Novel mutations of the HRAS gene and absence of hotspot mutations of the BRAF genes in oral squamous cell carcinoma in a Greek population

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Abstract. Oral squamous cell carcinoma (OSCC) is the sixth most common cancer in the world. The phosphatidylinositol 3 kinase (PI3K) signalling pathway has been reported to play an important role in OSCC. Since we have previously detected absence of hotspot PIK3CA gene mutations in the Greek population, we hypothesized that BRAF or HRAS may be activated as upstream effectors of the pathway. Furthermore, the status of the HRAS and BRAF mutations in OSCC has never been assessed before in the Greek population. Eighty-six primary paraffin-embedded tumors were screened for BRAF and HRAS hotspot mutations. In HRAS, two hotspot mutations in codon 12 (2.3%) and eight new genetic alterations were detected (8.6% overall). One new missense mutation, Alanine53Valine (Ala53Val), one silent mutation, two mutations in the 5'UTR region and four mutations in intron 1 were detected. No hotspot mutations in BRAF were found. A new silent mutation/polymorphism T1803C was detected at a percentage of 30%. This study is the first to report HRAS mutations in the Greek population. The results suggest that RAS is an important member of the PI3K signalling pathway and may play a role in the tumorigenesis of OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) is a subset of head and neck squamous carcinoma (HNSCC) involving the oral tongue, upper gingival, lower gingival and alveolus, floor of the mouth, buccal mucosa, retromolar triangle, lip mucosa and hard palate. OSCC is considered to be the sixth most common cancer in the world (1). Smoking and alcohol consumption are major risks for HNSCC but only a fraction of people with these habits actually develop oral cancer, which implies that other genetic factors contribute to the pathogenesis of the disease (2,3). Infection by human papilloma virus has also emerged as a risk factor for HNSCC (4).

The RAS family genes consist of three members, HRAS, KRAS and NRAS located on chromosomes 11, 12 and 1, respectively, encoding a 21 kDa membrane protein (p21), which possesses GTPase activity (5,6). HRAS has been shown to activate phosphatidylinositol 3 kinase (PI3K) more efficiently than KRAS and NRAS. Hotspots for RAS mutations are found in codons 12, 13 and 61, causing the mutant protein to lose its ability to exchange GTP with GDP thus remaining activated (7). RAS is also is an upstream regulator of PI3K. Thus an activated RAS could also activate the PI3K signalling pathway, which is shown to be activated in oral cancer (8,9). The incidence of RAS mutations varies greatly among different human tumors and different ethnicities. HRAS mutations are primarily found in cancers of the urinary tract and bladder. The incidence of RAS mutations in OSCC shows

racial disparity, being more frequent in India and South East Asia and less common in the West (10-12).

BRAF is one of the most commonly mutated oncogenes in human cancer. BRAF is a serine/threonine kinase of the RAF family, which consists of three genes, namely ARAF, BRAF and CRAF (5). More than 90% of the reported BRAF mutations occur in its kinase domain regarding a single nucleotide substitution V600E (also referred to as V599E according to the numbering system used) (13). These mutations lead to constitutive activation of BRAF, regardless of activation by RAS, which in turn leads to constitutive activation of MEK and ERK, leading to proliferation and survival of cancer cells. Furthermore, it has been widely documented that mutations affecting RAS and BRAF both in human cancers and in chemically induced mouse liver tumors seem to be mutually exclusive, suggesting the operation of alternative pathways for BRAF (14,15).

In view of the paucity of extensive studies regarding the status of BRAF and HRAS mutations in OSCC and to i) further assess the individual effect of the HRAS and BRAF mutations in OSCC, ii) to test whether their mutations are mutually exclusive, as in other cancers, and iii) to examine whether RAS is the activating factor of the PI3K signalling pathway previously reported (3,8,9) we performed a systematic molecular study in 86 clinically and histologically well-defined malignant tumors of oral squamous cell carcinoma, previously reported to lack PIK3CA hotspot mutations (15) and correlated the mutation status of HRAS and BRAF with age, gender, stage, histological grade and prognosis.

Materials and methods

Study population. Specimens from 86 consecutive patients, who underwent a major operation as part of their cancer treatment, were collected and retrieved from the Department of Oral and Maxillofacial Surgery at the Evangelismos Hospital, University of Athens, Athens, Greece, from January 2003 to December 2007. Most of them had advanced stages of the disease. In addition, tissue samples of the oral cavity from 80 healthy control volunteers, age and gender matched, were also collected from a similar population. We specifically chose samples from the primary tumor of each original specimen where cancer cells were identified by the pathologists. Demographic, preoperative and postoperative data were available for all patients. The patients were staged according to the International Union Against Cancer (16). The study protocol was approved by both the Ethics Committees of Evangelismos General Hospital and the Ethics Board of Medical School, University of Athens. All participants provided informed consent prior to entry to the study.

Extraction of genomic DNA. All samples were formalin-fixed and paraffin-embedded, and genomic DNA was extracted using the QIAmp extraction DNA kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. Concentration of the genomic DNA was assessed by the GenQuant spectrometer (Pharmacia Biotech Inc., Piscataway, NJ).

PCR analysis and DNA sequencing. Nested PCR was used for the detection of any point mutations in exons 2 and 3 of the HRAS gene, followed by direct sequencing. The sequences of the 2 oligonucleotide primer pairs were as follows: (1a) exon 2 forward 5'-ATCACTGGGTCATTAAGACAAGTGG-3' and exon 2 reverse 3'-CAGGCTCACCTCTATAGTGGGGTC-5', and (1b) exon 2 nested forward 5'-AGGTAGGCACGCTG CAGTCCTT-3' and exon 2 nested reverse 3'-AAATGGTTC TGGATCAGCTGGATG-5' (2a) exon 3 forward 5'-ATGAGA GGTACCAGGGAGAGGCT-3' and exon 3 reverse 3'-TGT ACTGGTGGATGTCCTCAAAAGACT-5' (2b) exon 3 nested forward 5'-ATTCCTACCGGAAGCAGGTGGTCAT-3' and exon 3 nested reverse 3'-AGACTTGGTGTTGTTGATGGCA AAC-5' (3a) exon 15 BRAF forward 5'-TCTTCATAATGCT TGCTCTGATAGG-3' exon 15 reverse 3'-CAGCATCTCAGG GCCAAAAATT-5' and (3b) exon 15 nested forward 5'-TAG GAAAATGAGATCTACTGTTTTCCTT-3' and exon 15 nested reverse 3'-TACCATCCACAAAATGGATCCAGAC-5'. The nested polymerase chain reaction (PCR) reaction was carried out in a total volume of 25 μl in a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA). The mixture of the PCR reaction contained 2.5 μl 10X reaction buffer, 3 μl dNTP (2.5 mM of each dNTP), 1.75 μl forward primer (10 μM), 1 μl reverse primer (25 pmol), 0.75 μl $MgCl_2$ (50 mM), 0.25 μl Platinum Taq DNA polymerase (5 U/μl), and 2 μl DNA template. For the nested PCR, DNA template was diluted 1:50 and one of the final concentration was used. Amplification was carried out with 5 min of initial denaturation at 94˚C followed by 36 cycles of denaturation at 94˚C for 45 sec, primer annealing at 55˚C for the BRAF and at 58˚C for the HRAS for 45 sec, and extension at 72˚C for 45 sec and final extension at 72˚C for 3 min. The PCR products were subsequently analyzed on a 4200 Two-Dye DNA Analysis System (LI-COR Biosciences, Lincoln, NE). (HRAS accession no. BC095471 and BRAF accession no. BC1120791).

Statistical analysis. All the available data were entered into a database using SPSS statistical software (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL). The χ^2 test was also used for a comparison of qualitative variables. The P-value was found to be significant when it was <0.05.

Results

Out of the 86 patients, 17 (19.8%) were stage I, 12 (14%) were stage II, 14 (16.3%) were stage III and 43 (50%) were stage IV, according to pTNM stage. Thirty-one patients (36%) had cancer of the oral tongue, 8 (9.3%) of the floor of the mouth, 25 (29.1%) of the lower gingival and alveolus, 4 (4.7%) of the upper gingival, 9 (10.5%) of the buccal mucosa, 6 (7%) of the retromolar triangle and 3 (3.5%) patients had cancer of the lip mucosa. The histological grade was well-differentiated in 17 (21%) of the patients, moderately-differentiated in 56 (69.1%) of the patients and poorly-differentiated in 8 (9.9%) of the patients. The clinical characteristics of the patients studied and the genetic alterations of the BRAF and HRAS gene are summarized in Table I.

The V600E hotspot mutation was not detected in any of the 86 specimens analyzed. Nevertheless, a new silent mutation/polymorphism was detected in 10 out of 86 specimens (8.6%) (accession no. BC1120791). This consists a novel finding and was entered in the Ensemble database (ssbraf-172036). It refers

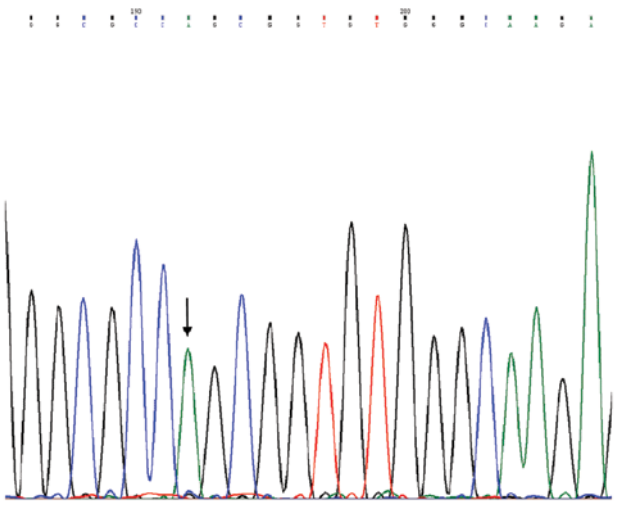

Figure 1. Gly12Ser. Hotspot codon 12 exon 2. Sequence chromatogram fragment of the HRAS gene exon 2 as determined by automated sequence analysis. The arrow indicates the substitution of a guanine by an adenine in a homozygous sample (accession no. BC095471.1).

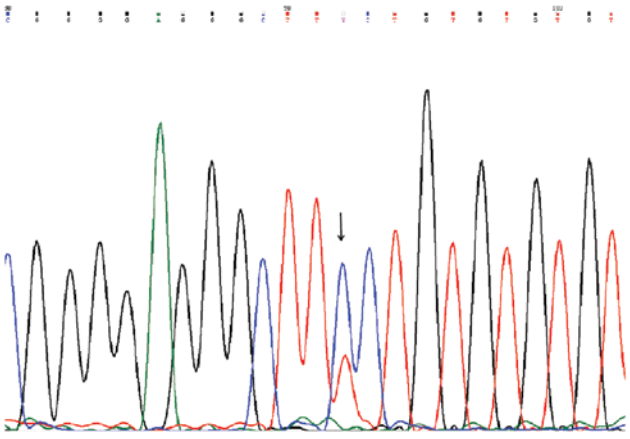

Figure 2. Phenylalanine78Phenylalanine(F78F) exon 3. Sequence chromatogram fragment of the HRAS gene as determined by automated sequence analysis. The arrow indicates the 2 peaks detected (both wild-type cytosine and substitution by a thymine) in a heterozygous sample (accession no. BC095471.1).

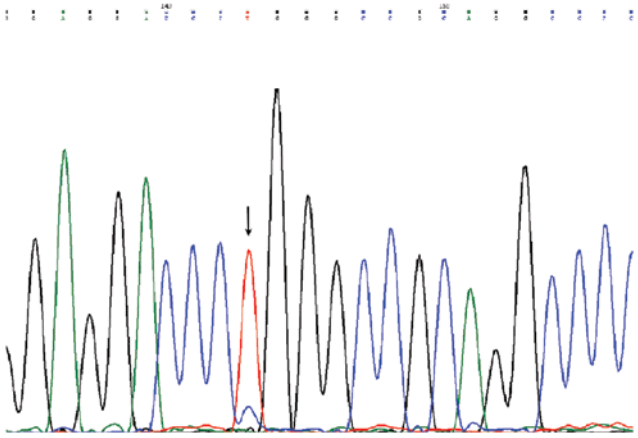

Figure 3. Ala18Val exon 2. Sequence chromatogram fragment of the HRAS gene as determined by automated sequence analysis. The arrow indicates the substitution of cytosine by a thymine in a homozygous sample (accession no. BC095471.1).

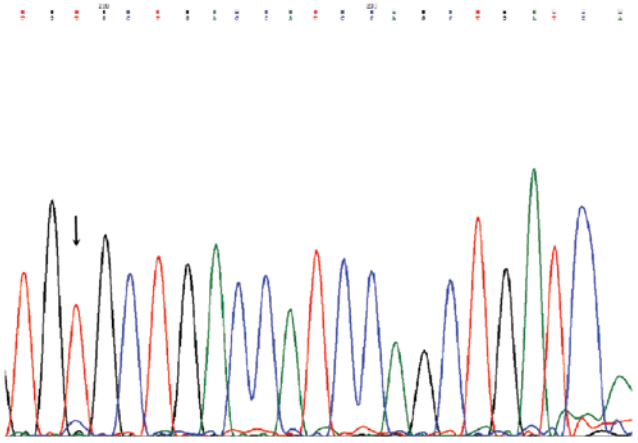

Figure 4. C149T exon 2. Sequence chromatogram fragment of the HRAS gene as determined by automated sequence analysis. The arrow indicates the substitution of cytosine by a thymine in a homozygous sample (accession no. BC095471.1).

to exon 15, where thymine is substituted by a cytosine (cDNA position T1803C) and results in a silent mutation. All specimens harbouring this polymorphism were heterozygous. This polymorphism was found in none of the 80 controls specimens ($P<0.001$).

Out of 86 tumors 2 (2.3%) had a hotspot mutation in codon 12 of the HRAS gene (Gly12Ser) (accession no. BC095471.1) (Fig. 1). Four new mutations were detected. Two of the new mutations harboured HRAS exon 2. Both were C→T transitions. One was a silent mutation (phenylalanine to phenylalanine, Phe78Phe) (Fig. 2) harbouring exon 3, while the other was a missense mutation (Alanine to Valine) Ala18Val harbouring exon 2 (accession no. BC095471.1) (Fig. 3). The specimen harbouring this missense mutation was homozygous. Another mutation harboured the 5'-UTR (Cytosine149Thymine) of the same exon (accession no. BC095471.1) (Fig. 4). It was a C/T (Cytosine/Thymine) transition and the specimen was homozygous for it. Another mutation was located in the intravening sequence 1 (G3542A). The specimen harbouring this mutation was heterozygous. It should be noted that one patient harboured both the new missense mutation Ala18Val and the 5'-UTR mutation homozygously. To our knowledge, there is no information available in the literature on these mutations. As these mutations are not stored in the COSMIC (Catalog Of Somatic Mutations In Cancer), a database of Sanger Institute, UK, this is the first report of such mutations in OSCC.

Furthermore, three already registered single nucleotide polymorphisms harbouring HRAS exon 2 were detected. The polymorphism rs61877782 was found in 2.3% of the specimens (2 of 86). The polymorphism rs41294870 was found in 3 out of 86 tumors (3%), whereas the polymorphism rs112587690 was found in 12 out of 86 tumors (14%).

Discussion

The mutation frequency of BRAF has been reported to be 59% in melanomas, 18% in colorectal cancers and 14% in

Table I. Clinical characteristics and HRAS and BRAF genetic alterations in patients with oral squamous cell carcinoma.

Case no.	Gender	Age	Tumor location	Histological grade	Stage	HRAS (accession no. BC095471)	BRAF (accession no. BC1120791)
1	M	56	Floor of mouth	Moderate	IV		
2	F	54	Floor of mouth	Moderate	IV		
3	F	54	Buccal mucosa	Moderate	IV		
4	F	71	Upper gingiva	-	II		ssBRAF-172036
5	F	69	Lower gingiva	Poor	IV		
6	M	66	Floor of mouth	Moderate	IV		
7	F	56	Lower gingiva	Moderate	IV		
8	M	43	Retromolar triangle	Well	I	rs112587690	
9	F	70	Buccal mucosa	Moderate	III		
10	F	78	Oral tongue	Moderate	II		
11	M	80	Lower gingiva	Moderate	IV		
12	F	26	Oral tongue	Well	III		
13	F	48	Oral tongue	Moderate	III		ssBRAF-172036
14	M	41	Buccal mucosa	Moderate	III		ssBRAF-172036
15	F	91	Lip mucosa	Moderate	III		ssBRAF-172036
16	F	65	Lip mucosa	Moderate	I		ssBRAF-172036
17	F	72	Lower gingiva	Moderate	I	rs112587690	
18	M	45	Floor of mouth	Well	I		
19	M	45	Lower gingiva	-	IV		
20	F	79	Oral tongue	Poor	IV		
21	M	67	Upper gingiva	Moderate	IV		
22	F	30	Oral tongue	Well	IV		
23	M	65	Retromolar triangle	Moderate	III		
24	M	65	Retromolar triangle	Moderate	III		ssBRAF-172036
25	F	75	Oral tongue	Moderate	IV		
26	F	-	Lower gingiva	Moderate	IV		
27	M	74	Retromolar triangle	Moderate	IV		
28	F	54	Buccal mucosa	-	IV	rs112587690	
29	M	69	Lower gingiva	Moderate	IV		
30	F	76	Retromolar triangle	Moderate	IV		
31	M	55	Lip mucosa	Moderate	I		ssBRAF-172036
32	M	51	Lower gingiva	Well	IV		
33	F	61	Oral tongue	Moderate	IV		
34	F	69	Oral tongue	Poor	III		ssBRAF-172036
35	F	87	Buccal mucosa	Well	I		
36	M	64	Upper gingiva	Well	I	rs112587690	
37	M	24	Oral tongue	Moderate	IV		
38	M	62	Oral tongue	Moderate	III		
39	M	57	Oral tongue	Moderate	III		
40	M	73	Floor of mouth	Moderate	IV		
41	F	74	Lower gingiva	Moderate	IV		
42	M	31	Oral tongue	Moderate	I		
43	M	55	Floor of mouth	Moderate	III		
44	F	-	Buccal mucosa	Moderate	I		
45	M	58	Floor of mouth	Moderate	IV		
46	F	63	Buccal mucosa	Moderate	II	rs112587690	
47	F	73	Retromolar triangle	Moderate	II		
48	M	44	Oral tongue	Well	IV		
49	F	78	Oral tongue	Moderate	IV		
50	M	61	Lower gingiva	Moderate	IV		
51	M	61	Lower gingiva	Moderate	IV		ssBRAF-172036

Table I. Continued.

Case no.	Gender	Age	Tumor location	Histological grade	Stage	HRAS (accession no. BC095471)	BRAF (accession no. BC1120791)
52	M	73	Lower gingiva	Moderate	IV		
53	M	37	Lower gingiva	Moderate	IV		
54	F	79	Lower gingiva	Well	IV		
55	M	46	Oral tongue	Poor	IV		
56	F	72	Oral tongue	Poor	II		
57	F	72	Lower gingiva	Moderate	I	G3542A	
58	F	48	Lower gingiva	Poor	IV	rs61877782	
59	F	26	Oral tongue	Well	III		
60	F	47	Oral tongue	Moderate	II		
61	M	46	Oral tongue	Moderate	II		ssBRAF-172036
62	M	57	Oral tongue	Poor	IV		
63	F	76	Lower gingiva	Moderate	III	F78F	
64	F	81	Oral tongue	Moderate	I	Hotspot codon 12	
65	M	65	Lower gingiva	Well	I		
66	F	73	Oral tongue	Moderate	II	rs61877782	
67	F	56	Lower gingiva	Moderate	IV		
68	F	68	Oral tongue	Well	I		
69	F	74	Buccal mucosa	Moderate	IV		
70	M	75	Oral tongue	Moderate	IV	rs112587690	
71	F	68	Upper gingiva	Moderate	IV	rs112587690	
72	F	66	Oral tongue	Moderate	II	Hotspot codon 12	
73	M	62	Lower gingiva	Moderate	I		
74	M	62	Floor of mouth	Well	I	rs41294870	
75	F	60	Lower gingiva	Poor	IV		
76	F	82	Lower gingiva	Moderate	IV		
77	F	75	Oral tongue	Well	I	rs41294870	
78	M	70	Buccal mucosa	Moderate	IV		
79	M	58	Oral tongue	Moderate	III	A18V,C149T	
80	M	50	Oral tongue	Well	I		
81	M	52	Oral tongue	Well	II	rs112587690	
82	M	57	Lower gingiva	Moderate	II	rs41294870	
83	M	64	Lower gingiva	Moderate	II	rs112587690	
84	M	51	Lower gingiva	Well	IV	rs112587690	
85	M	55	Oral tongue	-	IV	rs112587690	
86	M	69	Oral tongue	-	IV	rs112587690	

liver cancers (13). Weber *et al* analysed the BRAF mutation status in HNSCC of various sites in Germany by the use of PCR and sequencing and reported a 3% mutation frequency in their pharynx and hypopharynx specimens but none in specimens of the oral cavity (18). Davies *et al* analyzed 19 primary HNSCC using capillary-based modified heteroduplex mutation detection method in exons 11 and 15 and found no mutations (13). Al Sheikh Ali *et al* reported lack of BRAF mutations in 91 Japanese HNSCC as well as 12 HNSCC cell lines by SSCP and MASA analyses (19). Shelly *et al* conducted a study in canine oral cancer specimens and found no BRAF mutation in their cohort of samples (20). There is limited data regarding the implication of BRAF in OSCCs. Bruckman *et al* performed a study in the USA in OSCC tumors and reported a frequency of 2% (21).

RAS genes have been found to be activated in a wide variety of human tumors, including colon carcinoma and pancreatic carcinoma for which a 40 and 95% incidence of RAS gene activation has been demonstrated, respectively (22,23). However, the incidence of RAS gene mutation is low in breast cancer and stomach cancer (24,25). A high incidence of RAS mutation, 18-30% of cases from India and Vietnam, has been reported and it has been associated with areca nut chewing (3,10,26) whereas lower frequencies of 4-5% were reported from UK and Japan (27-29).

The present study showed lack of BRAF mutations in OSCC in the Greek population. This is in accordance with previous studies showing minor or no contribution of the BRAF mutations to the HNSCC (12,17,18) in the German and Japanese population and a minor contribution of the BRAF

gene to the OSCC in a study conducted in the USA (20). The new polymorphism (T1803C) was detected at a frequency of 8.6% in the cancer specimens and in no specimen in the control group. No correlation was found between the polymorphisms and age, gender, stage, histological grade and prognosis. Thus, further studies with larger sample sizes are necessary in order to elucidate statistical differences and delineate any potential role of this silent mutation/polymorphism.

Hotspot mutations in the HRAS gene were found in two cases (2.3%), which consists a relatively low frequency, all of them harbouring codon 12. Nevertheless, another four mutations were detected in our specimen, resulting in an overall frequency of HRAS genetic alterations of 4.7%. None of them have been previously reported. Although, only one of them resulted in an amino acid change (Ala18Val), further studies are needed in order to test whether the other mutations detected in the exon 2 (Phe78Phe), in the 5'-UTR region (untranslated region) or in the intronic region have any impact on the RNA splicing or the regulation of the gene and to further examine the function of the protein encoded. Nevertheless, these genetic alterations probably demonstrate the stress imposed on the gene by carcinogens.

Since no BRAF mutations were detected, no specimen harboured the BRAF and HRAS hotspot mutations. Furthermore, the same specimens have been previously examined for PIK3CA hotspot mutations, but none have been detected (15). Therefore, it is possible that the upstream effector HRAS may contribute to the activated PI3K signalling pathway as previously reported (7,8).

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