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Genetic study of the *BRAF* gene reveals new variants and high frequency of the V600E mutation among Iranian ameloblastoma patients

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Background: Ameloblastoma is a benign, slow-growing and locally invasive tumor. It is one of the most prevalent odontogenic tumors, with an incidence rate of 1% of all oral tumors and approximately 18% of odontogenic tumors. A group of genes have been investigated in patients with ameloblastoma. The *BRAF* V600E mutation has been implicated as the most common mutation in ameloblastoma. The presence or absence of this mutation has been associated with several clinicopathological properties, including location, age at diagnosis, histology, and prognosis. Although some populations have been investigated so far, little data are available on the Iranian population. The current research was launched to study the *BRAF* V600E mutation among a cohort of Iranian patients with ameloblastoma.

Methods: In this clinicopathological and molecular biology study, a total of 19 formalin-fixed, paraffin-embedded tissues were studied. DNA extraction was performed, followed by PCR-sequencing of exons 10 and 15 of the *BRAF* gene to identify mutations. *In silico* analysis was performed for the identified variants. Results were analyzed by *T* test, Chi-square, and Fisher's exact test.

Results: Totally, 12 of 19 samples (63%) harbored the p. V600E hotspot mutation. In addition, we identified several variants, two of which were novel. The c.1769T>G (p. V590G) and c.1751C>T (p.L584F) as the novel variants showed a possible damaging effect by *in silico* analysis. No variant was found within exon 10.

Conclusions: Our study confirms the role of *BRAF* mutations in ameloblastoma in the Iranian patients studied.

KEYWORDS
ameloblastoma, *BRAF*, gene, Iran

1 | INTRODUCTION

Ameloblastoma is a benign, slow-growing and locally invasive tumor. It is one of the most prevalent odontogenic tumors with an incidence rate of 1% of all oral tumors and approximately 18% of odontogenic tumors. It is able to perforate the cortical bone of the jaw to cause facial asymmetry.[1-3] Ameloblastoma is known to originate from the remaining tooth lamina and to reduce enamel epithelium, epithelial cell rests of Malassez, or the basal layer cells. It often affects the mandible (80%-85% of cases).[2,3] Ameloblastomas are categorized into unicystic, multicystic (solid), peripheral, and malignant subtypes. The conventional solid or multicystic subtype, with an

incidence of 86% of cases, is more infiltrative with a higher rate of recurrence.[1] Their histological variations consist of follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types, among which the follicular and plexiform types are the most common types.[4]

A group of genes have been investigated in patients with ameloblastoma. However, further investigations are required to detect the mechanisms of oncogenesis and molecular pathogenesis. Mutations influencing several genes within the MAPK pathway are currently known to occur in a large number of cases. The biological importance of these mutations is pronounced by their high frequency and pattern of mutual exclusivity. The hedgehog and FGFR-MAPK pathway components have been reported to be expressed during tooth development and in ameloblastomas.[5-7] Moreover, analysis using gene expression microarrays, immunohistochemistry, and quantitative RT-PCR have, in particular, indicated the differential expression of Hedgehog pathway genes in ameloblastomas.[8,9]

Both in vitro and anecdotal clinical data suggest MAPK pathway inhibition as a promising treatment option for ameloblastoma.[10] Variable sensitivity of primary ameloblastoma cells to EGFR-targeted drugs in vitro has been reported.[9] The *BRAF* V600E mutation (15 of 24 samples, 63%) was found in the cell line overexpressing EGFR but resistant to EGFR inhibition. These data provide a novel insight into the poorly recognized molecular pathogenesis of ameloblastoma and offer a rationale for testing the drugs targeting EGFR or mutant *BRAF* as novel therapies for ameloblastoma.[9]

More than 40 different mutations have been reported in the *BRAF* gene related to human cancer.[11] Interestingly, the *BRAF* V600E mutation has been implicated as the most common mutation (90% of all *BRAF* mutations) in ameloblastoma resulting in constitutive activation of the gene.[12] The presence or absence of this mutation has been associated with several clinicopathological properties, including location, age at diagnosis, histology, and prognosis.[10]

Although there are several studies to address the prevalence of the *BRAF* V600E mutation in ameloblastoma patients,[5,9,13,14] they do show that the mutation frequency differs in different populations; Iran is a big country with a population about 80 million people. However, there is no report on the role of *BRAF* in ameloblastoma. Therefore, the current research was launched to study the *BRAF* V600E mutation among a cohort of Iranian patients with ameloblastoma.

2 | MATERIALS AND METHODS

2.1 | Subjects

This study was approved by the Institutional Review Board of Isfahan University of Medical Sciences. Altogether, 23 families were recruited from the Department of Oral Pathology and Al Zahra Pathology Laboratory affiliated to Isfahan University of Medical Sciences and Department of Pathology, Shahid Beheshti University of Medical Sciences. All the centers had protocols for taking informed written consent from the patients before banking their archive samples. All families were of Persian ethnicity. A detailed clinical history was taken.

2.2 | DNA extraction

Totally, 23 formalin-fixed, paraffin-embedded tissues were recruited in this study. Tissue sections were deparaffinized with xylene followed by genomic DNA extraction using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. The concentration and purity of DNA were measured by Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1.2% agarose gel, and 50 ng of the genomic DNA was used for PCR.

2.3 | Mutation screening of *BRAF*

Sequencing of the selected *BRAF* gene exons 10 and 15 was performed to identify mutations. The primers for the exons were designed to include exon-intron boundaries. The exons were amplified using 2× Master Mix Red (Ampliqon, Copenhagen, Denmark) by standard PCR programs (Table 1). Primers were designed using Oligo (version 6.7.1.0 National Biosciences Inc.). Primer sequences and PCR conditions are available upon request.

PCR conditions were as follows: Each reaction contained 1 μL MgCl2 (50 mM), 2.5 μL Taq PCR buffer (10×), 0.5 μL of each of the primers (10 PM), 0.1 μL Taq DNA polymerase (5 U/μL), 0.5 μL dNTP

TABLE 1 Demographic and pathological features of the studied sample

Sample #	Age	Sex	Type	Variant			
3	87	M	FOLI	V600E			
5	58	M	FOLI	V600E			
6	46	M	PLXI	V600E			
9	51	M	PLXI	V600E			
10	52	F	PLXI				
12	30	M	PLXI	V600E			
15	16	F	PLXI				
16	30	M	PLXI	V600E			
17	58	F	FOLI		G606E		
20	78	M	FOLI	V600E			
22	35	M	FOLI				
23	50	F	PLXI	V600E			
24	22	M	FOLI	V600E	G606E		
25	31	M	FOLI				
26	37	F	FOLI	V600E			
28	33	M	FOLI				
29	41	M	FOLI	V600E	G606E		
30	45	F	FOLI			L584P[a]	V590G[a]
33	26	F	PLXI	V600E			

MAN, mandible; MAX, maxilla; F, female; M, male; FOLI, follicular; PLXI, plexiform; ACAN, acanthomatous.

[a]New variant.

mix (10 mM), and 1 μL DNA (about 50 ng). The total volume was adjusted to 25 μL by ddH_2O. A touchdown thermal program was performed as follows: one cycle of denaturation at 95°C for 3 minutes; five touchdown cycles of denaturation at 94°C for 40″, annealing at 64°C for 40″ in the first cycle with 1°C reduction per cycle, and extension at 72°C for 45″; 27 cycles of denaturation at 94°C for 45″, annealing at 60°C for 40″, and extension at 72°C for 45″; and a final extension step of 72°C for 7 minutes. PCR products were investigated using agarose gel electrophoresis and sequenced bidirectionally using ABI 3130XL automated sequencer (Applied Biosystems, Foster City, California, USA). Sequences were compared with the reference genomic sequence NG_007873.3 for variant detection using the SeqMan software version 5.00© (DNASTAR, Madison, WI, USA).

2.4 | Pathogenicity analyses

Pathogenicity of the identified variants was studied based on the nature of variants, evolutionary conservation of substituted amino acid. After identification of each variant, Ensemble, dbSNP, and 1000 genome database, NHLBI GO exome sequencing project (ESP), exome aggregation consortium (ExAC), and the Human Gene Mutation Database (HGMD) as well as the literature were investigated for previously known variants. Sequence variant numbering was based on the reference transcript sequence NM_004333 for the *BRAF* gene. All novel variants were named according to the guidelines of the human variation society (http://www.hgvs.org/). Possible pathogenic effects of the novel variants were checked by MutationTaster (http://www.mutationtaster.org/). In addition, FATHMM (http://fathmm.biocompute.org.uk/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/seq_submit.php), PANTHER (http://pantherdb.org/) SIFT (http://sift.jcvi.org/), ConSurf (http://consurf.tau.ac.il/2016/), and KinMut2 (http://kinmut2.bioinfo.cnio.es/input) were used for *in silico* prediction of the variant effects.

3 | RESULTS

We used 23 patients with multicystic ameloblastoma lesion, and DNA was extracted. However, upon PCR, only 19 samples showed satisfactory results after amplification on agarose gel after different conditions were examined. Demographic and pathological features of the studied samples are shown in Table 1. None of the samples had positive family history.

3.1 | Sequencing results for the *BRAF* gene

Only 19 of the 23 samples showed acceptable results in molecular studies and were included. From the 19 samples that were sequenced, 12 had an exon 15 heterozygous mutation named c.1799T>A (p.V600E); therefore, the prevalence of the mutation among ameloblastoma patients was 63%. Figure 1A shows this

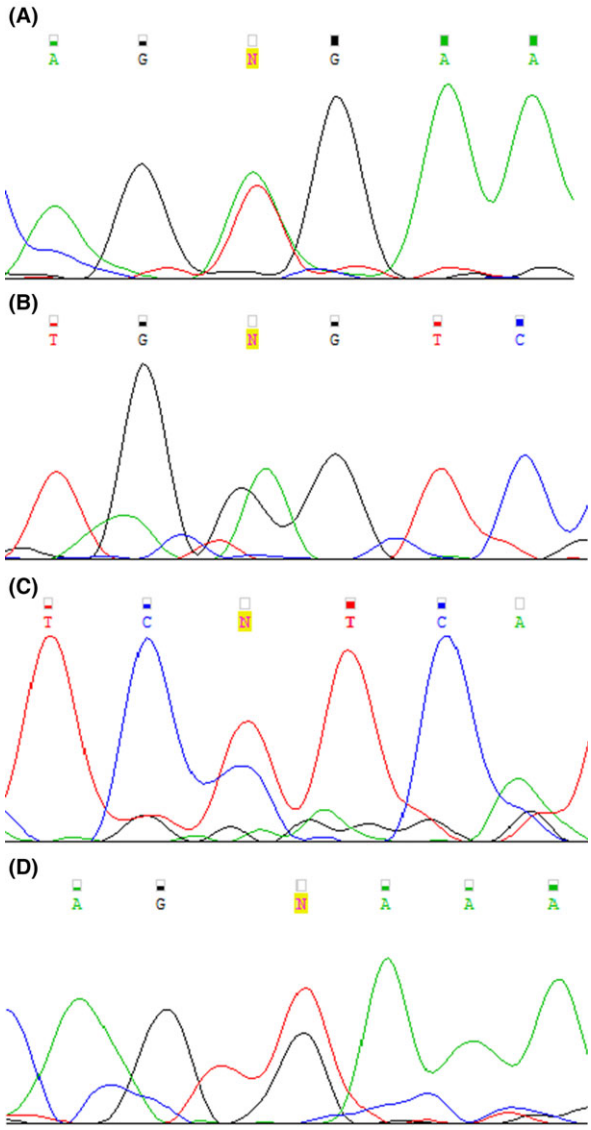

FIGURE 1 Chromatograms of variants identified in exon 15 in the ameloblastoma patients. (A) represents substitution of T to A at position 1799 of coding DNA (p.V600E), (B) shows the substitution of G to A at position 1817 of coding DNA (G606E), (C) indicates a novel variant in which C is replaced by T at coding DNA position 1751 (L584P), and (D) illustrates replacement of T to G at position 1769 at coding DNA level (V590G)

variant. The pathogenicity prediction of all identified variants was checked via online software tools (Table 2).

In terms of tissue distribution of the mutation, six cases were of plexiform and six were of follicular type. Thus, there was no significant difference in the distribution of the mutation. The average age of the patients harboring the mutation was 43.6 years vs those without the mutation (38.6 years of age). In addition, we identified three other variants including G606E, L584P, and V590G (Figure 1B-D), two of which, L584P and V590G were novel. The variants V590G and G606E showed possible damaging effects by *in silico* analysis. The novel variants, L584P and V590G, also were suggested to be of pathogenic consequence by the software tools except for KinMut2 tool for L584P.

TABLE 2 List of variants found in *BRAF* among ameloblastoma patients and their pathogenicity investigation using software prediction tools

	c.1799T>A (V600E)		c.1817G>A (p.G606E)		c.1751C>T (p.L584F)		c.1769T>G (p.V590G)	
Variant	**Score**	**Prediction**	**Score**	**Prediction**	**Score**	**Prediction**	**Score**	**Prediction**
SIFT	0	Damaging	0	Damaging	0.04	Damaging	0	Damaging
PROVEAN	−4.781	Deleterious	−7.376	Deleterious	−6.470	Deleterious	−6.215	Deleterious
MutationTaster2	121	Disease causing	98	Disease causing	22	Disease causing	109	Disease causing
Polyphen2.0	0.971	Probably damaging	0.493	Possibly damaging	0.964	Probably damaging	1.000	Probably damaging
ConSurf	9	Conserved	8	Conserved	9	Conserved	9	Conserved
PANTHER	910	Probably damaging	910	Probably damaging	1036	Probably damaging	1036	Probably damaging
FATHMM	−1.83	Cancer	−1.80	Cancer	−2.33	Cancer	−2.45	Cancer
KinMut2	0.939	Disease	0.962	Disease	−0.654	Neutral	0.769	Disease

4 | DISCUSSION

Ameloblastoma is a benign, locally invasive tumor with complex etiology.[1-3] In this descriptive study, Iranian patients with ameloblastoma were analyzed for the presence of the *BRAF* V600E mutation. Totally, 12 of 19 patients were found to carry this mutation. The *BRAF* gene has been reported to be mutated in some human cancers including colorectal cancer,[15-17] malignant melanoma,[18-24] patients with Langerhans cell histiocytosis,[25] papillary thyroid carcinoma,[26] non-small-cell lung carcinoma, non-Hodgkin lymphoma, and lung adenocarcinoma.[11] The *BRAF* mutations have been implicated in ameloblastoma. In 2014, three separate reports demonstrated recurrent MAPK mutations in ameloblastoma, with the most common mutation being *BRAF V600E*. The findings reported in these studies suggest a new paradigm for the diagnostic classification and treatment of ameloblastomas. Two of these reports indicated *BRAF* mutations at a similar frequency (64% and 63%; 54/84 and 15/24), while a third showed a lower frequency (46%; 13/28).[5,9,14]

Diniz et al. evaluated *BRAF* V600E in multicystic, unicystic, and desmoplastic ameloblastomas. In their study, 17 ameloblastoma samples were included. Fourteen of 17 (82%) ameloblastomas showed *BRAF* V600E mutation, 5/6 (83%) in unicystic, 7/9 (78%) in multicystic, and 2/2 in desmoplastic ameloblastomas.[13] In our study on multicystic ameloblastoma, 12 of 19 samples harbored the p. V600E hotspot mutation. Therefore, the prevalence of this mutation among the studied Iranian ameloblastoma cases is reported to be 63%. Sweeney et al. argued that *BRAF*-mutated tumors may have indications for location, histologic pattern (plexiform vs follicular), and possible prognosis. In this regard, 80% of ameloblastomas with the plexiform histologic pattern were *BRAF* wild-type.[13] However, Brown et al. reported no relationship between follicular/plexiform pattern and genotype. Plexiform histology was significantly more prevalent among *BRAF* wild-type tumors (62%) than among *BRAF*-mutated tumors (35%; P=.02).[5] In our study, in term of tissue distribution of the mutation, six of eight cases were of plexiform and six of 11 were of the follicular type (P=.63). Thus, there was no significant difference in the distribution of the mutation. This could be due to small sample size and population-related differences. Sweeney et al. found that *BRAF* mutations occurred in younger patients with a mean age at diagnosis of 34.5 years compared to 53.6 in *BRAF* wild-type cases (P=.0001).[13] In our study, the average age of the patients harboring the mutation was 43.6 years vs those without the mutation (38.6 years of age) (P=.385). Thus, no significant relationship was found between the average age and carrying the mutation.

Sweeney et al.[14] found that *BRAF* mutations are predominant in tumors of the mandible (75%). In a study, *BRAF* mutations were shown to occur much more frequently in the mandible and only rarely in the maxilla (5.6%), while 43% of *BRAF* wild-type tumors arose in the maxilla. 64% of *BRAF* wild-type tumors arose in the maxilla as well.[5] In our study, no significant difference was found between tumor types and the position of the jaw (mandibular and maxillary) (P=.99).

Cancer is a multistep process. Simultaneous occurrence of two variants (V600E with G606E and L584F with V590G) in the same gene may simply be a reflection of the instability of the genome. According to standards for reporting sequence variants in cancer, V600E is a variant with potential significance (second tier with evidence of level C) in ameloblastoma. The clinical significance of the novel variants is unknown.[27] Notably, the V590G, L584F, and G606E exist in the same exon as V600E, thereby affecting the same domain (protein kinase domain). Available evidence supports that c.1751C>T (p.L584F) and c.1769T>G (p.V590G) are classified in variants with uncertain significance category according to the ACMG guidelines.[28] Alternatively, none or one of these variants might have potential functional implications. Extending the sample size, recurrence of the variants in other ameloblastoma tumor samples, qPCR, and kinase activity assessment could unravel the importance of these variants in ameloblastoma. Currently, it is not known whether the simultaneous occurrence of the variants within the *BRAF* gene would reflect additional selective advantage for the tumor or they may simply reflect tumor multistep nature and genomic instability.

5 | CONCLUSION

In summary, for the first time in Iran, we studied *BRAF* mutations in a cohort of Iranian patients with ameloblastoma. We also report several variants some of which may have pathogenic effect. Our data confirm a significant role of the *BRAF* gene mutations among Iranian ameloblastoma patients.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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