

Consistent absence of BRAF mutations in salivary gland carcinomas

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Abstract

Introduction: Malignant salivary gland tumors are rare entities. Despite advances in surgery, radiation therapy and chemotherapy, the rate of the mortality and five-year survival has not been improved markedly over the last few decades. The activation of EGFR-RAS-RAF signaling pathway contributes to the initiation and progression of many human cancers, promising a key pathway for therapeutic molecules. Thus, the objective of this study was to evaluate BRAF mutations in salivary gland carcinomas. **Methods:** We designed PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) and screened 50 salivary gland carcinomas (SGCs) including mucoepidermoid carcinoma (MEC), adenoid cystic carcinoma (AdCC) and polymorphous low grade adenocarcinoma (PLGA) for the BRAF V600E mutation. **Results:** PCR-RFLP analyses demonstrated no mutation in BRAF exon 15 for SGC samples at position V600, which is the most commonly mutated site for BRAF in human cancer. **Conclusions:** According to our results SGCs didn't acquire BRAF mutations that result in a constitutive activation of the signaling cascade downstream of EGFR, hence SGCs can be a good candidate for anti EGFR therapies.

Key Words: BRAF, EGFR, Salivary gland carcinoma.

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Introduction

Malignant salivary gland tumors are rare, representing less than 5% of all head and neck cancers (1, 2). Despite advances in surgery, radiation therapy and chemotherapy, the rate of the mortality and five-year survival has not been improved markedly over the last few decades(3).

Mucoepidermoid carcinoma (MEC), adenoid cystic carcinoma (AdCC) and polymorphous low grade adenocarcinoma (PLGA) are the most frequent salivary gland carcinomas (SGCs). As histological vary wildly between different subtypes of salivary gland tumors, differentiation can be difficult.

Several genetic alterations such as activation of the EGFR-RAS-RAF cascade pathway contribute to the initiation and progression of cancers (4-7).

The RAS-RAF-MEK-ERK-MAP kinase signaling pathway is pivotal for the control of cell proliferation and differentiation. The signal of transduction in epidermal growth factor receptor (EGFR) pathway enables cancer cells to proliferate and metastasis (8-12). BRAF gene encodes a serine/threonine kinase that plays an important role in the mitogen-activated protein kinase signaling pathway (13).

The mainly common gene mutation is the transversion of thymine-to-adenine at the nucleotide position 1799 in exon 15, resulting in a valine-to-glutamate substitution at residue 600 (c. 1799 T>A, p.V600E). This missense mutation was demonstrated to activate kinase activity of the protein by simulating phosphorylation (14).

New targeted therapy options in salivary gland carcinomas can be developed by detecting molecular pathways in these cancers (5).

The aim of the present study was to determine the BRAF V600E mutation frequency in SGCs of the frequent histological types and design PCR- RFLP (Polymerase Chain Reaction -Restriction Fragment Length Polymorphism) as an effective screening method.

Thus, the objective of this study was to evaluate BRAF mutations in salivary gland carcinomas.

Materials and Methods

Tissue specimens and extraction of genomic DNA

In this retrospective study we analyzed the paraffin embedded biopsies of patients with salivary gland carcinomas. Specimens from patients and normal control subjects were retrieved from the Qaem and Omid Hospitals and Department of Oral and Maxillofacial Pathology, Mashhad University of Medical Sciences, Iran. Tumor samples were obtained from 50 patients (48% males and 52% females with a

mean age at diagnosis 55 years) treated with the histological diagnosis of a SGC including 24 MECs, 23 AdCCs and 3 PLGAs according to WHO classification (15). A whole number of 20 non-neoplastic and normal salivary glands were obtained from the normal tissue adjacent to the oral lesion samples (control group). Colorectal cancer sample with BRAF V600E mutation was used as positive control (Fig 2).

Histological type and quality verification were interpreted by two pathologists and when there was a disagreement a third pathologist reviewed the features (Fig1).

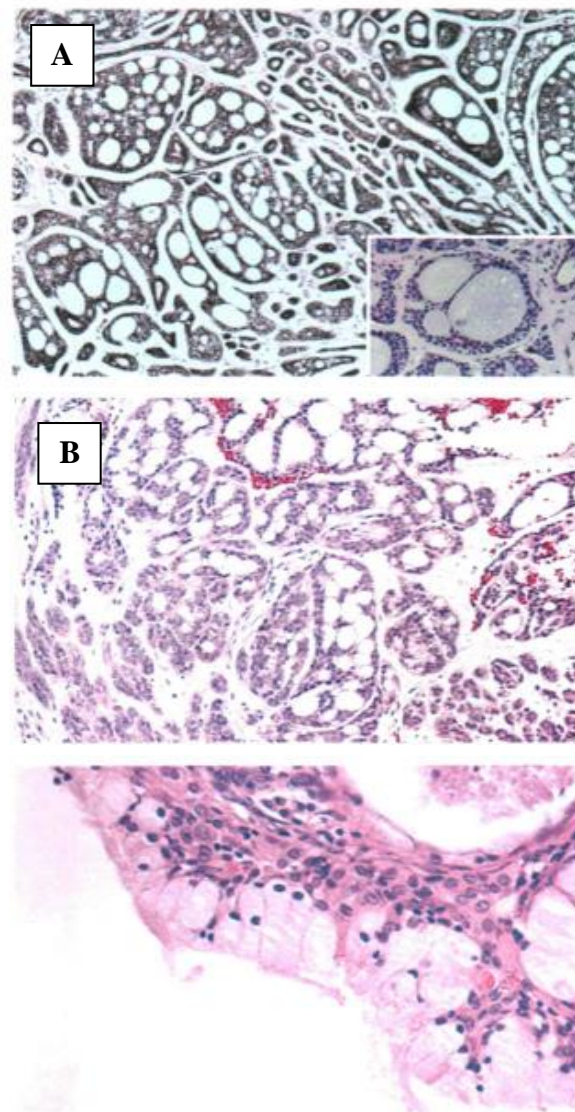


Figure 1: (A) Adenoid cystic carcinoma with islands of hyperchromatic cells forming cribriform and tubular structures, H & E, original magnification 100X. (B) Polymorphous Low-grade adenocarcinoma with cribriform arrangement of uniform tumor cells with pale-staining nuclei, H&E, original magnification 100X. (C) Mucoepidermoid carcinoma: low grade tumor with numerous large mucous cells surrounding a cystic space, H&E, original magnification 400X.

Parotid was the most common site of MECs, whereas minor salivary glands were most frequently involved in PLGAs and AdCCs.

All samples were formalin-fixed, paraffin-embedded and genomic DNA was extracted using the QIAamp extraction DNA kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. Concentration of genomic DNA was assessed by a Nano drop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

Mutation Analysis

The primers (Mt-F and Mt-R) were designed to introduce a BtsI restriction site in the wild allele for the analysis of codon 600 (Table 1). The PCR was carried out in a 25- μ L volume containing 10-PCR buffer, 1.5 mmol/L of MgCl₂, 0.2mol/L of each primer of Mt-F and Wt-R, 0.1 mmol/L of dNTP, 0.625 unit of Taq polymerase (Ampli-Taq Gold; Perkin-Elmer, Foster City, CA) and 50 ng of DNA. PCR conditions were as follows: 95°C for 11 minutes and 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds,

and finally 5 minutes at 72°C. Aliquots (5- μ L) of the first-stage PCR were digested with 10 units of the restriction enzyme BtsI (New England Biolabs, Inc, Beverly, MA) at 37°C for 3 hours; 1- μ L aliquots of the intermediate BtsI digests were then used in a second-stage PCR, under the same conditions as the first-stage PCR but with Mt-F and Mt-R primer (Table 1). Next, 25- μ L of the products which were obtained after the second stage PCR was digested with BtsI at 37°C for more than 6 hours. If there was a mutation in codon 600 of the *BRAF* gene, the second stage PCR product was cleaved into 112 base pair (bp) and 18 bp fragments, whereas if there was no codon 599 mutation, we expected to see fragment lengths of 78 bp, 34 bp, and 18 bp. All products were visualized on 3% agarose gels stained with ethidium bromide. If the first PCR step does not seem to be necessary, it may be possible to omit it without losing sensitivity in the detection of *BRAF* codon 600 mutations.

Table 1. Primer Sequence and PCR Conditions for PCR/RFLP Analysis

Gene	Primer Name	Primer Sequence (5'-3')	Position*	Annealing Temperature (°C)
Mt-F		TAAAAATAGGTGATTTTGGTCTAGCTGC	1011446-1011473	
BRAF	Wt-R	CCAAAAATTTAATCAGTGGAAAAATA	1011342-1011364	58
Mt-R		AAAAATTTAAGCAGTGGAAAAATAGC	1011344-1011369	

* The nucleotide numbering for *BRAF* refers to accession NT_007914.12

Results

According to RFLP results 34bp, 78bp and 112bp should be detected in mutant samples, whereas for normal samples 34bp and 78bp should be observed. Using our designed enriched RFLP analyses, no mutation was detected in BRAF exon 15 for SGC samples at position V600, which is the most commonly mutated site for BRAF in human cancer. Colorectal cancer sample with BRAF V600E mutation was used as positive control (Fig 2).

It should be mentioned that the similar results was also obtained in 20 normal tissue samples collected from the salivary glands (control group).

By sequencing the appropriate DNA, the nature of each mutations detected by RFLP analysis was confirmed (Fig 3).

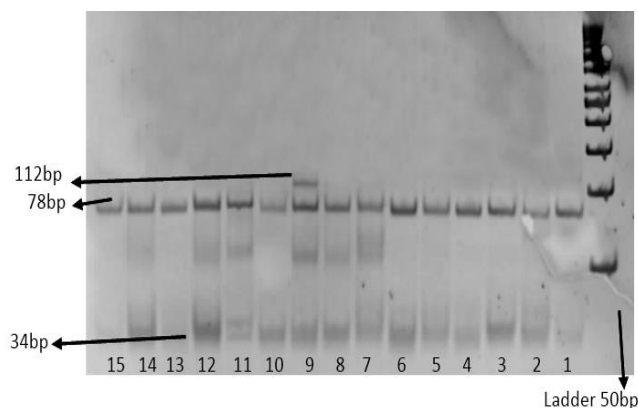


Figure 2. BRAF codon 600 restriction fragment length polymorphism analysis

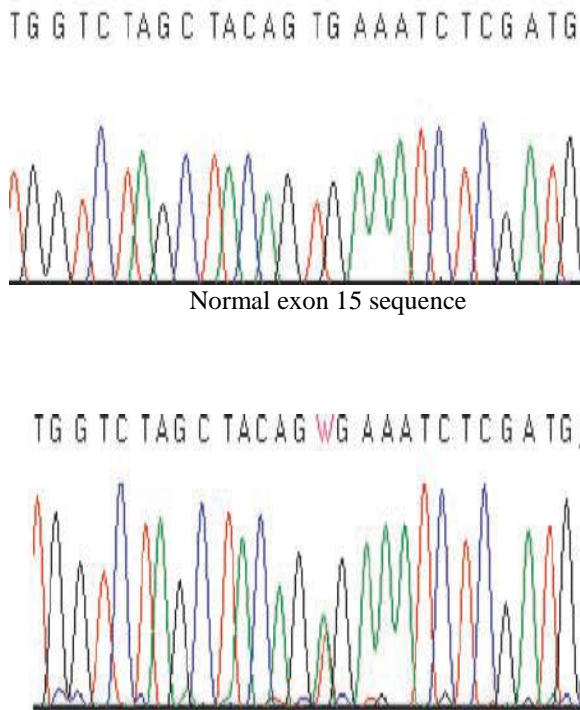


Figure 3. BRAF mutation in exon 15, position 600 substitution of A for the T. GTG → GAG Mutant Allele

Discussion

The activation of the EGFR-RAS-RAF pathway has been implicated in cancer development and its therapeutic potential has been shown. The EGFR broadcast signals to cancer cells result in reproduce and metastasize and BRAF is the downstream signaling molecule. Anti-EGFR therapies disrupt the cancer-triggering signaling cascade, promising therapeutic target for various types of human cancers (16, 17).

The epidermal growth factor receptor (EGFR) targeting monoclonal antibody, cetuximab, has been shown to improve the efficacy of standard chemotherapy regimens used in the first-line treatment of several common cancers (18-20).

The present study represents an evaluation of BRAF mutation in the SGCs. The oncogenic role of BRAF as well as promising results for targeted inhibitors of BRAF have been extensively shown in variety of malignancies (16).

Mutations of BRAF have been demonstrated in approximately 15% of all human cancers (3).

BRAF somatic mutation presents in 66% of malignant melanoma and approximately 10% of colorectal cancers. A hotspot for BRAF mutation is conversion of valine 599 to glutamic acid (V599E) and accounts for 80% of the BRAF mutations in colorectal cancers (21, 22).

BRAF V600E mutation is most prominent in melanoma (40–60%), low grade serous ovarian carcinoma (35%), papillary thyroid carcinoma (45%), and colorectal adenocarcinoma (5–15%). Other *BRAF* mutations include V600K and V600D/R, accounting for 16–29% and 3% of all *BRAF* mutations in melanoma, respectively. Another activating *BRAF* mutation that is almost exclusively found in pilocytic astrocytomas is the *KIAA1549-BRAF* fusion, found in 66–100% of these tumors (23-29).

Nardi et al investigated BRAF mutations in salivary duct carcinoma, a rare subtype of SGCs with histological resemblance with breast duct carcinoma. They found BRAF mutations in a subset of salivary duct carcinoma that were AR-positive and Her2-negative (17).

In contrast to melanoma and colorectal cancers, the absence of BRAF mutations was reported in cervical and endometrial cancer (10). It has been also demonstrated that BRAF mutations are rare in head and neck squamous cell carcinomas (HNSCCs) (20). The results of Weber et al showed, only 3% of HNSCCs showed activating BRAF mutations, while Lopez et al reported no mutations in both sinonasal adenocarcinoma and sinonasal SCC (30, 31).

Dashe et al (4) evaluated BRAF mutations in SGCs including AdCC, MEC, acinic cell carcinoma (ACC), myoepithelial carcinoma and ex pleomorphic adenocarcinoma. Their findings showed no incidence of BRAF mutations in studied groups.

In consistent with the results of Dashe et al, we also demonstrated that BRAF mutations in contrast to their presence in a variety of human cancers, are absent in SGCs.

Conclusion

According to our findings SGCs didn't acquire mutations that result in a constitutive activation of the signaling cascade downstream of EGFR, hence SGCs can be a good candidate for anti EGFR therapies.

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