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IMMUNOHISTOCHEMICAL EXPRESSION OF DE NOVO METHYLTRANSFERASES (DNMT3A AND DNMT3B) IN AMELOBLASTOMA AND ODONTOGENIC CYSTS

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ABSTRACT

Background: Ameloblastoma (AME), radicular cyst (RC), dentigerous cyst (DC), and odontogenic keratocyst (OKC) are the most frequently occurring odontogenic lesions. De novo DNA methylation takes place by the action of DNA methyl transferases (DNMT) 3A and 3B. Epigenetic studies revealing the expression of methyltransferases in odontogenic cysts and tumors are sparse and miss the relevance of the expression to clinical outcomes. This study examined the expression of DNMT3A and DNMT3B in the most common odontogenic cysts and AME and linked the expression to clinical variables.

Methods: Immunohistochemical staining was performed on 75 paraffin-embedded tissue sections, 15 of each of NOM, RC, DC, OKC, and AME. The immunopositive epithelial cells were counted, statistically analyzed, and correlated to clinical features.

Results: DNMT3A was more highly significantly expressed in odontogenic cysts than DNMT3B while the expression was the highest in AME with no significant difference between both markers. DNMT3A correlated significantly with disrupted cortical integrity in RC ($p<0.001$) and AME ($p=0.038$), besides, male gender ($p=0.001$) and multilocularity ($p=0.001$) in OKC. DNMT3B was only highly expressed in DC in females ($p=0.031$).

Conclusion: DNMT3A has more prevailing action in de novo methylation of RC, DC, and OKC than DNMT3B. It might have a role in the excessive growth of RC and the aggressiveness of AME and OKC.

KEY WORDS: Ameloblastoma; de novo methylation; DNA methyltransferases; epigenetics; odontogenic cysts

Abbreviations: Ameloblastoma; (AME), area under curve; (AUC), cytosine-phosphate-guanine; (CpG), dentigerous cyst; (DC), DNA methyltransferase; (DNMT), normal mucosa; (NOM), odontogenic keratocyst; (OKC), radicular cyst; (RC), World health organization; (WHO).

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INTRODUCTION

DNA methyltransferases (DNMT) are a group of enzymes linked to either preservation or de novo methylation of the genome (Jones, 2012). A crucial step in gene silencing during tumorogenesis is the addition of a new methyl group to the promoter cytosine-phosphate-guanine (CpG) dinucleotide island by DNMT3A and DNMT3B. During the past years, the role of methylation in carcinogenesis and its impact on tumor behavior and progress has been widely explored (Lu et al., 2020). Changes in methylation patterns have been related to the pathogenesis of several tumors including ovarian cancer (Bai et al., 2012), breast cancer (Ben et al., 2012), and oral squamous cell carcinoma (Daniel et al., 2010). However, studies regarding epigenetic events in odontogenic lesions are still scarce.

The World health organization (WHO) classified odontogenic lesions into tumors, inflammatory and developmental cysts. The most commonly occurring epithelial odontogenic tumor is ameloblastoma (AME) which is known for its locally aggressive behavior and high recurrence. Radicular cyst (RC) is a frequent inflammatory cyst related to the apex of non-vital teeth. Dentigerous cyst (DC) and odontogenic keratocyst (OKC) are developmental cysts where OKC is locally destructive with a high recurrence rate and DC has a tendency to change into AME and other malignant neoplasms (Soluk-Tekkeşin and Wright, 2018).

Epigenetic modifications in odontogenic lesions like cell cycle genes and tumor suppressor genes silencing were already observed (Moreira et al., 2009 a; Moreira et al., 2009b; Khojasteh et al., 2013; Guimarães et al., 2015). Although few reports previously studied DNMTs expression in odontogenic tumors and a number of cysts (Cavaliéri et al., 2010; Guimarães et al., 2015), cysts in these studies were of small sample size, missed relevance of the expressions with clinical variables, besides some differences in results between studies.

Exploring de novo methyltransferases in AME and the most common odontogenic cysts helps us understand the size of gene silencing in these lesions and the differences in the role played by epigenetics in their biology. Thus, we conducted this study to detect and compare the immunohistochemical expression of DNMT3A and DNMT3B in RC, DC, OKC, and AME in addition to correlating the expression with clinical parameters.

MATERIAL AND METHODS

Samples

By adopting an alpha (α) level of 0.05 (5%), a beta (β) level of 0.2 (i.e. power=80%) and an effect size (W) of (0.922) calculated based on the results of a previous study (Cavaliéri et al., 2010). The predicted total sample size (n) was found to be (25) samples (5samples per group). Sample size calculation was performed using G*Power version 3.1.9.7 (Faul et al., 2007). As the calculated number is a minimum estimation of the required sample size, the sample size was increased to (75) samples (15 samples/ group).

Seventy- five formalin- fixed specimens were included in this study (15 of each of): RC, DC, OKC, AME, and, normal mucosa (NOM) taken from the gingiva during implant surgery. All the tissues were obtained from the pathology archive of the Oral Pathology Department, Faculty of Dentistry, Ain Shams University, Cairo, Egypt. All blocks were coded and the patient's information was anonymous. The study was approved by the ethics committee of the Faculty of Dentistry, Ain Shams University with a waiver from consent (FDASU-Rec ER092216). The patients' clinical data was summarized in **Table 1**.

Immunohistochemistry and Analysis

We performed immunohistochemical staining using the streptavidin-peroxidase method. Proper formalin-fixed and paraffin-embedded 4-μm thick sections were obtained and mounted on positively charged slides. The slides were deparaffinized in xylene, rehydrated in a graded alcohol series, and washed in tap water. For antigen retrieval, the sections were placed in a microwave oven with a

TABLE (1) Patients' Characteristics

		RC		DC		OKC		AME	
Age	**Range**	12-63		14-62		14-35		19-62	
	Mean± SD	37.4±12.5		33.2±13.35		27.4±6.5		43.5±13.4	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Sex	**M**	9	60.0%	12	80.0%	11	73.3%	7	46.7%
	F	6	40.0%	3	20.0%	4	26.7%	8	53.3%
Site	**Maxilla**	6	40.0%	6	40.0%	14	93.3%	0	0.0%
	Mandible	9	60.0%	9	60.0%	1	6.7%	15	100.0%
X-ray	**Unilocular**	15	100.0%	15	100.0%	8	53.3%	1	6.7%
	Multilocular	0	0.0%	0	0.0%	7	46.7%	14	93.3%
Cortical integrity	**Intact**	6	40.0%	13	86.7%	2	13.3%	2	13.3%
	Disrupted	9	60.0%	2	13.3%	13	86.7%	13	86.7%

citric acid solution, pH 6.0, for 15 min. Endogenous peroxidase activity was blocked with 6% H2O2 in methanol for 15 min. The sections were incubated with primary antibodies against DNMT3A (SAB5701326, 1:50, Sigma, St. Louis, MO, USA)) and DNMT3B (HPA001595, 1:100, Sigma, St. Louis, MO, USA)) at 4°C overnight, followed by incubation with ADVANCETM/HRP (Dako, Carpinteria, CA, USA), and diaminobenzidine tetrahydrochloride (DAB) chromagen (Sigma, St. Louis, MO, USA) for the visualization of antigen-antibody complexes. All sections were counterstained with Harris Haematoxylin.

Two independent pathologists carefully examined the slides and captured at least 3 microscopic fields of each specimen at original magnification of 40X using (Canon EOS 650D) camera mounted on a light microscope (BX60, Olympus, Japan). DNMT3A and DNMT3B immunoreactivity was assessed in the nucleus and cytoplasm of epithelial cells. Only the cells showing nuclear reaction were counted (Daniel et al, 2010) and the percentage of positive cells to the total number of cells was calculated.

Statistical Analysis

The collected data was tabulated using Microsoft Excel version 2007. Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Data was summarized using median and inter-quartile range in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests. For comparison of paired measurements within the same group, the non-parametric Wilcoxon signed rank test was used. For comparing categorical data, Chi-square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is less than 5. ROC curve was constructed with area under curve (AUC) analysis performed to detect the best cut-off value of significant parameters. P-values less than 0.05 were considered statistically significant.

RESULTS

The immunohistochemical stain of DNMT3A and DNMT3B in NOM, RC, DC, OKC, and AME are shown in **Fig.1.** All groups showed positive nuclear and/or cytoplasmic epithelial immunoreactivity of DNMT3A and DNMT3B. DNMT3A expression in NOM was predominantly cytoplasmic or negative. Diffuse nuclear and cytoplasmic stain of DNMT3A was detected in RC and DC while OKC revealed

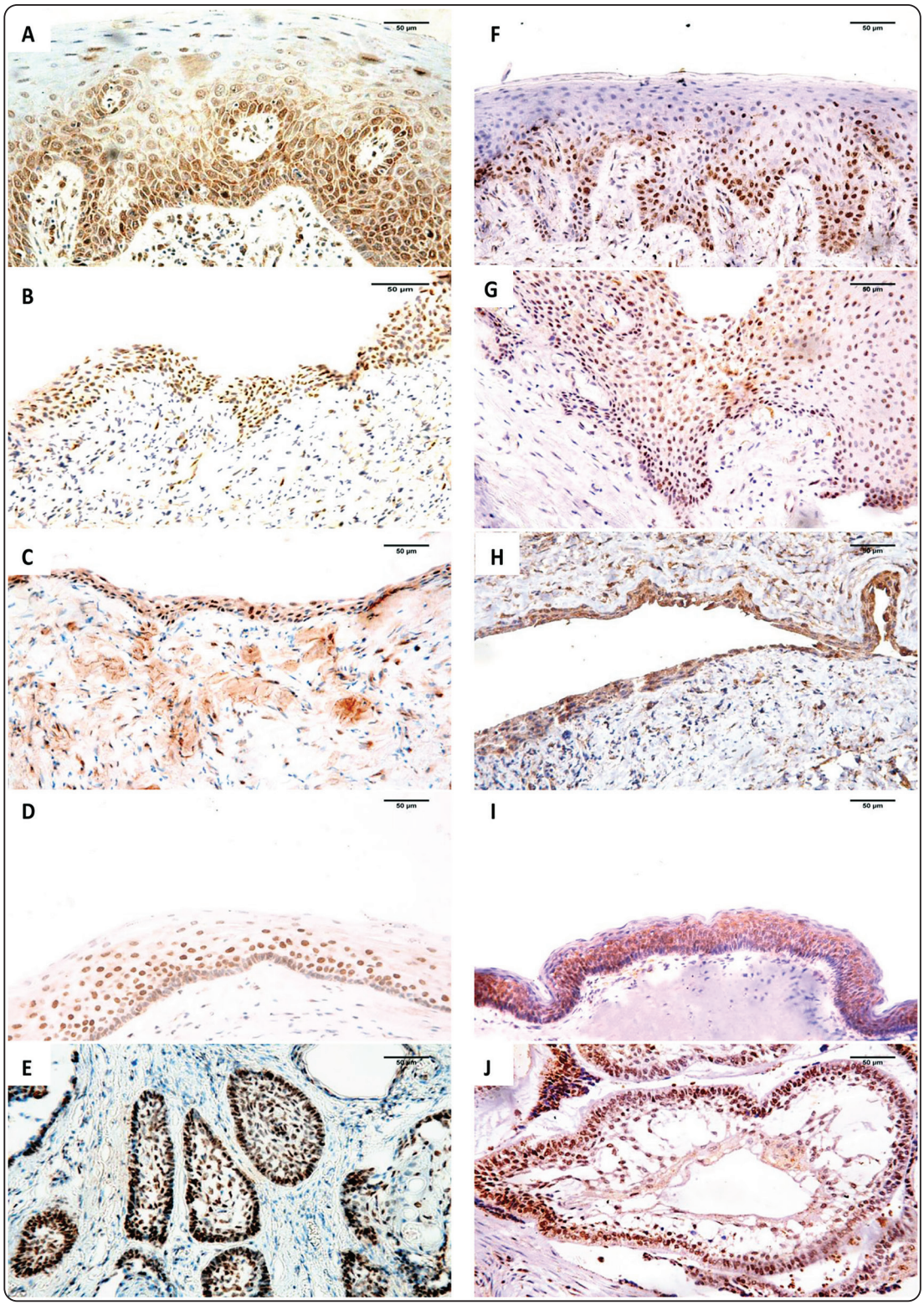

Fig. (1) Immunohistochemical reaction of DNMT3A in (A,B,C,D, E) and DNMT3B in (F,G,H,I,J), (A) Nuclear, cytoplasmic and negative expressions of epithelium in NOM, (B,C,D) Nuclear and cytoplasmic expressions in RC, DC and OKC (E) Nuclear expression of AME, (F) Nuclear and negative reactions in NOM, (G) Nuclear, cytoplasmic and negative reactions in RC, (H,I) Cytoplasmic expression in DC and OKC (J) Nuclear expression in AME (H and E, Original magnification, 40X).

basal cytoplasmic expression and supra-basal prickle cell nuclear expression. Regarding DNMT3B, NOM showed only basal and supra-basal nuclear stain. Diffuse nuclear and cytoplasmic expression was identified in RC, while diffuse and supra-basal cytoplasmic stain was found in DC and OKC respectively. Moreover, diffuse nuclear expressions of DNMT3A and DNMT3B in ameloblast- like cells and stellate reticulum -like cells were detected in AME.

Comparing the expression of both markers between groups revealed a statistically significant increase in the median percentage of positive cells of DNMT3A in all groups in relation to the control with the highest immunoreactivity in AME (82.07, p <0.001) and DC (80.26, p <0.001) (**Table 2, Table 3, Fig.2A**). Concerning the expression of DNMT3B, only AME group showed a statistically significant increase in median nuclear reactivity (91.28) from NOM (p=0.024) (**Table 2, Table 3, Fig.2A**). RC, DC, and OKC revealed higher immunohistochemical expression of DNMT3A than DNMT3B, however, DNMT3B was higher in NOM and AME with no significant difference between both markers in AME group (**Table 2, Fig.2A**).

TABLE (2) Comparing the positive cell percentage of DNMT3A and DNMT3B between all groups and comparing the difference of expression of DNMT3A and DNMT3B in each group

		NOM	RC	DC	OKC	AME	*P*-value
DNMT3A	**Median**	17.58	58.23	80.26	56.71	82.07	<0.001
DNMT3B		31.79	22.61	10.45	10.76	91.28	<0.001
***P*-value**		0.003	0.023	0.002	0.005	0.078	

P<0.05 is statistically significant

TABLE (3) P value between one group and the other

	DNMT3A (*P*- value)	DNMT3B (*P*-value)
NOM VS RC	0.035	0.084
NOM VS DC	<0.001	<0.001
NOM VS OKC	0.136	<0.001
NOM VS AME	<0.001	0.024
RC VS DC	0.133	0.019
RC VS OKC	0.541	0.022
RC VS AME	0.003	<0.001
DC VS OKC	0.034	0.960
DC VS AME	0.154	<0.001
OKC VS AME	<0.001	<0.001

P<0.05 is statistically significant

The cell positivity rate compared among different clinical variables identified more DNMT3A positive cells in RC and AME with disrupted than intact cortical integrity (p<0.001, p= 0.038 respectively) with cut off points (36.4018, 70.5350 respectively) and OKC in males than females with multilocular than unilocular radiographic appearance (p=0.001) with cut-off points (18.5273, 55.9444) respectively. On the other hand, only females detected more nuclear reactivity of DNMT3B than males in DC (p=0.031) and cut-off point (12.3044) (**Table 4, Fig 2B**).

TABLE (4) Comparison among clinical and radiographic features in RC, DC, OKC and AME groups

		DNMT3A (*P*- value)				DNMT3B (*P*- value)			
		RC	**DC**	**OKC**	**AME**	**RC**	**DC**	**OKC**	**AME**
Sex	**M** **F**	0.272	0.448	0.001	0.054	0.113	0.031	0.489	0.613
Site	**Maxilla** **Mandible**	0.181	0.529	0.667	---	0.689	0.689	1	---
Cortical Integrity	**Intact** **Disrupted**	<0.001	0.686	0.229	0.038	0.529	0.229	0.800	0.686
X-ray	**Unilocular** **Multilocular**	---	---	0.001	0.267	---	---	0.463	0.267

P<0.05 is statistically significant

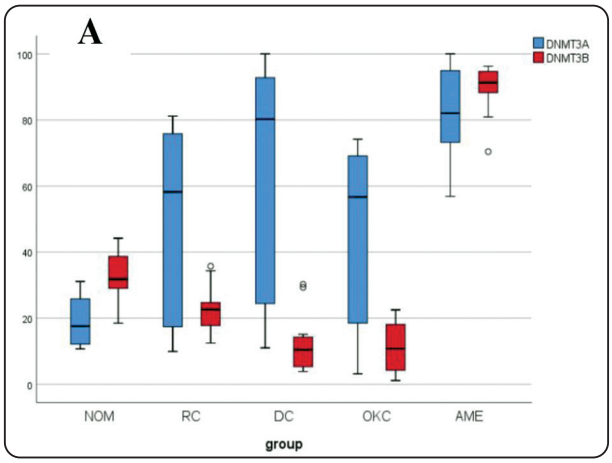

Fig. (2) (A): Graphic distribution of positive cells (%) for each antibody in NOM, RC, DC, OKC and AME,

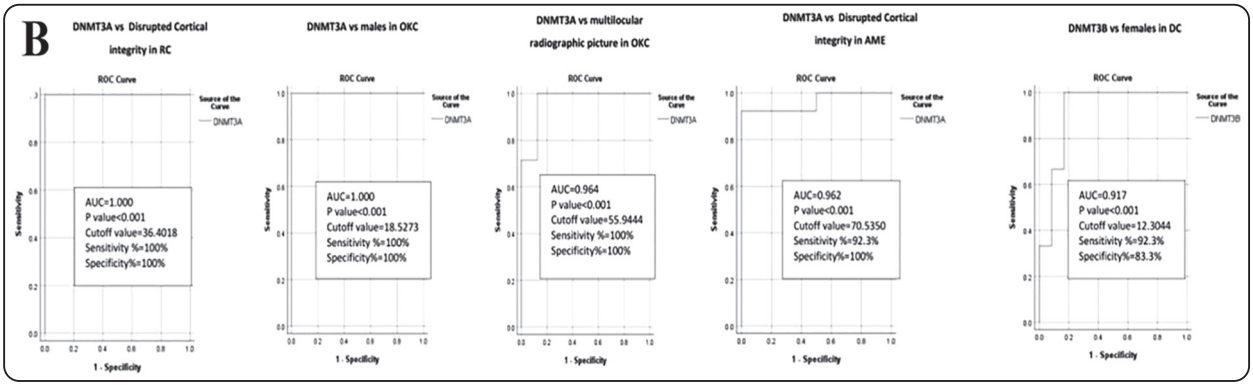

Fig. (2) (B): The cut-off points determined by ROC curve tests only in categorical variables comparison with a significant difference in RC, DC, OKC and AME groups.

DISCUSSION

The study of epigenetic changes in different cysts and neoplasms deepens our knowledge of the different mechanisms involved in the initiation, progression, and aggressiveness of the lesions. A proper understanding of the association of epigenetics with clinical behavior paves the way for more promising therapeutic targets.

As a positive control, NOM revealed similar results to that of Guimarães et al, (2015) who observed cytoplasmic expression of DNMT3A and nuclear expression, in the spinous layer, of DNMT3B. DNMT enzymes were only considered active when detected in the nucleus, as previously reported (Daniel et al., 2010). Regarding RC, our DNMT3A results did not agree with that of Cavaliéri et al. (2010) who did not find nuclear expression of DNMT3A in any of their RC samples. Guimarães et al. (2015) reported more than 50% nuclear expression of DNMT3B in 30% of their study cases, while our median nuclear positivity was 22.61%. The nuclear positivity in the epithelium of RC may be attributed to the inflammation-induced epigenetic switch. This phenomenon was observed in some tumors where the inflammatory mediators secreted from the microenvironment changed the expression of the DNMT in the tumor cells (Qian et al., 2008; Cardenas et al., 2014).

Interestingly the median nuclear reactivity of DNMT3A in DC cases was comparable to that of AME. This finding explains that DNMT3A may have a role in the transition of some DCs to AME; however, this needs further research on DC and unicystic AME samples. So far, no previous reports revealing the expression of DNMT3A in DC could be reached. Only a median of 10.45% nuclear expression of DNMT3B was found in this study, however, one previous study reported that 50% of the cases expressed more than 25% to 50% nuclear reactivity (Guimarães et al., 2015).

The suprabasal nuclear DNMT3A expression of OKC is in agreement with Cavaliéri et al, (2010) who noticed the same distribution of positivity in 3 out of 10 samples; however, Guimarães et al. (2015) only detected supra-basilar cytoplasmic reaction. The predominant supra-basilar cytoplasmic expression of DNMT3B in our results contrasts that of Guimarães et al. (2015) who detected more than 75% nuclear reaction above the basal cell layer in 100% of cases.

Concerning AME, the expression of de novo methyl transferases in AME agrees with Amaral-Silva et al. (2021) who found a diffuse nuclear expression in the central and peripheral cells with higher median nuclear positivity for DNMT3A than DNMT3B, however, no detectable difference in the expression of both enzymes was observed in our study. In contrast, Cavaliéri et al. (2010) detected only cytoplasmic reactivity of DNMT3A in AME samples. Guimarães et al. (2015) also claimed that no AME cases expressed more than 25% nuclear expression for DNMT3A, nevertheless, the expression of DNMT3B was predominantly nuclear in 100% of cases.

The significant high expression of both enzymes in AME compared to most cysts was supported by the previous studies (Cavaliéri et al., 2010; Guimarães et al., 2015), a finding proving the action of the methyl transferases in silencing tumor suppressor genes and triggering the aggressive behavior of AME (Sandoval et al., 2018). The overall results of this paper disclosed a prevailing nuclear staining of DNMT3A in all odontogenic cysts compared to that of DNMT3B suggesting a more significant role in methylation of these lesions. In a study comparing both enzymes in odontogenic lesions, Guimarães et al. (2015) claimed that DNMT3B had more involvement in the epigenetic events of odontogenic tumors and OKC.

The disparities in DNMT expressions in different studies could be linked to the epigenetic changes between ethnicities (Salas et al., 2021). One study revealed that the prevalence of certain types of cancer in definite ethnic groups was related to

differences in gene methylation (Jordan et al., 2022). Another study revealed changes in the expression of DNMT3A in systemic lupus erythematosus patients between African and European Americans (Wiley et al., 2013).

To the best of our knowledge, this is the first study correlating the expressions of DNMT3A and DNMT3B with clinical parameters in odontogenic cysts. Comparing the expression of DNMT3A and DNMT3B with clinical and radiographic features, our results suggest that RC with over-expressed DNMT3A may have more tendencies to enlarge causing cortical plate disruption. AME with DNMT3A over-expression can have a more locally aggressive behavior. Moreover, an over-expressed DNMT3A occurs mostly in the male gender and multilocular radiographic presentation of OKC indicating more aggressiveness. Regarding DNMT3B, no correlation with clinical and radiographic features was recognized except for the high expression in females with DC. In their study on AME, Amaral-Silva et al. (2021) did not find any correlation between de novo methyl transferases and aggressiveness, site, or sex; however, they detected a link between DNMT3B and risk of recurrence.

The aggressive behavior of AME and OKC with aberrant high DNMT3A expression may be associated with the silencing of some tumor suppressor genes. Previous reports detected p21 hypermethylation in OKC (Moreira et al., 2009a) besides p21 and p16 hypermethylation in AME (Moreira et al., 2009 b; Khojasteh et al., 2013), nevertheless, neither of these studies correlated the change in methylation profile with clinical behavior.

CONCLUSION

In conclusion, DNMT3A may have a prevalent role in the epigenetic events of RC, DC, and OKC, while both DNMT3A and DNMT3B have a nearly equal contribution in AME methylation. DNMT3A may be a useful epigenetic marker predicting RC enlargement and aggressiveness of OKC and AME. However, we recommend further research to confirm the cut-off values. In addition, further molecular studies are needed to assess the relation of specific gene silencing to DNMT expression and clinical features.

Declaration of Competing Interest

No competing interest

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