Scientific Research Publishing

Open Journal of Pathology, 2020, 10, 93-107
https://www.scirp.org/journal/ojpathology
ISSN Online: 2164-6783
ISSN Print: 2164-6775

RASSF1A Methylation Status and BRAF V600E Immunohistochemical Expression in Odontogenic Lesions

Chatchaphan Udompatanakorn[1,2], Naomi Yada[1*], Kou Matsuo[1]

[1]Division of Oral Pathology, Department of Health Promotion, Kyushu Dental University, Kitakyushu, Japan
[2]Department of Oral Surgery and Oral Medicine, Faculty of Dentistry, Srinakharinwirot University, Bangkok, Thailand
Email: *r12yada@fa.kyu-dent.ac.jp

How to cite this paper: Udompatanakorn, C., Yada, N. and Matsuo, K. (2020) *RASSF1A* Methylation Status and BRAF V600E Immunohistochemical Expression in Odontogenic Lesions. *Open Journal of Pathology*, **10**, 93-107.
https://doi.org/10.4236/ojpathology.2020.103009

Received: April 21, 2020
Accepted: June 16, 2020
Published: June 19, 2020

Abstract

Background: The etiology and pathogenesis of odontogenic lesions remain to be determined. Previous studies have identified epigenetic and genetic alterations that may be relevant to lesions progression and development. Hypermethylation of the Ras association domain family protein 1A (RASSF1A) has been observed in a variety of human cancers. However, the methylation status of RASSF1A in odontogenic lesions remains unknown. Thus, the aim of this study was to investigate the prevalence of RASSFA promoter hypermethylation and v-raf murine sarcoma viral oncogene homolog B V600E mutant (BRAF V600E) expression as well as the correlations between these alterations and clinicopathological features of patients with odontogenic lesions. **Methods:** We subjected 66 formalin-fixed, paraffin-embedded odontogenic lesions [ameloblastoma (AM), 21; ameloblastic carcinoma (AC), 6; odontogenic keratocyst (OKC), 19; and dentigerous cyst (DC), 20] to methylation-specific polymerase chain reaction to determine RASSF1A hypermethylation and immunohistochemistry to detect BRAF V600E protein expression. **Results:** We observed RASSF1A hypermethylation in 20% (4/20; methylation could not be detected in one lesion), 100% (6/6), 26.3% (5/19), and 5% (1/20) of AM, AC, OKC, and DC samples, respectively. RASSF1A methylation was significantly more frequently observed in AC relative to AM, OKC, and DC ($p < 0.001$). Moreover, 85.7% (18/21) and 83.3% (5/6) AM and AC samples, respectively, were BRAF V600E-positive, whereas all OKC and DC sample were BRAF V600E-negative. No correlations of RASSF1A methylation and BRAF V600E expression with clinicopathological features were observed. **Conclusions:** Concomitant RASSF1A methylation and positive BRAF V600E expression are commonly observed in AC, which may contribute to AC tumorigenesis.

Keywords

Odontogenic Lesions, DNA Methylation, *RASSF1A*, Immunohistochemistry, BRAF V600E

1. Introduction

Odontogenic tumors and cysts are a heterogeneous group of diseases derived from the cells of the tooth forming apparatus and their remnants [1] [2]. Ameloblastoma (AM) is among the most common benign odontogenic tumors and is clinically characterized by features such as slow infiltrative growth mainly involving the mandible, an asymptomatic presentation, and high recurrence rate when treated with conservative surgery [2] [3]. In contrast, ameloblastic carcinoma (AC) is a rare malignant odontogenic tumor. It combines the histological features of AM with malignant cytologic features such as cellular pleomorphism, mitotic activity, and nuclear hyperchromatism. AC may cause extensive local destruction of the jawbone as well as lung metastasis in approximately a third of patients [2] [4]. Odontogenic keratocysts (OKCs) and dentigerous cysts (DCs) are common developmental odontogenic cysts. DCs arise from the crowns of impacted or unerupted teeth and are usually slow growing and asymptomatic [5]. In contrast, OKC has a unique presentation characterized by locally aggressive behavior and tendency to recur in cases of incomplete resection or in the presence of daughter cysts [2] [6].

Ras association domain family 1 isoform A (RASSF1A) is an important tumor suppressor gene located in the 3p21.3 region of the human genome [7]. RASSF1A has been implicated in the RAS signaling pathway and is involved in various biological events, such as cell cycle arrest and apoptosis, and may also reduce tumorigenicity in cancer cell lines [8]. Extensive investigations have revealed that RASSF1A is frequently hypermethylated in several tumor types, such as small cell lung cancer, breast cancer, prostate cancer (~88% - 99% of samples), but is less frequently methylated in oral squamous cell carcinoma and ovarian cancer (~22% - 40% and 26% - 30% of samples, respectively) [8] [9].

BRAF is a member of the rapidly accelerated fibrosarcoma (RAF) family of serine/threonine kinases [10]. Specifically, this kinase activates the RAF/MEK/ERK cascade to induce cell cycle progression and cell growth [11] [12]. BRAF is commonly mutated in a variety of human cancers [10]. The most frequent mutation is a missense thymine to adenine transversion in exon 15, resulting in amino acid change in BRAF protein at codon 600 from valine (V) to glutamic acid (E) (V600E) [12]. BRAF V600E mutations have been reported in various tumors, such as malignant melanomas, thyroid papillary carcinomas, and right-sided colorectal cancers [10] [12].

Currently, the etiology and pathogenesis of odontogenic lesions remain un-

clear [13], although previous studies have reported various transcriptional changes and genetic alterations relevant to lesion progression and development [14] [15] [16]. In AM, for example, BRAFV600E mutation has been detected in 62% - 83% of both multicystic and unicystic cases [17], and strong agreement was observed between immunohistochemical analyses of BRAF V600E and the molecular detection of BRAFV600E mutation [18]. BRAF V600E expression has been detected in 46% - 89% of cases of mandibular AM [19] [20] [21] [22]. However, the potential correlations between BRAF V600E expression and clinical variables in AM remain controversial. In 2017, Fregnani *et al.* reported a significant association between positive BRAF V600E expression and recurrent AM, whereas other studies found no such correlation [19] [20] [21] [22]. Similarly, the frequency of BRAFV600E mutation in OKC remains uncertain [23] [24].

To date, a few published studies have explored epigenetic alterations in odontogenic lesions [25] [26] [27] [28]. To our knowledge, however, no information is available regarding the RASSF1A methylation statuses of these lesions and the corresponding associations with clinical variables. This study aimed to assess the frequency of RASSF1A promoter methylation and BRAF V600E immunohistochemical expression in various types of odontogenic lesions (AM, AC, OKC, and DC) and to explore potential correlations between these changes and clinicopathological variables. Potentially, the study findings could provide a better understanding about the roles of RASSF1A and BRAFin the development of odontogenic lesions.

2. Material and Methods

2.1. Sample Collection and Histological Evaluation

This study was approved by the research ethics committee of Kyushu Dental University, Kitakyushu, Fukuoka, Japan (reference number: 17 - 50). We included a total of 66 odontogenic lesions (AM, 21; AC, 6; OKC, 19; DC, 20) in this study. All samples were formalin-fixed/paraffin-embedded (FFPE) biopsy and/or resection specimens retrieved from the Division of Oral Pathology at Kyushu Dental University from January 2009 to June 2018. Two oral pathologists (NY and KM) confirmed the histopathological diagnoses based on the World Health Organization classification of odontogenic cysts and tumors [2]. We based conclusive diagnoses of AC samples on malignant morphological features and a Ki-67 proliferation index > 10% [29]. The clinicopathological characteristics of all samples are summarized in **Table 1**.

2.2. DNA Extraction, Bisulfite Modification, and Methylation-Specific Polymerase Chain Reaction (MS-PCR)

We extracted genomic DNA from all 66 FFPE odontogenic lesions using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Next, we modified 20 µl of DNA with sodium bisulfite to convert unmethylated cytosines to uracil while preserving methylated cytosines,

Table 1. Clinicopathological characteristics of the odontogenic lesions included in the study (N = 66).

Characteristics	AM n (%)	AC n (%)	OKC n (%)	DC n (%)
Samples	21 (100)	6 (100)	19 (100)	20 (100)
Sex				
Male	11 (52.4)	2 (33.3)	8 (42.1)	14 (70)
Female	10 (47.6)	4 (66.7)	11 (57.9)	6 (30)
Age, years				
≤50	14 (66.7)	2 (33.3)	9 (47.4)	15 (75)
>50	7 (33.3)	4 (66.7)	10 (52.6)	5 (25)
Mean ± SD	46.2 ± 22.22	55.3 ± 14.62	51.9 ± 21.60	42.2 ± 11.41
Location				
Maxilla	0 (0)	0 (0)	4 (21.1)	6 (30)
Mandible	21 (100)	6 (100)	15 (78.9)	14 (70)
Histological subtype				
Follicular	13 (61.9)	4 (66.7)	N/A	N/A
Plexiform	8 (38.1)	2 (33.3)	N/A	N/A
Histology				
Daughter cyst present	N/A	N/A	9 (47.4)	N/A
No daughter cyst	N/A	N/A	10 (52.6)	N/A
Status				
Primary	15 (71.4)	2 (33.3)	16 (84.2)	20 (100)
Recurrent	6 (28.6)	4 (66.7)	3 (15.8)	0 (0)

AM: Ameloblastoma; AC: Ameloblastic carcinoma; OKC: Odontogenic keratocyst; DC: Dentigerous cyst; N/A: Not available; SD: Standard deviation

using the EZ DNA Methylation-GoldTM Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Bisulfite-converted DNA was eluted in a total volume of 12.5 µl and stored at –20˚C until use.

We detected the methylation status of the RASSF1A promoter region using MS-PCR with specific primers, as described previously by Helmbold *et al.* [30]. The primers for methylated sequence (forward: 5'-GTGTTAACGCGTTGCGTATC-3' and reverse: 5'-AACCCCGCGAACTAAAAACGA-3') produced a 93-bp product, while the primers for unmethylated sequences (forward: 5'-TTTGGTTGGAGTGTGTTAATGTG-3' and reverse: 5'-CAAACCCCACAAACTAAAAACAA-3') produced a 105-bp product. The MS-PCR total reaction volume of 25 µl contained 200 ng of bisulfite-modified DNA (template), 12.5 µl of 2× MS-PCR buffer, 1.5 µl each of forward and reverse primers (15 pmol each), 0.25 µl of SYBR® Green I, 6.25 µl of distilled water, and 1 µl of MS-PCR enzyme (Takara Episcope MS-PCR Kit, Shiga, Japan). The

thermal cycling conditions were 95˚C for 10 min; 40 cycles at 94˚C for 30 s (Denaturation), 60˚C for 30 s (Annealing), and 72˚C for 30 s (Extension); and a final extension at 72˚C for 10 min. We used universal unmethylated and methylated DNA (Qiagen) as positive controls for unmethylated and methylated genes, respectively. A reaction in which DNA was replaced by water was used as a negative control. We separated all PCR products on 2% Tris-borate EDTA agarose gels, stained with ethidium bromide, and evaluated them under ultraviolet light.

2.3. Immunohistochemistry

For immunohistochemical analysis, all FFPE odontogenic lesions were cut into 4–μm sections, deparaffinized, rehydrated, and immersed in 3% hydrogen peroxide solution to quench endogenous peroxidase activity. We then incubated the sections in 10 mM EDTA buffer (pH 8.0) for 40 min at 98˚C for antigen retrieval and treated them with normal horse serum for 20 min at room temperature to eliminate nonspecific binding. Next, we incubated the tissues at 4˚C overnight with a mouse monoclonal primary antibody of BRAF V600E (1:50, sc-5284, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After washing with PBS, we incubated the sections with a biotinylated secondary antibody (R.T.U. Vectastain Universal ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 30 minand rinsed them with PBS. Subsequently, we incubated the sections for 30 min with Vectastain ABC-reagent (R. T. U. Vectastain Universal ABC kit) and washed them in PBS. Finally, the samples were incubated in 3,3'-diaminobenzidine (DAB) solution for 3 minto visualize the immunolabeled proteins, counterstained with Mayer's hematoxylin, dehydrated, and mounted. We used melanocytic nevi tissue as a positive control for BRAF V600E expression [31]. The presence of clear cytoplasmic BRAF V600E staining in the odontogenic epithelium was considered a positive immunostaining result. Faint diffuse staining and/or staining of monocytes, macrophages, and plasma cells was considered a negative result [17].

2.4. Statistical Analysis

We examined differences in the RASSF1A methylation frequency and BRAF V600E immunohistochemical expression frequency between AM, AC, OKC, and DC using Fisher's exact test. We also examined correlations of the RASSF1A methylation status and BRAF V600E immunohistochemical status with various clinicopathological characteristics of the samples using Fisher's exact test. A p value of <0.05 was considered statistically significant.

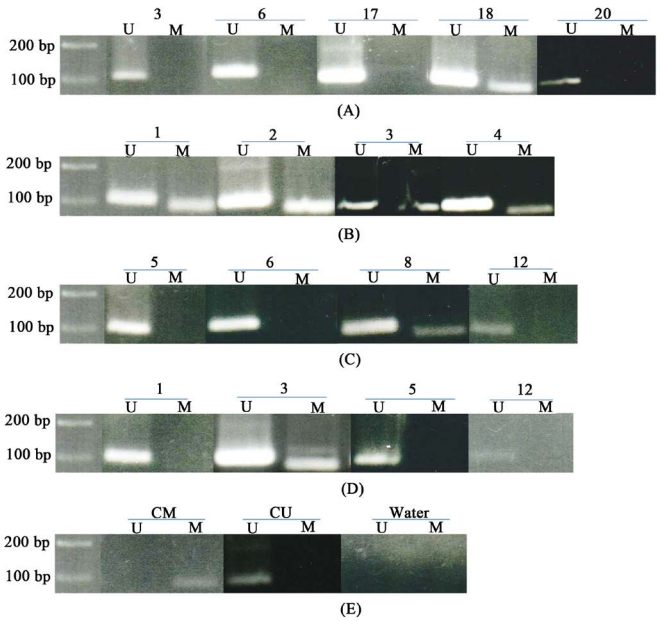
3. Results

3.1. RASSF1A Methylation Statuses of Odontogenic Lesions and Associations with Clinicopathological Features

To investigate the frequency of RASSF1A methylation in odontogenic lesions,

we analyzed the RASSF1A promoter methylation statuses of 21 AM, 6 AC, 19 OKC, and 20 DC samples by MS-PCR. Notably, we detected partial methylation in 4/20 (20%; one sample could not be detected), 6/6 (100%), 5/19 (26.3%), and 1/20 (5%) AM, AC, OKC, and DC samples, respectively. Notably, the frequency of RASSF1A methylationwas significantly higherin AC samples than in AM, OKC, and DC samples ($p < 0.001$), and significantly higher in OKC and AM samples than in DC samples ($p < 0.001$ and $p = 0.004$, respectively) (Table 2; Figure 1).

RASSF1A hypermethylation in the odontogenic lesions did not correlate significantly with patient sex or age (≤50 vs. >50 years) or with tumor status (primary/recurrent) or location ($p > 0.05$ for all). In AM and AC samples, RASSF1A promoter hypermethylation also did not correlate with histological subtype (follicular/plexiform; $p > 0.05$). In OKC samples, no significant correlation was observed between RASSF1A promoter hypermethylation and histology (daughter cyst present/absent; $p > 0.05$). Table 3 lists the results of the correlation analysis of RASSF1A methylation with clinicopathological features in AM samples (data not shown for AC, OKC, and DC samples).



The RASSF1A promoter methylation statuses in AM (lane A), AC (lane B), OKC (lane C), and DC (lane D) samples were analyzed using methylation-specific PCR. Methylation-(M) and unmethylation-(U) specific PCR products (93 and 105 bp, respectively) were resolved on 2% agarose gelwith a 100-bp marker. Universal unmethylated and methylated DNA (CU and CM) were used as positive controls. A reaction lacking DNA was used as a negative control (lane E). In AM (lane A), samples no. 3, 6, 17, and 20 exhibited an unmethylated RASSF1A promoter, while sample No. 18 exhibited partial methylation. In AC (lane B), all samples exhibited partial methylation of the RASSF1A promoter. In OKC (lane C), samples No. 5, 6, 12 exhibited an unmethylated RASSF1A promoter, while sample no.8 exhibited partial methylation. In DC (lane D), samples no. 1, 5, 12 exhibited an unmethylated RASSF1A promoter, while sample No. 3 exhibited partial methylation.

Figure 1. Representative *RASSF1A* methylation-specific PCR analyses of ameloblastoma (AM), ameloblastic carcinoma (AC), odontogenic keratocyst (OKC), and dentigerous cyst (DC) specimens.

Table 2. Frequency of *RASSF1A* methylation and BRAF V600E immunohistochemical expression in odontogenic lesions (N = 66).

No.	Genetic/epigenetic alterations	Odontogenic lesions				P value[b]
		AM (n = 21)	AC (n = 6)	OKC (n = 19)	DC (n = 20)	
1	*RASSF1A* methylation	4/20 (20%)[a]	6/6 (100%)	5/19 (26.3%)	1/20 (5%)	<0.001[†], 0.004*
2	BRAF V600E expression	18/21 (85.7%)	5/6 (83.3%)	0/19 (0%)	0/20 (0%)	<0.001[‡]

AM: Ameloblastoma; AC: Ameloblastic carcinoma; OKC: Odontogenic keratocyst; DC: Dentigerous cyst. [a]Data were obtained for 20 samples; *RASSF1A* methylation could not be detected in one sample. [b]Fisher's exact test; $p < 0.05$ indicates statistical significance. [†]Comparisons of ameloblastic carcinoma with other odontogenic lesions and of odontogenic keratocyst with dentigerous cyst. *Comparison of ameloblastoma with dentigerous cyst. [‡]Comparison of ameloblastoma and ameloblastic carcinoma with odontogenic keratocyst and dentigerous cyst.

Table 3. Analysis of the associations of *RASSF1A* methylation and BRAF V600E expression with clinicopathological variables in ameloblastoma samples (N = 21).

Characteristics (n)	RASSF1A methylated n (%)[a]	P value	BRAF V600E IHC positivity n (%)	P value*
Sex				
Male (11)	2/10 (20)	1.0	8/11 (72.7)	0.21
Female (10)	2/10 (20)		10/10 (100)	
Age, years				
≤50 (14)	2/13 (15.4)	0.59	13/14 (92.9)	0.25
>50 (7)	2/7 (28.6)		5/7 (71.4)	
Location				
Anterior mandible (3)	1/3 (33.3)	0.51	3/3 (100)	1.0
Posterior mandible (18)	3/17 (17.6)		15/18 (83.3)	
Histological subtype				
Follicular (13)	3/12 (25)	0.62	11/13 (84.6)	1.0
Plexiform (8)	1/8 (12.5)		7/8 (87.5)	
Status				
Primary (15)	3/14 (21.4)	1.0	12/15 (80)	0.53
Recurrent (6)	1/6 (16.7)		6/6 (100)	

IHC: immunohistochemistry; [a]Data were obtained for 20 samples; *RASSF1A* methylation could not be detected in one sample. *Fisher's exact test; $p < 0.05$ indicates statistical significance.

3.2. BRAF V600E Immunohistochemical Expression in Odontogenic Lesions and Associations with Clinicopathological Features

Positive BRAF V600E immunostaining was observed in 18/21 (85.7%), 5/6 (83.3%), 0/19 (0%), and 0/20 (0%) of AM, AC, OKC, and DC samples, respec-

tively (Table 2). Both AM and AC samples exhibited moderate to strong BRAF V600E cytoplasmic staining in both the central and peripheral ameloblast-like cells. In contrast, we observed negative or non-specific immunostaining (*i.e.*, faint diffuse staining) in the epithelial linings of all OKC and DC samples. Non-specific immunostaining of macrophages and plasma cells was also detected in some odontogenic lesions. The frequency of BRAF V600E expression was significantly higher in AM and AC samples than in OKC and DC samples ($p < 0.001$ for all) (Table 2; Figure 2).

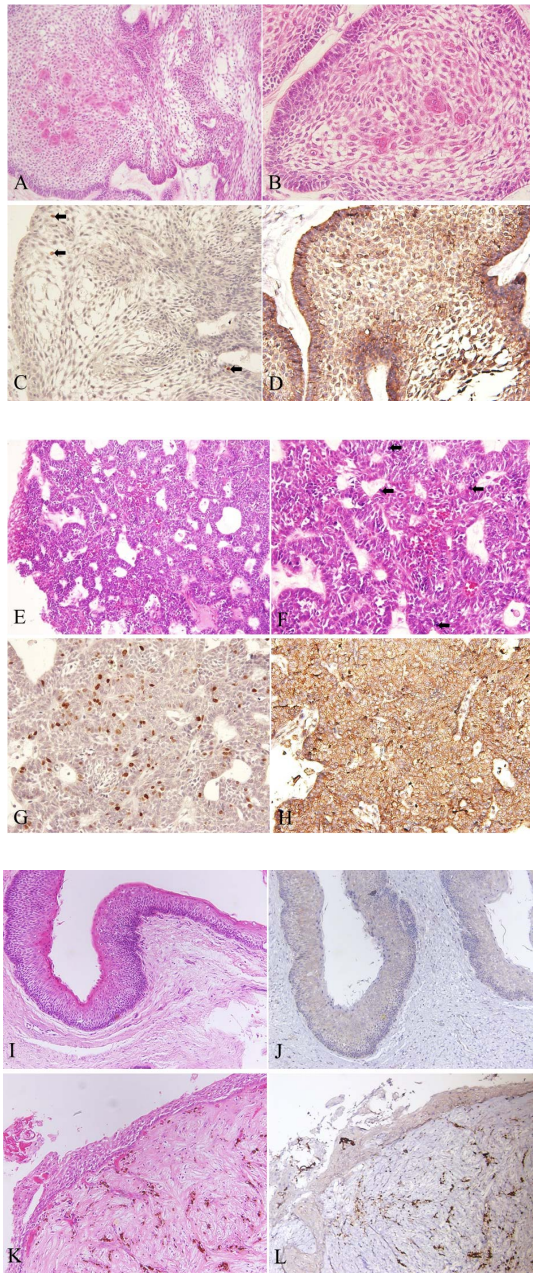
In these odontogenic lesions, we did not observe any associations between BRAF V600E expression and patient sex or age or tumor status (primary/recurrent) or location ($p > 0.05$ for all). Moreover, BRAF V600E expression was not correlated with the histological subtype (follicular/plexiform) in AM and AC samples ($p > 0.05$ for both), or with histology (daughter cyst present/absent) in OKC samples ($p > 0.05$). Table 3 summarizes the results of correlation analysis between BRAF V600E expression and various clinicopathological variables in AM samples (data not shown for AC, OKC, and DC samples).

4. Discussion

The pathogenesis of odontogenic tumors and cysts has not been well established, although epigenetic and genetic alterations appear to play important developmental roles [13]. In this study, we investigated the potential correlations of the RASSF1A promoter methylation status and BRAF V600E immunohistochemical expression with various clinicopathological characteristics in AM, AC, OKC, and DC samples. Despite differences in both markers between the various lesion types, we did not identify any significant correlations with clinicopathological features.

It has been suggested that RASSF1A heterodimerizes Novel Ras effector 1 and may mediate the tumor-suppressive effects of Ras cascade [7]. As noted previously, RASSF1A hypermethylation is a common event in the development of several cancers [8]. In this study, we reported aberrant RASSF1A hypermethylation in all six tested AC samples, but low to moderate RASSF1A methylation frequencies in other odontogenic lesions. The methylation of other tumor suppressor genes has also been reported in odontogenic lesions. For example, p16 methylation was observed in 25% - 53% of AM and 100% of AC cases [26] [28]. The presence of p16 alteration in AM may predispose the lesions to malignant features in the future [28]. p21 methylation was present in 30% of OKC samples, suggesting that p21 methylation might implicate in the OKC development [25].

In this study, we observed a much higher frequency of RASSF1A methylation in AC samples (100% cases) relative to AM, OKC, and DC samples (20%, 26.3%, 5% cases, respectively) ($p < 0.001$). Recently, Zhao *et al.* reported a significantly higher frequency of RASSF1A methylation in malignant solitary pulmonary nodules (SPNs) than in benign SPNs (70% vs.14%) [32]. Other studies also reported higher frequencies of RASSF1A promoter methylation in patients with



Representative photomicrographs of hematoxylin and eosin (H & E)-stained ameloblastoma (AM) (A and B), ameloblastic carcinoma (AC) (E and F), odontogenic keratocyst (OKC) (I), and dentigerous cyst (DC) samples (K). In AM, islands of odontogenic epithelium showed columnar peripheral cells, whereas the central cells were stellate reticulum like cells with focal areas of squamous metaplasia (A and B). Sheets of malignant epithelial cells in AC showing a plexiform pattern (E). One focal area of AC showed cytological features of malignancy, such as cellular pleomorphism, nuclear hyperchromatism, and mitotic figures (arrows) (F). Representative immunohistochemical staining of Ki-67 in AM (C) and AC (G) samples. Higher Ki-67 expression was observed in AC (labeling index: 17.7%) than in AM (labeling index: 2.3%) (arrows). Representative immunohistochemical staining of BRAF V600E in AM (D), AC (H), OKC (J), and DC (L). Moderate to strong BRAF V600E cytoplasmic immunostaining was observed in AM and AC samples, while non-specific immunostaining (faint diffuse staining) was observed in OKC and DC samples. Non-specific staining of macrophages and plasma cells was observed in the DC samples (L). Original magnifications, ×100 (A, E, I, J, K, L) and ×200 (B, C, D, F, G, H).

Figure 2. Staining of representative odontogenic lesion samples.

high-grade and advanced-stage nasopharyngeal carcinoma, breast cancer, and ovarian cancer, suggesting that RASSF1A promoter methylation may lead to a more aggressive tumor phenotype and disease progression [33] [34] [35].

We further investigated the associations between the RASSF1A methylation statuses of the odontogenic lesions and various clinicopathological variables, including patient sex or age, tumor status or location, histological subtype (AM and AC), and histology (OKC). We did not observe significant associations of RASSF1A hypermethylation with any of these variables, although this might be attributable to the small sample size. To our knowledge, this was the first study to evaluate the potential correlations between the RASSF1A methylation statuses of odontogenic lesions and clinicopathological features. Consequently, we cannot directly compare our results to those of other studies. Further research is needed to assess the associations between clinicopathological characteristics and RASSF1A promoter hypermethylation in odontogenic tumors and cysts.

Previous studies demonstrated that the overexpression of RASSF1A promotes apoptosis and cell cycle arrest in various cancer cell lines [8]. Joo *et al.* demonstrated that exogenous RASSF1A upregulated p27 and downregulated cyclin D1 expression and decreased retinoblastoma protein phosphorylation (Ser 608) in human gastric cancer cell lines, thus inhibiting cell proliferation [36]. Moreover, Wang *et al.* demonstrated that exogenous RASSF1A could increase the percentage of cells in the G0/G1 phase of cell cycle, inhibit cell proliferation, and induce apoptosis in a nasopharyngeal carcinoma cell line [33]. Therefore, the epigenetic silencing of RASSF1A by promoter methylation has been implicated in the development of many human cancers [33]. Some recent studies have identified the biological functions of RASSF1A methylation in tumorigenesis. Schirosi *et al.* reported a positive correlation between RASSF1A methylation and cytoplasmic and nuclear β-catenin expression, which resulted in the proliferation and metastasis of colorectal cancer cells [37]. Dubois *et al.* suggested that RASSF1A methylation could downregulate GEF-H1 and RhoB activity and thus promote the nuclear accumulation of YAP, resulting in the metastasis of non-small cell lung cancer cells [38].

Reports suggest that BRAF V600E mutation plays a key role in the tumorigenesis of various cancers, particularly melanomas, thyroid cancers, and hematopoietic malignancies, and a less frequent role in colorectal and lung adenocarcinomas [39]. Recent studies demonstrated that activating BRAF V600E mutations occur in 62% - 83% of AM and AC cases [16] [17]. Moreover, BRAF V600E immunohistochemistry is highly sensitive and specific for the detection of BRAF V600E mutation in thyroid cancers and ameloblastomas, with a level of reliability comparable to that of molecular techniques [18] [40]. Previous studies reported BRAF V600E expression in 46% - 89% of mandibular AM cases [19] [20] [21] [22] consistent with our observations of positive BRAF V600E immunostaining in 85.7% and 83.3% cases of mandibular AM and AC, respectively. In contrast, the frequency of BRAF V600E mutation in OKC samples remains uncertain. Cha *et al.* reported frequent BRAF V600E mutation (~63%) in OKC

samples [23], while Franca *et al.* and Zhang *et al.* detected BRAF V600E mutation in very few samples (0% - 3.5%) [24] [41]. In our study,all OKC and DC samplesexhibited negative or non-specific epithelial BRAF V600E immunostaining. Our results were consistent with the results obtained by Pereira *et al.*, who reported non-specific BRAF V600E immunostaining in the epithelium and plasma cells in some dentigerous and radicular cysts [17]. Taken together, immunohistochemical BRAF V600E expression was observed at higher frequencies in AM and AC samples than in OKC and DC samples ($p < 0.001$).

Moreover, we did not observe any correlations of immunohistochemical BRAF V600E expression with patient sex or age, tumor location or status, or histological subtype in the AM and AC samples. This finding was consistent with a report by Kurppa *et al.* [19]. However, other studies of AM cases have reported significant correlations of BRAF V600E expression with recurrent disease, a younger age (≤50 years), and tumor location in the posterior region of mandible (vs. the anterior region) [20] [21] [22]. These inconsistent results might be attributable to small sample sizes and ethnic differences. Additional large-scale studies are needed to clarify the associations of BRAF V600E expression with clinicopathological features of AM patients.

Finally, previous studies have reported a correlation between RASSF1A methylation and KRAS/BRAF mutation in some tumors. Specifically, RASSF1A promoter hypermethylation occurs predominantly in sporadic colorectal, pancreatic, and thyroid cancers that habor wild type KRAS/BRAF [42] [43]. Interestingly, KRAS/BRAF mutation may occur concomitantly with RASSF1A hypermethylation in malignant melanomas and sporadic MSI colorectal cancers, suggesting a possible synergistic effect [44] [45]. In this study, we observed that 83.3% of tested AC lesions harbored concomitant RASSF1A methylation and BRAF V600E-positive expression, suggesting a synergistic result between the inactivating of RASSF1A and the genetic alteration of BRAF V600E expression. The majority of AM cases (70%) exhibited a preferential single genetic alteration leading to positive BRAF V600E expression. Our observations of infrequent RASSF1A methylation and BRAF V600E expression in OKC and DC samples suggest that genetic/epigenetic alterations in these genes may not play a vital role in the pathogenesis of these lesions.

A limitation of this study is the small sample size. Further studies with larger samples are needed to confirm our preliminary results and elucidate the role of RASSF1A methylation in the tumorigenesis of odontogenic lesions. In addition, we tried to confirm the results of RASSF1A methylation status presented in this study by analyzing the RASSF1A expression with reverse-transcription PCR (RT-PCR) and immunohistochemistry. However, in the majority of RASSF1A unmethylated cases, RASSF1A expression could not be detected by both techniques. It is possible that the routine decalcification procedure may diminish RASSF1A mRNA and protein in our samples. Thus, the selection of a weaker acid-decalcifying agent might provide better results of molecular and immunohistochemical studies of this gene.

5. Conclusion

In summary, we investigated the RASSF1A tumor suppressor gene methylation statuses of various odontogenic lesion types, and observed that RASSF1A promoter methylation was significantly more frequent in AC than in other odontogenic lesions. However, we did not observe a significant correlation between RASSF1A hypermethylation in these lesions and clinicopathological variables. Moreover, we confirmed that positive BRAF V600E expression was frequently observed in mandibular AM and AC lesions (>80% of tested cases), but was absent in OKC and DC lesions. However, BRAF V600E expression did not correlate with clinicopathological features in either AM or AC cases.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Raj, A., Ramesh, G., Nagarajappa, R., Pandey, A. and Raj, A. (2017) Prevalence of Odontogenic Lesions among the Kanpur Population: An Institutional Study. *Journal of Experimental Therapeutics and Oncology*, **12**, 35-42.

[2] Takata, T. and Slootweg, P.J. (2017) Introduction to Odontogenic and Maxillofacial Bone Tumours. In: El-Naggar, A.K., Chan, J.K.C., Grandis, J.R., Takata, T. and Slootweg, P.J., Eds., *WHO Classification of Head and Neck Tumours*, 4th Edition, IARC, Lyon, 205.

[3] Chae, M.P., Smoll, N.R., Hunter-Smith, D.J. and Rozen, W.M. (2015) Establishing the Natural History and Growth Rate of Ameloblastoma with Implications for Management: Systematic Review and Meta-Analysis. *PLoS ONE*, **10**, e0117241. https://doi.org/10.1371/journal.pone.0117241

[4] Cox, D.P., Muller, S., Carlson, G.W. and Murray, D. (2000) Ameloblastic Carcinoma Ex Ameloblastoma of the Mandible with Malignancy-Associated Hypercalcemia. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, **90**, 716-722. https://doi.org/10.1067/moe.2000.109076

[5] Jaafari-Ashkavandi, Z., Mehranmehr, F. and Roosta, E. (2019) MCM3 and Ki67 Proliferation Markers in Odontogenic Cysts and Ameloblastoma. *Journal of Oral Biology and Craniofacial Research*, **9**, 47-50. https://doi.org/10.1016/j.jobcr.2018.09.003

[6] Stoelinga, P.J.W. (2019) Keratocystic Odontogenic Tumor (KCOT) Has Again Been Renamed Odontogenic Keratocyst (OKC). *International Journal of Oral & Maxillofacial Surgery*, **48**, 415-416. https://doi.org/10.1016/j.ijom.2018.07.020

[7] Pfeifer, G.P. and Dammann, R. (2005) Methylation of the Tumor Suppressor Gene *RASSF1A* in Human Tumors. *Biochemistry* (*Moscow*), **70**, 576-583. https://doi.org/10.1007/s10541-005-0151-y

[8] Donninger, H., Vos, M.D. and Clark, G.J. (2007) The *RASSF1A* Tumor Suppressor. *Journal of Cell Science*, **15**, 3163-3172. https://doi.org/10.1242/jcs.010389

[9] Wen, G., Wang, H. and Zhong, Z. (2018) Associations of *RASSF1A*, *RARβ*, *and CDH1* Promotor Hypermethylation with Oral Cancer Risk a PRISMA-Compliant Meta-Analysis. *Medicine* (*Baltimore*), **97**, e9971.

https://doi.org/10.1097/MD.0000000000009971

[10] Cohen, Y., Xing, M., Mambo, E., Guo, Z., Wu, G., Trink, B., Beller, U., Westra, W.H., Ladenson, P.W. and Sidransky, D. (2003) *BRAF* Mutation in Papillary Thyroid Carcinoma. *Journal of the National Cancer Institute*, **95**, 625-627. https://doi.org/10.1093/jnci/95.8.625

[11] Chang, F., Steelman, L.S., Lee, J.T., Shelton, J.G., Navolanic, P.M., Blalock, W.L., Franklin, R.A. and McCubrey, J.A. (2003) Signal Transduction Mediated by the Ras/Raf/MEK/ERK Pathway from Cytokine Receptors to Transcription Factors: Potential Targeting for Therapeutic Intervention. *Leukemia*, **17**, 1263-1293. https://doi.org/10.1038/sj.leu.2402945

[12] Ducreux, M., Chamseddine, A., Laurent-Puig, P., Smolenschi, C., Hollebecque, A., Dartigues, P., Samallin, E., Boige, V., Malka, D. and Gelli, M. (2019) Molecular Targeted Therapy of *BRAF*-Mutant Colorectal Cancer. *Therapeutic Advances in Medical Oncology*, **11**, 1-15. https://doi.org/10.1177/1758835919856494

[13] Lapthanasupkul, P., Juengsomjit, R., Poomsawat, S. and Arayapisit, T. (2018) Expression Profile of Polycomb Group Proteins in Odontogenic Keratocyst and Ameloblastoma. *Actahistochemica*, **120**, 215-220. https://doi.org/10.1016/j.acthis.2018.01.009

[14] Kumamoto, H. (2006) Molecular Pathology of Odontogenic Tumors. *Journal of Oral Pathology & Medicine*, **35**, 65-74. https://doi.org/10.1111/j.1600-0714.2006.00380.x

[15] Li, T.J. (2011) The Odontogenic Keratocyst: A Cyst or a Cystic Neoplasm? *Journal of Dental Research*, **90**, 133-142. https://doi.org/10.1177/0022034510379016

[16] Diniz, M.G., Gomes, C.C., de Sousa, S.F., Xavier, G.M. and Gomez, R.S. (2017) Oncogenic Signaling Pathways in Benign Odontogenic Cysts and Tumors. *Oral Oncology*, **72**, 165-173. https://doi.org/10.1016/j.oraloncology.2017.07.021

[17] Pereira, N.B., Pereira, K.M., Coura, B.P., Diniz, M.G., de Castro, W.H., Gomes, C.C. and Gomez, R.S. (2016) *BRAF* V600E Mutation in the Diagnosis of Unicystic Ameloblastoma. *Journal of Oral Pathology & Medicine*, **45**, 780-785. https://doi.org/10.1111/jop.12443

[18] Brown, N.A., Rolland, D., McHugh, J.B., Weigelin, H.C., Zhao, L., Lim, M.S., Elenitoba-Johnson, K.S. and Betz, B.L. (2014) Activating *FGFR2-RAS-BRAF* Mutations in Ameloblastoma. *Clinical Cancer Research*, **20**, 5517-5526. https://doi.org/10.1158/1078-0432.CCR-14-1069

[19] Kurppa, K.J., Caton, J., Morgan, P.R., Ristimäki, A., Ruhin, B., Kellokoski, J., Elenius, K. and Heikinheimo, K. (2014) High Frequency of BRAF V600E Mutations in Ameloblastoma. *Journal of Pathology*, **232**, 492-498. https://doi.org/10.1002/path.4317

[20] Fregnani, E.R., Perez, D.E., Paes de Almeida, O., Fonseca, F.P., Soares, F.A., Castro-Junior, G. and Alves, F.A. (2017) BRAF-V600E Expression Correlates with Ameloblastoma Aggressiveness. *Histopathology*, **70**, 473-484. https://doi.org/10.1111/his.13095

[21] do Canto, A.M., da Silva Marcelino, B.M.R, Schussel, J.L., Wastner, B.F., Sassi, L.M., Corrêa, L., de Freitas, R.R., Hasséus, B., Kjeller, G., Junior, C.A.L and Braz-Silva, P.H. (2019) Immunohistochemical Analysis of BRAF V600E Mutation in Ameloblastomas. *Clinical Oral Investigation*, **23**, 779-784. https://doi.org/10.1007/s00784-018-2494-y

[22] Kelppe, J., Thoren, H., Ristimaki, A., Haglund, C., Sorsa, T. and Hagstrom, J. (2019) BRAF V600E Expression in Ameloblastomas-A 36-Patient Cohort from Helsinki

University Hospital. *Oral Diseases*, **25**, 1164-1174. https://doi.org/10.1111/odi.13072

[23] Cha, Y.H., Cho, E.S., Kang, H.E., Ko, J., Nam, W., Kim, H.J., Kim, N.H., Kim, H.S., Cha, I.H. and Yook, J.I. (2017) Frequent Oncogenic *BRAFV600E* Mutation in Odontogenic Keratocyst. *Oral Oncology*, **74**, 62-67. https://doi.org/10.1016/j.oraloncology.2017.09.016

[24] Franca, J.A., de Sousa, S.F., Diniz, M.G., Pereira, T.D.S.F., de Resende, T.A.C., Santos, J.N.D., Gomez, R.S. and Gomes, C.C. (2018) Absence of *BRAFV600E* Mutation in Odontogenic Keratocysts. *Journal of Oral Pathology & Medicine*, **47**, 186-191. https://doi.org/10.1111/jop.12671

[25] Moreira, P.R., Guimaraes, M.M., Guimaraes, A.L., Diniz, M.G., Gomes, C.C., Brito, J.A. and Gomez, R.S. (2009) Methylation of *p*16, *p*21, *p*27, *RB*1 and *p*53 Genes in Odontogenic Keratocysts. *Journal of Oral Pathology & Medicine*, **38**, 99-103. https://doi.org/10.1111/j.1600-0714.2008.00718.x

[26] Moreira, P.R., Guimaraes, M.M., Gomes, C.C., Diniz, M.G., Brito, J.A., de Castro, W.H. and Gomez, R.S. (2009) Methylation Frequencies of Cell-Cycle Associated Genes in Epithelial Odontogenic Tumours. *Archives of Oral Biology*, **54**, 893-897. https://doi.org/10.1016/j.archoralbio.2009.07.006

[27] Farias, L.C., Gomes, C.C., Rodrigues, M.C., de Castro, W.H., Lacerda, J.C., Ferreira, E.F. and Gomez, R.S. (2012) Epigenetic Regulation of Matrix Metalloproteinase Expression in Ameloblastoma. *BMC Clinical Pathology*, **12**, Article No. 11. https://doi.org/10.1186/1472-6890-12-11

[28] Khojasteh, A., Khodayari, A., Rahimi, F., Ghaderian, M.H., Jafarian, M., Nayebi, A., Akbarzadeh Najar, R., Tabatabayipanah, A. and Jahangirnia, A. (2013) Hypermethylation of *p*16 Tumor-Suppressor Gene in Ameloblastic Carcinoma, Ameloblastoma, and Dental Follicles. *Journal of Oral and Maxillofacial Surgery*, **71**, 62-65. https://doi.org/10.1016/j.joms.2012.04.033

[29] Martinez-Martinez, M., Mosqueda-Taylor, A., Carlos-Bregni, R., Pires, F.R., Delgado-Azanero, W., Neves-Silva, R., Aldape-Barrios, B. and Paes-de Almeida, O. (2017) Comparative Histological and Immunohistochemical Study of Ameloblastoma and Ameloblastic Carcinomas. *Medicina Oral Patologia Oraly Cirugia Bucal*, **22**, e324-e332. https://doi.org/10.4317/medoral.21901

[30] Helmbold, P., Lahtz, C., Herpel, E., Schnabel, P.A. and Dammann, R.H. (2009) Frequent Hypermethylation of *RASSF1A* Tumour Suppressor Gene Promoter and Presence of Merkel Cell Polyomavirus in Small Cell Lung Cancer. *European Journal of Cancer*, **45**, 2207-2211. https://doi.org/10.1016/j.ejca.2009.04.038

[31] Kiuru, M., Tartar, D.M., Qi, L., Chen, D., Yu, L., Konia, T., McPherson, J.D., Murphy, W.J. and Fung, M.A. (2018) Improving Classification of Melanocytic Nevi: *BRAF V600E* Expression Associated with Distinct Histomorphologic Features. *Journal of the American Academy of Dermatology*, **79**, 221-229. https://doi.org/10.1016/j.jaad.2018.03.052

[32] Zhao, J., Cui, X., Huang, X., Lu, R., Chen, P. and Zhang, Z. (2019) Methylation of *RUNX3* and *RASSF1A* and the Risk of Malignancy in Small Solitary Pulmonary Nodules. *Journal of Cancer Research and Therapeutics*, **15**, 899-903. https://doi.org/10.4103/jcrt.JCRT_790_18

[33] Wang, T., Liu, H., Chen, Y., Liu, W., Yu, J. and Wu, G. (2009) Methylation Associated Inactivation of *RASSF1A* and Its Synergistic Effect with Activated K-Ras in Nasopharyngeal Carcinoma. *Journal of Experimental and Clinical Cancer Research*, **28**, 160. https://doi.org/10.1186/1756-9966-28-160

[34] Hagrass, H.A., Pasha, H.F., Shaheen, M.A., Abdel Bary, E.H. and Kassem, R. (2014) Methylation Status and Protein Expression of *RASSF1A* in Breast Cancer Patients. *Molecular Biology Reports*, **41**, 57-65. https://doi.org/10.1007/s11033-013-2837-3

[35] Rezk, N.A., Mohamed, R.H., Alnemr, A.A. and Harira, M. (2018) Promoter Methylation of *RASSF1A* Gene in Egyptian Patient with Ovarian Cancer. *Applied Biochemistry and Biotechnology*, **185**, 153-162. https://doi.org/10.1007/s12010-017-2648-4

[36] Joo, M.K., Kim, K.H., Park, J.J., Yoo, H.S., Choe, J., Kim, H.J., Lee, B.J., Kim, J.S. and Bak, Y.T. (2015) CpG Island Promoter Hypermethylation of Ras Association Domain Family 1*A* Gene Contributes to Gastric Carcinogenesis. *Molecular Medicine Reports*, **11**, 3039-3046. https://doi.org/10.3892/mmr.2014.3055

[37] Schirosi, L., Mazzotta, A., Opinto, G., Pinto, R., Graziano, G., Tommasi, S., Fucci, L., Simone, G. and Mangia, A. (2016) β-Catenin Interaction with *NHERF*1 and *RASSF1A* Methylation in Metastatic Colorectal Cancer Patients. *Oncotarget*, **7**, 67841-67850. https://doi.org/10.18632/oncotarget.12280

[38] Dubois, F., Keller, M., Calvayrac, O., Soncin, F., Hoa, L., Hergovich, A., Parrini, M.C., Mazières, J., Vaisse-Lesteven, M., Camonis, J., Levallet, G. and Zalcman, G. (2016) *RASSF1A* Suppresses the Invasion and Metastatic Potential of Human Non-Small Cell Lung Cancer Cells by Inhibiting YAP Activation through the GEF-H1/RhoB Pathway. *Cancer Research*, **76**, 1627-1640. https://doi.org/10.1158/0008-5472.CAN-15-1008

[39] Dumaz, N., Jouenne, F., Delyon, J., Mourah, S., Bensussan, A. and Lebbe, C. (2019) Atypical *BRAF* and *NRAS* Mutations in Mucosal Melanoma. *Cancers*, **11**, 1133. https://doi.org/10.3390/cancers11081133

[40] Singarayer, R., Mete, O., Perrier, L., Thabane, L., Asa, S.L., Van Uum, S., Ezzat, S., Goldstein, D.P. and Sawka, A.M. (2019) A Systematic Review and Meta-Analysis of the Diagnostic Performance of BRAF V600E Immunohistochemistry in Thyroid Histopathology. *Endocrine Pathology*, **30**, 201-218. https://doi.org/10.1007/s12022-019-09585-2

[41] Zhang, R., Yang, Q., Li, T., Qu, J., Hong, Y. and Liu, P. (2020) The *BRAF p.V600E* Mutation Is a Common Event in Ameloblastomas But Is Absent in Odontogenic Keratocysts. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 1-7. https://doi.org/10.1016/j.oooo.2019.04.001

[42] van Engeland, M., Roemen, G.M., Brink, M., Pachen, M.M., Weijenberg, M.P., de Bruïne, A.P., Arends, J.W., van den Brandt, P.A., de Goeij, A.F. and Herman, J.G. (2002) *K-Ras* Mutations and *RASSF1A* Promoter Methylation in Colorectal Cancer. *Oncogene*, **21**, 3792-3795. https://doi.org/10.1038/sj.onc.1205466

[43] Xing, M., Cohen, Y., Mambo, E., Tallini, G., Udelsman, R., Ladenson, P.W. and Sidransky, D. (2004) Early Occurrence of *RASSF1A* Hypermethylation and Its Mutual Exclusion with *BRAF* Mutation in Thyroid Tumorigenesis. *Cancer Research*, **64**, 1664-1668. https://doi.org/10.1158/0008-5472.CAN-03-3242

[44] Reifenberger, J., Knobbe, C.B., Sterzinger, A.A., Blaschke, B., Schulte, K.W., Ruzicka, T. and Reifenberger, G. (2004) Frequent Alterations of Ras Signaling Pathway Genes in Sporadic Malignant Melanomas. *International Journal of Cancer*, **109**, 377-384. https://doi.org/10.1002/ijc.11722

[45] Oliveira, C., Velho, S., Domingo, E., Preto, A., Hofstra, R.M., Hamelin, R., Yamamoto, H., Seruca, R. and Schwartz, S.Jr. (2005) Concomitant *RASSF1A* Hypermethylation and *KRAS*/*BRAF* Mutations Occur Preferentially in MSI Sporadic Colorectal Cancer. *Oncogene*, **24**, 7630-7634. https://doi.org/10.1038/sj.onc.1208906