Consistent absence of BRAF mutations in salivary gland carcinomas

Nooshin Mohtasham[1, 2], Hossein Ayatollahi[3], Narges Ghazi [2, 4], Amirhossein Jafarian[5], Azam Roshanmir[6], Farnaz Mohajertehran[4]

[1]Oral & Maxillofacial Diseases Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[2]Department of Oral and Maxillofacial Pathology, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran
[3]Department of Hematology and Blood Bank, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[4]Dental Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[5]Department of Pathology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[6]Department of Oral and Maxillofacial Pathology, School of Dentistry, Golestan University of Medical Sciences, Golestan, Iran

Received 18 December 2016 and Accepted 29 January 2017

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**.**

Mohtasham N, Ayatollahi H, Ghazi N, Jafarian A, Roshanmir A, Mohajertehran F. Consistent absence of BRAF mutations in salivary gland carcinomas**.** J Dent Mater Tech 2017; 6(2): 73-8.

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).

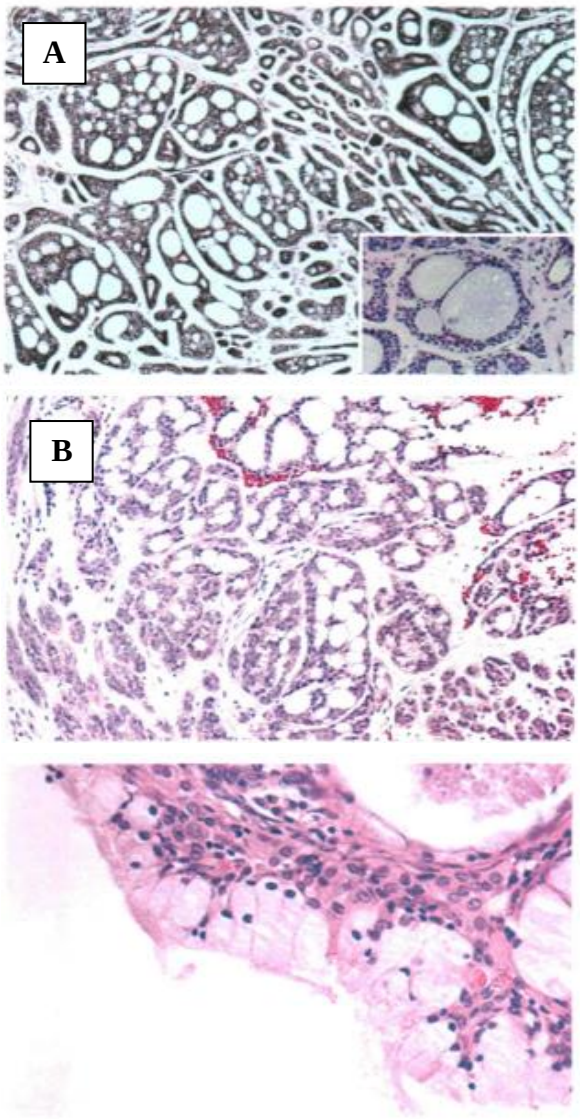


Figure 1: (A) Adenoid cystic carcinoma with islands of hyperchromatic cells forming cribriform and tubular structures, H & E, original magnification 100X. (**B)** Polymorphous Low-grade adenocarcinoma with cribriform arrangement of uniform tumor cells with pale-staining nuclei, H&E, original magnification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

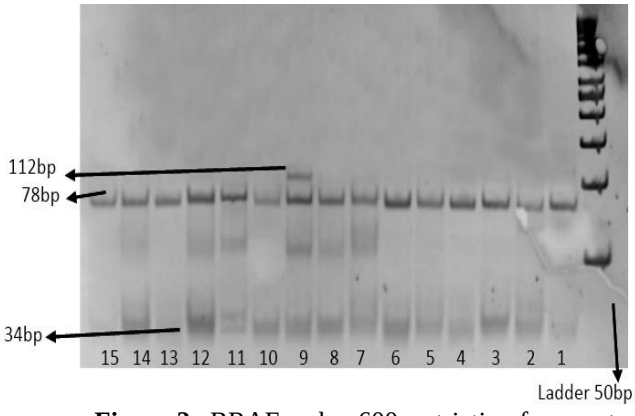
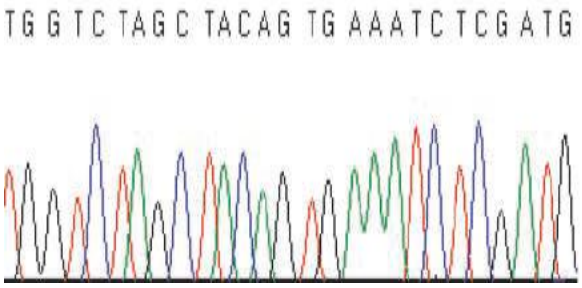


Figure 2. BRAF codon 600 restriction fragment length polymorphism analysis



Normal exon 15 sequence

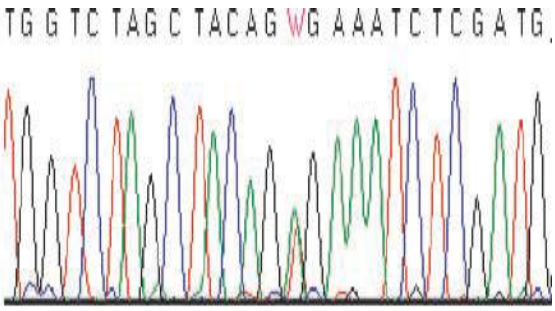


Figure 3. BRAF mutation in exon 15, position 600 substitution of A for the T. GTG → GAG Mutant Allele

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 varine 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 adenocacinoma 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.

Acknowledgement

This study was supported by a grant from the Vice Chancellor of Mashhad University of Medical Sciences, Iran.

References

1. Milano A, Longo F, Basile M, Iaffaioli RV, Caponigro F. Caponigro, Recent advances in the treatment of salivary gland cancers: emphasis on molecular targeted therapy. Oral Oncology 2007; 43(8):729–734.

2. 2-. Saghravanian N, Ghazi N, Saba M. Clinicopathologic evaluation of salivary gland neoplasms: a 38-year retrospective study in Iran. Ann Diagn Pathol 2013;17(6):522-5
3. Boyrie S, Fauquet I, Rives M, Genebes C, Delord JP. Cystadenocarcinoma of the parotid: case report of a BRAF inhibitor treatment.Springerplus 2013 ;18(2):679.
4. Domingues R, Mendonca E, Sobrinho L, Bugalho MJ. Searching for RET/ PTC rearrangements and BRAF V599E mutation in thyreoid aspirates might contribute to establish a preoperative diagnosis of papillary thyreoid carcinoma. Cytopathol 2005;16(1):27–31.
5. Dahse R ,Kromeyer-Hauschild K, Berndt A, Kosmehl H. No incidence of BRAF mutations in salivary gland carcinomas—implications for anti-EGFR therapies. J BioMed Biotechnol 2009 Research International 2009; 5:736-740.
6. Penko K, Livezey J, Fenton C, Patel A, Nicholson D, Flora M, Oakley K, Tuttle RM, Francis G BRAF mutations are uncommon in papillary thyreoid cancer of young patients. Thyroid 2005;15(4):320–325.
7. Mayr D, Hirschmann A, Löhrs U, Diebold J. KRAS and BRAF mutations in ovarian tumors: a comprehensive study of invasive carcinomas, borderline tumors and extraovarian implants. Gynecol oncol 2006;103(3):883-887.
8. Dibb NJ, Dilworth SM, Mol CD. Switching on kinase: oncogenic activation of BRAF and the PDGFR family. Nat Rev Cancer 2004; 4(9):718–727.
9. Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D, Marais R; Cancer Genome Project. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004,116 (6):855– 867.
10. O'Neill E, Kolch W. Conferring specificity on the ubiquitous Raf/MEK signalling pathway.Br J Cancer 2004; 90(2):283– 288.
11. 11 Pappa KI, Choleza M, Markaki S, Giannikaki E, Kyroudi A, Vlachos G, Voulgaris Z, Anagnou NP. Consistent absence of BRAF mutations in cervical and endometrial cancer despite KRAS mutation status. Gynecol oncol 2006; 100(3):596-600 .
12. 12-Jin Y-M, Li B-J, Qu B, Du Y-J. BRAF, K-ras and BAT26 mutations in colorectal polyps and stool.World J Gastroenterol, 2006, 12(32):5148.
13. 13- Kadiyska TK, Konstantinova DV, Atanasov VR, Kremensky IM, Mitev VI. Frequency and application of the hot spot BRAF gene mutation (p. V600E) in the diagnostic strategy for Hereditary Nonpolyposis Colorectal Cancer. Cancer detect prev 2007;31(3):524-526.
14. 14- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al.Mutations of the BRAF gene in human cancer. Nature 2002;417:949–954.
15. 15- Barnes L, Eveson W, Reichart P,Sidransky W. "Pathology and genetics of head and neck tumours"in World Health Organization Classification of Tumours, IARC Press, Lyon, France.2005; 210-242.
16. 16- Cros J, Sbidian E, Hans S, Roussel H, Scotte F, Tartour E, Brasnu D, Laurent-Puig P, Bruneval P, Blons H, Badoual C. Expression and mutational status of treatment-relevant targets and key oncogenes in 123 malignant salivary gland tumours. Ann Oncol 2013; 24(10):2624-2629
17. 17- Nardi V, Sadow PM, Juric D, Zhao D, Cosper AK, Bergethon K, Scialabba VL, Batten JM, Borger DR, Iafrate AJ, Heist RS, Lawrence DP, Flaherty KT, Bendell JC, Deschler D, Li Y, Wirth LJ, Dias-Santagata D. Detection of novel actionable genetic changes in salivary duct carcinoma helps direct patient treatment. Clin Cancer Res 2013;19(2):480-490.
18. 18- Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, De Raucourt D, Bokemeyer C, Schueler A, Amellal N, Hitt R. F.Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2008; 359(11):1116-1127.
19. 19- Van Cutsem E, Köhne CH, La´ng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zubel A, Celik I, Rougier P, Ciardiello F. Cetuximab

Plus Irinotecan, Fluorouracil, and Leucovorin as First-Line Treatment for Metastatic Colorectal Cancer: Updated Analysis of Overall Survival According to Tumor KRAS and BRAF Mutation Status.J Clin Oncol 2011; 29(15):2011-2019.

20. 20- Walts AE, Pao A, Sacks W, Bose S. BRAF genetic heterogeneity in papillary thyroid carcinoma and its metastasis.Hum Pathol 2014; 45(5): 935–941.
21. 21- Cornejo KM, Hutchinson L, Deng A, Tomaszewicz K, Welch M, Lyle S, Dresser K, Cosar EF. BRAF/KRAS gene sequencing of sebaceous neoplasms after mismatch repair protein analysis. Hum Pathol 2014; 45(6):1213–20.
22. 22- Kuan SF, Navina S, Cressman KL, Pai RK. Immunohistochemical detection of BRAF V600E mutant protein using the VE1 antibody in colorectal carcinoma is highly concordant with molecular testing but requires rigorous antibody optimization., Hum Pathol 2014;45(3): 464–72.
23. 23- Dimmler A, Geddert H, Faller G. EGFR, KRAS, BRAF-mutations and microsatellite instability are absent in goblet cell carcinoids of the appendix. Pathol Res Pract 2014; 210 (5): 274–78.
24. 24- Pakneshan S, Salajegheh A, Smith RA, Lam AK. Clinicopathological relevance of BRAF mutations in human cancer. Pathol 2013; 45(5):346–356.
25. 25-Arkenau HT, Kefford R, Long GV.Targeting BRAF for patients with melanoma.Br J Cancer 2011; 104:392–98.
26. 26- Kudchadkar R, Paraiso KH, Smalley KS, Targeting mutant BRAF in melanoma: current status and future development of combination therapy strategies. Cancer J 2012; 18(2):124–131.
27. 27- Jones DT, Kocialkowski S, Liu L, Pearson DM, Ichimura K, Collins VP.Oncogenic RAF1 rearrangement and a novel BRAF mutation as alternatives to KIAA1549:BRAF fusion in activating the MAPK pathway in pilocytic astrocytoma. Oncogene 2009; 28(20):2119–2310.
28. 28- Lawson AR, Tatevossian RG, Phipps KP, Picker SR, Michalski A, Sheer D, et al. RAF gene fusions are specific to pilocytic astrocytoma in a broad paediatric brain tumour cohort. Acta Neuropathol 2010;120:271–310
29. 29-Thiel A, Ristimäki A. Toward a Molecular Classification of Colorectal Cancer: The Role of BRAF. Front Oncol 2013; 15(3): 281.
30. 30- Weber A, Langhanki L, Sommerer F, Markwarth A, Wittekind C, Tannapfel A. Mutations of the BRAF gene in squamous cell carcinoma of the head and neck.Oncogene 2003; 22(30):4757-4759.
31. 31- López F, García Inclán C, Pérez-Escuredo J, Alvarez Marcos C, Scola B, Suárez C, Llorente JL, Hermsen MA. KRAS and BRAF mutations in sinonasal cancer.Oral Oncol 2012;48(8):692-697.

Corresponding Author
Narges Ghazi
Department of Oral & Maxillofacial Pathology,
School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran.
Address: Vakil-Abad Blv, Dentistry Faculty, Mashhad, Iran
Tel: +98-513-8829501
Fax: +98-513- 8829500
Email: ghazin@mums.ac.ir