Yes	Yes	No
45	F	77	R buccal vestibule	well	Yes	Yes	Yes	No