Expression of WNT1 in ameloblastoma and its significance

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Abstract. The present study aimed to measure the expression of WNT1 in ameloblastoma (AB). Immunohistochemistry was used to observe changes in WNT1 expression in 80 AB samples, 10 keratocystic odontogenic tumor (KCOT) samples and 10 normal oral mucosa (NOM) samples. Western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were used to measure WNT1 protein and mRNA expression, respectively, in 30 AB samples, 5 KCOT samples, 5 NOM samples and 3 tooth germ samples. Ectopic cytoplasmic expression of WNT1 was detected in AB; 88.8% (71/80) of the samples were WNT1-positive. The western blotting results demonstrated that compared with NOM (0.57±0.05), WNT1 expression was significantly higher in AB tissue (1.74±0.36, $P<0.05$), whereas it was not significantly different between AB and KCOT samples (0.80±0.06, $P>0.05$). RT-qPCR revealed that the level of *WNT1* gene expression in AB was increased 2.43-fold compared with normal mucosa, and 1.77-fold compared with tooth germ tissue. In conclusion, WNT1 protein and mRNA expression were increased in AB, and there was ectopic cytoplasmic expression. This indicates that WNT1 may serve an important role in AB occurrence and development.

Introduction

The development of ameloblastoma (AB) may be similar to tooth germ development, as it is likely derived from the epithelial lining of the dental follicle, the epithelial residue of the tooth plate and the basal cells of the oral mucosa (1).

From *C. elegans* to humans, *WNT* genes are conserved across many species. Previous studies have demonstrated that WNT1 is a key signal molecule for controlling cell growth and proliferation. It transfers regulatory information between cells, and serves an important role in stem cell differentiation and neural development. Kumamoto and Ooya (2) reported that WNT pathway abnormalities induce tumor occurrence, as WNT1 activation phosphorylates β-catenin in the nucleus to activate the classic WNT pathways, inducing cell proliferation and inhibiting apoptosis to generate anomalous growth.

As the effect of the WNT signaling pathway in the process of tooth development is well characterized, and as multiple associated factors are expressed during different developmental periods of the tooth germ (3), we hypothesized that this pathway may be associated with the development of AB. Therefore, immunohistochemistry, western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were used to examine WNT1 expression in AB, with the aim of providing a basis for diagnosing and treating AB.

Materials and methods

Tissue samples. Immunohistochemical specimens were obtained from paraffin blocks archived at the Department of Oral Pathology, Stomatological Hospital of China Medical University (Shenyang, China), between June 2009 and April 2013. The study protocol was approved by the Medical Ethics Committee of Stomatological Hospital of China Medical University. The study was performed in accordance with the Declaration of Helsinki. All specimens were analyzed subsequent to obtaining verbal informed patient consent, This was not a retrospective study. There were 80 cases of AB (Table I) with no chemotherapy or radiotherapy administered prior to tissue extraction, 10 of keratocystic odontogenic tumor (KCOT) and 10 samples of normal oral mucosa (NOM). There were 40 male and 40 female patients with AB; the age range was 18-79 years and the median age was 49 years. All specimens were classified according to the 2005 World Health Organization (WHO) criteria (4). The areas the tumor tissue was extracted from included the mandible (65 cases), maxilla (12 cases) and gingiva (3 cases). The histological types were as follows: 76.3% (61/80) solid/polycystic, 1.6% (13/80) unicystic, 3.8% (3/80) desmoplastic and 3.8% (3/80) peripheral. Of the solid type, 42 cases were typed as follicular, 5 as plexiform, 7 as follicular/plexiform, 3 as acanthomatous, 2 as basal cell and 2 as keratinous.

The fresh tissue samples obtained between January 2004 and December 2009 for western blotting and RT-qPCR

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were surgical resection specimens from the Department of Oral and Maxillofacial Surgery, Stomatological Hospital of China Medical University. The samples included 30 cases of AB (Table II), 5 cases of KCOT, and 5 NOM samples. In addition, 3 cases of tooth germ tissue were abortuses from the Department of Obstetrics of the People's Liberation Army no. 202 Hospital (Shenyang, China). The samples were cryopreserved at -86˚C and were classified according to the 2005 WHO criteria. There were 16 male and 14 female patients with AB; the age range was 16-72 years, and the median age of which was 44 years. The areas involved included the mandible (24 cases), maxilla (3 cases), gingiva (1 case), left parapharyngeal space palate (1 case) and left cheek (1 case). The histological type was as follows: 83.3% (25/30) were solid/polycystic, 3.3% (1/30) unicystic, 3.3% (1/30) desmoplastic and 10% (3/30) peripheral. Of the solid type, 14 were follicular, 5 plexiform, 3 acanthomatous, 2 basal cell and 1 follicular/plexiform.

Immunohistochemistry. Sections (5 μm) were obtained from the paraffin-embedded tissues of the first group. Following the inactivation of endogenous peroxidase by quenching with H_2O_2, sections were treated overnight with a goat anti-WNT1 polyclonal primary antibody (dilution, 1:400; Abcam, Cambridge, MA, USA; cat no. ab15251) at 4˚C. The streptavidin-peroxidase (SP) method was used; an SP kit (Zymed; Thermo Fisher Scientific, Inc., Waltham, MA, USA) contained biotinylated secondary antibodies that were incubated for 10 min at 37˚C; sections were washed three times with PBS buffer between steps. One drop (38 μl) of liquid Diaminobenzidine (DAB, Maxim Biotech, Inc., Fuzhou, China) chromogen was added to 1 ml of stable DAB substrate buffer (cat no. ab64238; Abcam, Cambridge, MA, USA) in the mixing vial. This DAB chromogen reagent was added at room temperature for 5 to 15 min until the color turned brown. Samples in which the primary antibody had been replaced with PBS buffer were used as the negative control.

Immunohistochemical reactivity for WNT1 was evaluated based on brown staining in the cytoplasm. The evaluation considered (A) the intensity of staining: Colorless, 0; light yellow, 2; brown-yellow, 2; brown, 3; and (B) the proportion of stained tumor cells: <25%, 1; 25-75%, 2; >75%, 3. The score for each case was derived as A x B, and cases were classified according to their score: 0-1, negative (-); 2-4, weakly positive (+); 4-7, moderately positive (++); and >7, strong positive (+++), as previously described (5).

Western blot. Pre-made protein cracking fluid (20 mmol/l Tris-HCL pH 7.4, 150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS. 1% deoxycholic acid socium, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 μg/ml aprotinin, 1 mmol/l Na_3VO_4) was used to extract proteins and extracted proteins were separated using SDS-PAGE (12% separation polyacrylamide gel and 4% polyacrylamide concentration glue). The amount of protein per lane was 38.8 μg. The coomassie brilliant blue method (6) was used for protein quantification. The proteins were transferred to a nitrocellulose membrane (Biomol; Enzo Life Sciences, Inc., Farmingdale, NY, USA) and blocked with Tween-20 in Tris-buffered saline containing 5% non-fat dry milk overnight at 4˚C. The goat anti-human WNT1 polyclonal antibody (dilution, 1:400; cat no. ab15251; Abcam) was used as the primary antibody; the secondary antibody was alkaline phosphatase-labeled anti-goat IgG (dilution, 1:1,000; cat no. A0208; Seikagaku Corporation, Tokyo, Japan). β-tubulin was used as the internal control antibody (dilution, 1:1,000; cat no. A01030; Abbkine Scientific Co., Ltd., Wuhan, China). The 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium Alkaline Phosphatase Color Development kit was used for visualization (cat no. C3206; Beyotime Institute of Biotechnology, Jiangsu, China). AlphaView 2.0 gel imaging analysis software (ProteinSimple; Bio-Techne, Minneapolis, MN, USA) was used for quantitative analysis.

RT-qPCR. TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA, and cDNA was synthesized with reverse transcription using the PrimeScript One Step RT-PCR kit Ver.2 (cat no. RR055A; Takara Biotechnology Co., Ltd., Dalian, China). PCR amplification of the cDNA was performed in 20 μl mixtures according to the protocol

Table I. Histopathological classification of the 80 AB tumor samples used in immunohistochemistry.

AB type	Cases, n
Solid/polycystic	61
Follicular	42
Plexiform	5
Follicular/plexiform	7
Acanthomatus	3
Basal cell	2
Keratinous	2
Unicystic	13
Desmoplastic	3
Peripheral	3

AB, ameloblastoma.

Table II. Histopathological classification of the 30 AB tumor samples used for western blotting and reverse transcription-quantitative polymerase chain reaction.

AB type	Cases, n
Solid/polycystic	25
Follicular	14
Plexiform	5
Follicular/plexiform	1
Acanthoma	3
Basal cell	2
Unicystic	1
Desmoplastic	3
Peripheral	1

AB, ameloblastoma.

Table III. *WNT1* and *ACTB* primers.

Gene	Primers	Sequence	Product size, bp
WNT1	Upstream	5'-CGGGCAACAACCAAAGTC-3'	107
	Downstream	5'-GCAGCAGCGTAGCAGAAAC-3'	
ACTB	Upstream	5'-AGTTGCGTTACACCCTTTC-3'	492
	Downstream	5'-TGTCACCTTCACCGTTCC-3'	

ACTB, β-actin.

Table IV. Difference in WNT1 expression between AB and NOM as determined by immunohistochemistry.

		WNT1 expression			
Tissue type	n	Positive	Negative	χ^2	P-value
AB	80	71	9	36.88	<0.001
NOM	10	0	10		

AB, ameloblastoma; NOM, normal oral mucosa.

Table V. Difference in WNT1 expression between AB and KCOT tissues as determined by immunohistochemistry.

		WNT1 expression			
Tissue type	n	Positive	Negative	χ^2	P-value
AB	80	71	9	1.325	0.25
KCOT	10	7	3		

AB, ameloblastoma; KCOT, keratocystic odontogenic tumor.

of the SYBR Premix Ex Taq™ II kit (cat no. RR820A; Takara Biotechnology Co., Ltd.). The thermocycling conditions were as follows: Initial denaturation stage at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, and then primer annealing and template amplification at 60˚C for 34 sec. Primer3 software was used to design the PCR primers, which were synthesized by Beijing Genomics Institute (Beijing, China) (Table III). β-actin (*ACTB*) was used as an internal control. qPCR results were analyzed using Rotor gene Real-Time Analysis software 2.3.1 (Corbett Life Science; Qiagen GmbH, Hilden, Germany). The comparative Cq ($2^{-\Delta\Delta Cq}$) method was used to determine the relative quantitative result (7).

Statistical analysis. Data are presented as the mean ± standard error of the mean. All experiments were conducted with a minimum of three repeats. Statistical analysis was performed using SPSS 17.0 software. χ^2 tests and one-way analysis of variance followed by Fisher's least significant difference tests were used for the comparisons. P<0.05 was considered to represent a statistically significant difference.

Results

WNT1 protein expression is increased in AB, as determined with immunohistochemistry. WNT1 expression in NOM was negative for all samples (Fig. 1A) while there was positive WNT1 expression in 70% of KCOT samples (7/10); WNT1 was expressed in the cytoplasm of the lining epithelial cells, and staining was generally weak-to-moderately positive (Fig. 1B). There was positive WNT1 expression in 89% of AB samples (71/80); staining was predominantly moderately or strongly positive in the outer periphery of the columnar or cuboidal cells of the dental epithelium, the cytoplasm of the central stellate reticular layer, and the inflammatory interstitial lymphocytes (Fig. 1C-F).

Table VI. Difference in WNT1 expression between different types of AB as determined by immunohistochemistry.

		WNT1 expression			
AB type	n	Positive	Negative	χ^2	P-value
Solid/polycystic	61	55	6	2.073	0.56
Unicystic	13	11	2		
Desmoplastic	3	2	1		
Peripheral	3	3	0		

AB, ameloblastoma.

As shown by Tables IV-VI, WNT1 expression in AB was significantly higher than in NOM (P<0.001), but not significantly different from in KCOT (P>0.05). There was no significant difference between AB types (P>0.05).

WNT1 protein expression is increased in AB, as determined with western blotting. The WNT1 protein expression level in each sample was determined with western blotting (Fig. 2A); expression levels in AB, KCOT and NOM were 1.74±0.36, 1.31±0.06, and 0.57±0.05 (Fig. 2B). WNT1 expression in AB was significantly higher than in NOM (P<0.05), but not significantly different from in KCOT (P>0.05).

WNT1 mRNA expression is increased in AB, as determined with RT-qPCR. RT-qPCR was used to assess the *WNT1* mRNA expression level in 30 AB, 3 tooth germ and 5 NOM tissue

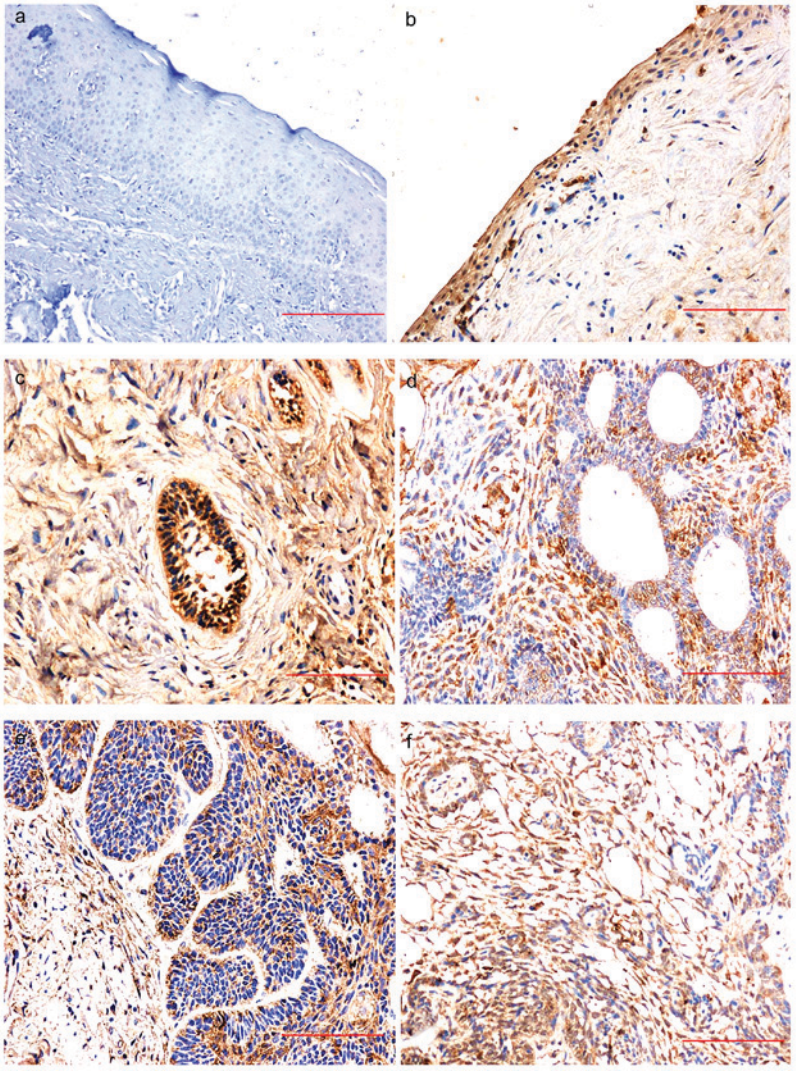

Figure 1. Immunohistochemical reactivity for WNT1. (a) Negative WNT1 expression in normal oral mucosa. (b) Positive WNT1 expression in keratocystic odontogenic tumor tissue. (c) Positive WNT1 expression in follicular AB. (d) Positive WNT1 expression in plexiform AB. (e) Positive WNT1 expression in acanthoma AB. (f) Positive WNT1 expression in basal cell/keratinous AB. Magnification, x200; scale bar, 50 μm. AB, ameloblastoma.

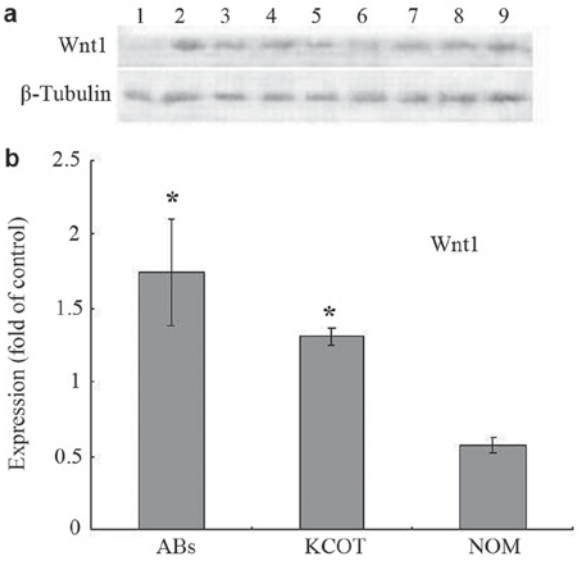

Figure 2. WNT1 western blotting results. (a) Lane 1, NOM; lane 2, KCOT; lane 3-8: AB. The molecular weight of WNT1 is 40 kDa. (b) Wnt1 was higher in ABs than in NOM, but not significantly different from that in KCOT. AB, ameloblastoma; NOM, normal oral mucosa. *P<0.05 vs. NOM.

samples (Fig. 3A and B). The *WNT1* PCR product melting curve peaks of all samples were at 83˚C, the dissolution temperature was uniform and the peaks were sharp, indicating that the product was specific. It was determined that the expression of *WNT1* was increased 2.43-fold in AB compared with NOM tissue samples and 1.77-fold in AB compared with tooth germ tissue samples (both P<0.05; Fig. 3C).

Discussion

The WNT signaling pathway is named for its initiating protein, WNT1; *WNT1* is the first member of the 19-member *WNT* gene family and encodes a 370-amino acid protein (8). Howe *et al* (9) reported that WNT1 induces overexpression of downstream target genes by activating the WNT/β-catenin signaling pathway, resulting in excessive cell growth. The diverse biological effects of WNT signaling include the regulation of cell proliferation, morphogenesis, and cell survival and death processes; abnormal activation of WNT signaling is associated with a variety of developmental disorders, degenerative diseases, types of cancer e.g. colon cancer (10), retinopathy (11),

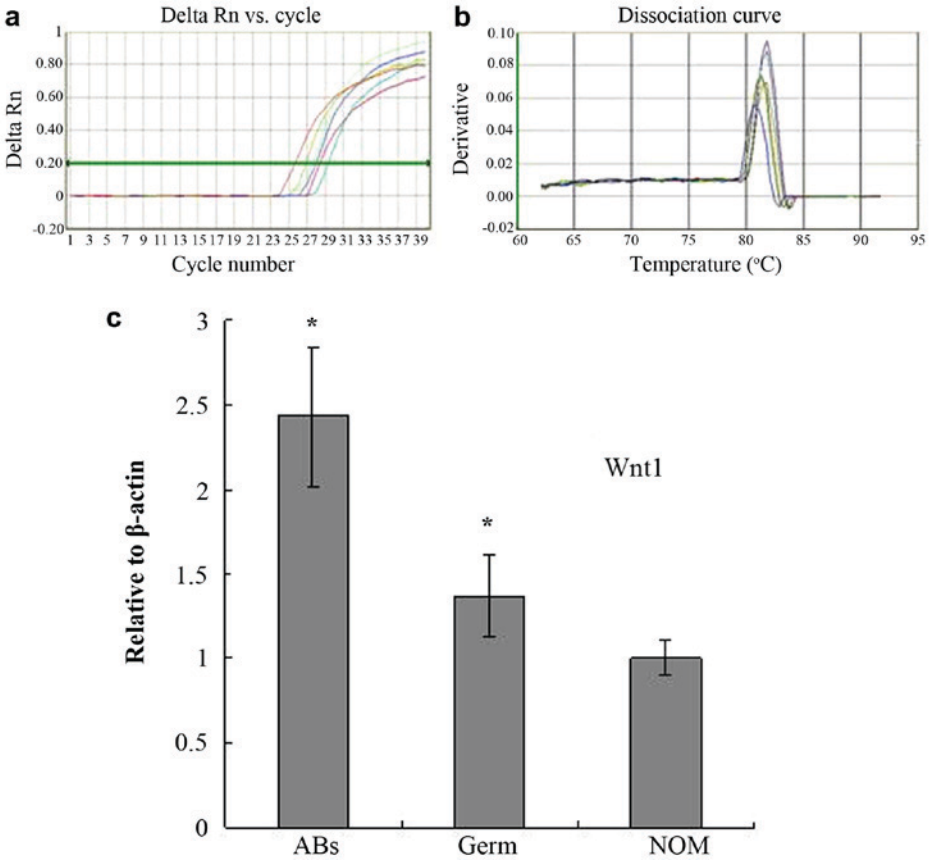

Figure 3. Reverse transcription-qPCR determination of *WNT1* expression levels in AB and NOM. (a) Amplification curve for qPCR. (b) Dissolution curve for qPCR. (c) *WNT1* mRNA expression level was higher in ABs than in NOM or tooth germ tissue. qPCR, quantitative polymerase chain reaction; AB, ameloblastoma; NOM, normal oral mucosa. *P<0.05 vs. NOM.

limbless malformations (12), and bone and cartilage diseases, including arthritis (13). WNT1 is a tumor-associated protein which is upregulated in breast cancer, hepatocellular carcinoma, oral carcinoma, colorectal cancer and other types of epithelial malignancy; it may be involved in the occurrence of these tumors (14-17). Joeng *et al* demonstrated that WNT1 effectively reduced β-catenin expression in the cytoplasm, and induced apoptosis by recovering the function of WNT inhibitory factor-1 (WIF-1) in colon cancer cells, increasing WNT siRNA or using anti-WNT monoclonal antibodies (18).

In the present study, WNT1 expression was positive in 71 cases of AB; of these, expression was strongly positive in 60 cases (84.5%) and weakly positive in 11 cases (15.5%), suggesting heterogeneity in WNT1 expression intensity in AB. In addition, WNT1 was expressed only in the cytoplasm of tumor cells, and not in the nucleus. WNT1 was expressed in the peripheral cells of tumor cell nests and the stellate reticular cell layer. We hypothesize that positive WNT1 expression in tumor-adjacent tissues may be associated with the invasiveness of AB. Positive WNT1 expression was also detected in cells surrounding the epithelial islands of follicular and acanthoma AB, reflecting the importance of WNT1 signaling in cell proliferation (19). However, it is difficult to compare the difference in WNT1 content using only immunohistochemical staining.

Western blotting demonstrated that WNT1 levels in the tumor tissue were significantly higher than in NOM tissue, suggesting that WNT1 expression was enhanced in AB. RT-qPCR was used to determine that *WNT1* mRNA levels in 30 cases of AB were 2.43 times higher than that in NOM tissue, suggesting that WNT1 expression is enhanced at mRNA level. As mRNA levels directly reflect gene expression, they support the hypothesis that *WNT1* gene activation is associated with AB. The mechanisms for the activation of the *WNT1* gene in AB has yet to be determined.

The *WNT* gene was designated as such as it contains a virus integration site; such integration sites are also present upstream of the *WNT1* gene promoter (20). Papillary ductal carcinoma of the breast is potentially induced by human papilloma virus infection, and high *WNT1* mRNA expression has been detected in tumors of this type (14). Integrating a viral gene into the viral integration site upstream of the *WNT1* gene promoter induces mammary tumor formation (20), suggesting that the activation of the *WNT1* gene in cancer may be associated with the transfection of viral genes. As AB development due to viral infection has never been reported, further studies are warranted to determine whether there are mutations at the *WNT1* gene regulatory site.

Immunohistochemical results of this study showed that the expression level of WNT1 was significantly higher in the AB than in normal oral mucosa tissues, combined with western blotting and RT-qPCR results suggest that WNT1 and the occurrence of ABs and its aggressive has a high correlation. Through the study of biological behavior of AB and the relationship between WNT1 expression, WNT1 could also be the diagnosis and evaluation of prognosis of effective index of AB. For this reason, the WNT1 as a target genes to treat AB is helpful.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GW performed the immunohistochemical, western blot analysis and RT-qPCR experiments and was a major contributor in writing the manuscript. MZ made substantial contributions to conception and design. YC and JJ collected and organized the data of 80 patients with ameloblastoma. XG and TW performed the statistical analysis of data. All authors reviewed the final manuscript and approved it for publication.

Ethics approval and consent to participate

The study protocol was approved by the Medical Ethics Committee of Stomatological Hospital of China Medical University. The study was performed in accordance with the Declaration of Helsinki. Informed verbal patient consent was obtained for the use of all specimens.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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