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ORIGINAL ARTICLE / *ARTICLE ORIGINAL*

Developmental Signaling Genes in Ameloblastoma

Gènes signalant le développement de l'améloblastome

K. Onyegbula · O.S. Onile · V.N. Okoje · C.I. Anumudu

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Abstract *Context*: This study investigated the presence or absence of *β-catenin* and *Patched1 (PTCH1)* genes involved in the developmental pathway in ameloblastoma, in order to clarify the genetic etiology of this tumor.
Aim: The aim of this study was to investigate whether *PTCH1* and *β-catenin* genes are involved in the development of ameloblastoma.
Subjects and Methods: Archived formalin-fixed paraffin-embedded specimens of 89 ameloblastoma cases from the year 2000 to 2010 were genotyped by polymerase chain reaction (PCR).
Results: A total of 21 (23.6%) of the 89 ameloblastoma cases were positive for *β-catenin* gene, where 14/21 (66.7%) cases were mandibular ameloblastoma. Plexiform 5/21 (23.8%) and cystic 5/21 (23.8%) ameloblastoma were the most regular histological type positive for *β-catenin*. However, *β-catenin* positive was more in the feminine gender (11/19, 57.9%) than the masculine (8/19, 42.1%). Only one case was positive for *PTCH1* gene and this was histologically a mandibular site and plexiform-type ameloblastoma.
Conclusions: This study suggested that *β-catenin* and *PTCH1* genes may play an important role in the pathogenesis of ameloblastoma.

Keywords Ameloblastoma · *β-catenin* · Odontogenic · Patched

Résumé Cette étude a examiné la présence ou l'absence de gènes de la *β-caténine* et *Patched1 (PTCH1)* impliqués dans le développement de l'améloblastome, afin de clarifier l'étiologie génétique de cette tumeur.
Objectif : L'objectif de cette étude était de connaître l'implication ou non des gènes de la *β-caténine* et *PTCH1* dans le développement de l'améloblastome.
Sujets et méthodes : Des échantillons archivés de 89 cas d'améloblastomes datant de 2000 à 2010 fixés au formol et inclus en paraffine ont été génotypés par réaction en chaîne par polymérase (PCR).
Résultats : Au total, 21 (23,6 %) des 89 cas d'améloblastome étaient positifs au gène de la *β-caténine*, où 14 cas sur 21 (66,7 %) étaient des améloblastomes mandibulaires. Les améloblastomes plexiformes (5 cas sur 21, 23,8 %) et kystiques (5 cas sur 21, 23,8 %) constituaient le type histologique positif à la *β-caténine* le plus courant. Cependant, les cas positifs à la *β-caténine* étaient plus fréquents chez les sujets féminins (11 cas sur 19, 57,9 %) par rapport aux sujets masculins (8 cas sur 19, 42,1 %). Seul un cas s'est révélé positif au gène *PTCH1* et, histologiquement, il s'agissait d'améloblastome mandibulaire et de type plexiforme.
Conclusions : Cette étude laisse supposer que les gènes de la *β-caténine* et *PTCH1* peuvent jouer un rôle important dans la pathogenèse de l'améloblastome.

Mots clés Améloblastome · *β-caténine* · Odontogène · Patched

Introduction

Ameloblastoma is the most common odontogenic tumor, which does not differentiate to form the enamel and arises from the odontogenic apparatus [1]. These tumors represent only 1% of all jaw tumors [2]. There is considerable variation in histological patterns, and classification within this context comprises follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types [3]. Of these, the follicular and plexiform types are the most common [4]. These histologic variants show no correspondence with either the clinical appearance of the tumor or its behavior, and different sections of the same lesion may show one or the other histologic type [5].

C.I. Anumudu (✉)
Department of Zoology, University of Ibadan, Ibadan, Nigeria
e-mail : cianumudu@yahoo.com

K. Onyegbula · O.S. Onile · C.I. Anumudu
Departments of Oral and Maxillo-facial Surgery, Ibadan, Nigeria

V.N. Okoje
Oral Pathology, University College Hospital, Ibadan, Nigeria

Ameloblastomas constitute almost half (48.9%) of the odontogenic tumors with female-to-male and maxilla-to-mandible ratios of 1:1.7 and 1:8, respectively [6]. Ameloblastoma is notorious for its recurrence although it is benign in nature. Thus, it is of great importance not only to the surgeons but also to the private practitioners of dentistry [7]. There are suggestions that alteration in signaling pathways that are important for normal tooth development such as tumor necrosis factor, fibroblast growth factor, sonic hedgehog, and wingless-type (Wnt) pathways could contribute to the etiology of ameloblastoma [3,8–11]. Wnts have been recognized in three distinct pathways, the canonical β-catenin pathway, the planar cell polarity pathway, and the Wnt Ca^{2+} pathway. In the absence of a Wnt ligand, cytoplasmic β-catenin is associated with adenomatous polyposis coli (APC) and axin, phosphorylated by glycogen synthase kinase (GSK3β) and casein kinase I (CKI), and polyubiquitinated by the β-tranducin repeat-containing protein (βTrCP) complex, targeting it for proteosomal degradation. Ubder this condition, transcription factors in the nucleus (lymphoid enhancer factor/T-cell factor-LEF/TCF) associated with transcriptional corepressors, such as Groucho; and the transcription of Wnt target genes is repressed. In the presence of a Wnt ligand, phosphorylation and degradation of β-catenin are inhibited, allowing it to accumulate in the cytoplasm and translocate into the nucleus. Nuclear β-catenin interacts with LEF/TCF family transcription factors and several other transcriptional coactivators to initiate transcription of target genes [12]. Aberrant β-catenin expression and APC missense mutation may play an important role for the pathogenesis of epithelial odontogenic tumors [13].

PTCH1 had been demonstrated to be expressed at various levels in ameloblastoma [14]. This protein is believed to be a receptor for a secreted molecule (sonic hedgehog). The human homologue of the Drosophila segment polarity gene *PTCH1* is a tumor suppressor gene within the sonic hedgehog pathway and this pathway has been shown to function in the patterning of limbs, the axial skeleton, the central nervous system, and tooth development.

Most studies in Nigeria have been concern with the demographical distribution, clinical features, and management of ameloblastoma [15–17], and not much has been done to investigate the genetics of the disease. The aim of this study was to investigate whether *Patched1 (PTCH1)* and *β-catenin* genes are implicated in the development of ameloblastoma.

Subjects and methods

Materials

Eighty-nine (89) blocks of formalin-fixed paraffin-embedded tissues samples diagnosed as ameloblastoma, deposited between the years 2000 and 2010 were retrieved from the archives of the Department of Oral Pathology, College of Medicine, University of Ibadan, Nigeria. A formalin-fixed paraffin-embedded control sample of normal salivary gland tissue was selected from the archives of the same institution. The control sample did not show any histological evidence of ameloblastic changes. Ethical approval was obtained from the University of Ibadan and University College Hospital (UI/UCH) joint ethical committee and Oyo State Ministry of Health, Oyo State, Nigeria.

Methods

All tissue samples were first re-embedded in fresh molten wax and six 5 μm sections were cut from each formalin-fixed paraffin-embedded tissue block using a microtome and subsequently placed in 2.5 ml Eppendorf tubes. The sections were then de-paraffinized by modifying the method of Coura *et al*. 2005 [18]. Two milliliters of xylene was added to the tissue sections in the Eppendorf tubes and incubated in a shaking water bath (Uniscope SM101, Surgifriend, UK) previously set at 55°C for 30 min, after which the xylene was carefully pipetted off and the process repeated twice.. Residual xylene was removed by adding 2 ml of absolute ethanol and incubating at 55°C for 5 min. The ethanol was pipetted off and the process repeated twice. Residual ethanol was removed by incubating at 55°C for 30 min. The tissue pellets then was resuspended by vortexing. This process was then followed by deoxyribonucleic acid (DNA) extraction from the tissue pellets.

DNA extraction

DNA was extracted from de-paraffinized tissue pellets using 70 μl of digestion buffer made up of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 0.45% (v/v) Tween-20, and 10 mg/ml proteinase-K (pH 8.3); and incubated in a Stuart orbital incubator S150 at 36°C overnight. After overnight incubation, proteinase-K was inactivated by incubating in a shaking water bath (Uniscope SM101, Surgifriend UK) previously set at 95°C for 10 min. It was thereafter vortexed and spun in a Hermle-2323 centrifuge at 7,000 rpm for 5 min. Subsequently, the supernatant was collected in autoclaved PCR tubes and stored at −20°C for DNA amplification.

DNA amplification

Extracted DNA samples from ameloblastoma cases were subjected to PCR amplification. Briefly, the amplification reactions were performed in a final volume of 25 μl containing 1.5 μl of template DNA, 0.5 μl deoxynucleotide triphosphates (dNTPs), 5 μl PCR buffer, 15.4 μl sterile water,

1.5 µl $MgCl_2$, 0.1 µl Taq polymerase, and 0.5 µl of each forward and reverse primers (Integrated DNA Technologies, Coralville, Iowa, USA). The primers used in the genotyping reactions are shown in Table 1. Samples containing the amplification mix were subjected to 35 amplification cycles. Conditions for the amplification were an initial denaturation at 94°C for 5 min, which was followed by a subsequent denaturation step at 94°C for 60 s; an annealing step at 50°C for 60 s and initial extension step at 72°C for 60 s which was followed by a final extension step at 72°C for 10 min. Ten microliter aliquots of PCR amplification products was subjected to electrophoresis on 1.5% agarose gel in a Maxfill electrophoretic tank followed by ethidium bromide staining and viewed on a photodocumentation system.

Results

In this study, DNA samples from ameloblastoma cases were amplified for *PTCH1* and *β-catenin*, by PCR using the *PTCH1.1* and *PTCH1.2* primers for *PTCH1* and *CTNNB* and *BCAT* primers for *β-catenin*.

Two primers (*CTNNB* and *BCAT*) were used in amplification of *β-catenin* while *PTCH1.1* and *PTCH1.2* were also used in the amplification of *PTCH1* gene to enhance the possibility of having more amplicons. PCR amplification of *β-catenin* (using *CTNNB* primer) gene amplicon of size 300 bp was positive in 11 samples (Fig. 1). Also 10 samples (Fig. 2) were positive for *β-catenin* (using *BCAT* primer) gene amplicon of size 100 bp by PCR while only one sample was positive for *PTCH1.2* gene of size 295 bp.

Thus a total of 11 (12.4%) and 10 (11.23%) ameloblastoma cases were positive for *CTNNB* and *BCAT* primers (*β-catenin* gene), respectively; while only one (1.12%) ameloblastoma case was positive for *PTCH1.2* gene (Tables 2, 3). The analysis of the clinical data revealed slightly different distribution among the genders; with 45 (50.56%) cases diagnosed in men, 42 (47.19%) cases in women, and in two (2.25%) cases the data was not specified. The age of the patients varied between 8 and 72 years, with the mean value upon diagnosis of 34 years. The mean age of men and women was 32.9 and 34.1 years, respectively.

Negative control sample (−ve) from normal salivary gland was included, which showed no positive amplification for all the genes.

Table 1 Primers used in the genotyping reactions.

Genes	Primer sequence	Size of PCR Product (bp)
CTNNB	Forward 5′-CCAATCTACTAATGCTAATACTG-3′ Reverse 5′-CTCCATTCTGACTTTCAGTAAGGC-3′	**300**
BCAT	Forward 5′-ATGGAACCAGACAGAAAAGCG-3′ Reverse 5′-CAGGATTGCCTTTACCACTCA-3′	**100**
PTCH1.1	Forward 5′-TCTGAGGTCCGCAGGGGGTG-3′ Reverse 5′-CCACCGCGAAGGCCCCAAAT-3′	–
PTCH1.2	Forward 5′-TCTGAGGTCCGCAGGGGGTG-3′ Reverse 5′-GGCATGGGCGCTGACGAGTT-3′	**295**

Fig. 1 Polymerase chain reaction (PCR) for the *β-catenin* gene using primer CTNNB yield eleven amplicons in lane 13, 18, 26, 45, 48, 52, 54, 55, 70, 83, and 85 from ameloblastoma cases and a negative control sample (normal salivary gland) in lane −ve. The DNA ladder 1 kb is in lane M

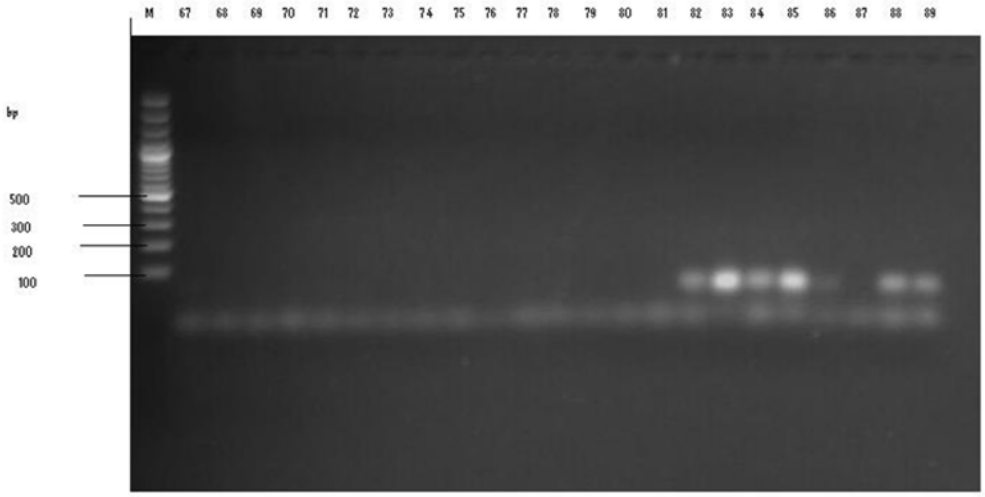

Fig. 2 PCR for the *β-catenin* gene using BCAT primer yielded seven amplicons in lane 82, 83, 84, 85, 86, 88, and 89 from ameloblastoma cases in lane 67–89. The DNA ladder 1 kb is in lane M

Table 2 Different histological types of ameloblastoma genotyped.

Types of ameloblastoma	Genes in number and percentage (*n*/%)			
	CTNNB	***BCAT***	***PTCH1.2***	***PTCH1.1***
Telangiectatic	0/0.0	0/0.0	0/0.0	0/0.0
Follicular	1/9.09	1/10	0/0.0	0/0.0
Plexiform	4/36.4	1/10	1/100	0/0.0
Acanthomatous	1/9.09	2/20	0/0.0	0/0.0
Cystic	3/27.3	2/20	0/0.0	0/0.0
Demoplastic	0/0	0/0.0	0/0.0	0/0.0
Mixed plexiform/follicular	0/0	1/10	0/0.0	0/0.0
Unspecified	2/18.18	3/30	0/0.0	0/0.0

Table 3 Different maxillofacial sites genotyped.

Ameloblastoma site	Genes in number and percentage (*n*/%)			
	CTNNB	***BCAT***	***PTCH1.2***	***PTCH1.1***
Mandible	7/63.6	7/70	1/100	0/0.0
Maxilla	0/0.0	1/10	0/0.0	0/0.0
Palate	0/0.0	0/0.0	0/0.0	0/0.0
Buccal cavity	1/9.09	0/0.0	0/0.0	0/0.0
Unspecified	3/27.3	2/20	0/0.0	0/0.0

Discussion

An attempt was made to demonstrate the presence of *PTCH1* and *β-catenin* genes in archived formalin-fixed paraffin-embedded ameloblastoma cases using the PCR method. Although ameloblastoma occurs in all age groups, this study showed that persons positive for *β-catenin* (*BCAT* and *CTNNB*) genes were more in the age group 11−20, 21−30, 31−40, and 41−50 years in descending order whilst persons in the age group ≥ 60 years show less predisposition. This was also the finding by Siriwardena *et al*. [13]. This present study also showed that *β-catenin* was amplified from ameloblastoma of almost all the histological types: follicular, plexiform, acanthomatous, cystic and a mixed plexiform/follicular ameloblastoma. This was similar to the report of Sekine *et al*. [19], and Milyake *et al*., [20] who found the expression and

mutation of *β-catenin* in follicular ameloblastoma, the expression and mutation of *β-catenin* in plexiform ameloblastoma have also been reported [21]. Nuclear localized β-catenin was observed in follicular and plexiform-type ameloblastomas [13], and these tumors are occasionally associated with gain-of-function mutation of *β*-catenin or loss-of-function mutation of APC. In this study it was observed that 67% of the mandibular ameloblastoma were positive for *β-catenin* which is in consonance with the report of Siriwardena *et al*. [13], that had showed 100% positive expression for *β-catenin*, while others ameloblastomas of maxilla (10%), buccal cavity (9.09%), and palate (0%) showed less expression for *β-catenin* gene.

Twenty-two cases amplified for *β-catenin* gene using two different primers (*BCAT* and *CTNNB*) for PCR, thereby showing about 23.6% cases of ameloblastoma positive for the *β-catenin* gene. It was observed that plexiform (36.4%) and cystic (27.3%) ameloblastoma were the most regular histological type of ameloblastoma positive for *β-catenin*, followed by the acanthomatous, follicular, and a mixed plexiform/follicular type. This study also showed that ameloblastoma samples from persons of both sexes were positive for *β-catenin* gene with a slight majority in females 11/19 (57.9%) than males 8/19 (42.1%), while the gender of two samples was not specified. Similar findings have shown four females and two males positive for *β-catenin* in ameloblastoma [13]. This implies that females are likely to be more predisposed to the manifestation of this tumor compared to males. However, some authors did not think that the tumor had a gender predilection [22–25]. These data therefore indicate the possible role of *β-catenin* gene in the etiopathogenesis of ameloblastoma. The amplification of *β-catenin* gene in certain cell types of ameloblastoma in this study suggested that Wnt signaling activity differs in the different ameloblastoma cellular component and that different members of the Wnt-family may be variously involved in the development of primary and recurrent lesion [26].

None of the DNA samples from all 89 ameloblastoma cases amplified for *PTCH1.1* primer in the PCR, and only one sample was amplified for *PTCH1.2* primer. The plexiform and mandibular ameloblastoma amplified for *PTCH1* gene (Tables 2, 3) which is similar to the result of Kawabata *et al*. [21], who also reported the possible relationship between the CGG8 allele in PTCH1 and the risk for ameloblastoma. *PTCH1* gene mutation was found in sporadic odontogenic keratocysts, medulloblastoma, breast cancer, colon cancer, meningioma, and also in ameloblastoma [14,27,28].

Conclusion

Our findings suggest that *β-catenin* and *PTCH1* genes might possibly be involved in the etiopathogenesis of ameloblastoma. It would be a further step in the characterization of these genes, to determine their sequences for mutational changes. This would foster our understanding of the role played by these genes in the disease.

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Conflict of interest : K. Onyegbula, O.S. Onile, V.N. Okoje and C.I. Anumudu have no conflict of interest to declare.

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