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Original Article

Expression of E-cadherin, vimentin and β-catenin in ameloblastoma and association with clinicopathological characteristics of ameloblastoma

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Abstract: Objective: Ameloblastoma shows invasive growth and is susceptible to recurrence. This study aimed to detect the expression of E-Cadherin, Vimentin and β-Catenin (proteins related to epithelial mesenchymal transformation (EMT) in the ameloblastoma and to explore association with clinicopathological characteristics of ameloblastoma. Methods: Immunohistochemistry was employed to detect the protein expression of E-Cadherin, Vimentin and β-Catenin in the ameloblastoma, and its association with clinicopathological characteristics of ameloblastoma was further evaluated. Results: E-Cadherin expression was reduced or absent on the membrane of the peripheral columnar and cubic epithelial cells; Vimentin expression was high in the interstitium as well as epithelial cells in ameloblastoma; E-Cadherin expression was negatively related to Vimentin expression. β-catenin expression on the membrane of ameloblastoma epithelial cells reduced, but its ectopic expression was observed in the cytoplasm and/or nucleus. Conclusion: EMT related proteins E-Cadherin, β-catenin and Vimentin are involved in the occurrence and development of ameloblastoma and these proteins can be used as biomarkers of invasiveness of ameloblastoma.

Keywords: Ameloblastoma, E-cadherin, vimentin, β-catenin, epithelial mesenchymal transition

Introduction

The tumorigenesis has involvement of multiple factors, and molecular changes, accumulation of gene defect and the subsequent clonal selection and expansion may cause the tumorigenesis [1]. Molecular changes usually occur before the cellular changes and clinicopathological alterations, and thus detection of these molecular changes is crucial for the early diagnosis and prognosis prediction of tumors [2]. Under physiological condition, odontogenic epithelium may develop into teeth, but under certain conditions, odontogenic epithelium or relevant tissues and residual epithelium may serve as a resource of intra-osseous tumor or cyst. Ameloblastoma is a benign tumor derived from odontogenic epithelium, has slow growth and may not cause evident pain and other discomfort. Ameloblastoma is often diagnosed when jaw bulging occurs at late stage to compress the surrounding tissues and nerves causing facial deformity and dysfunction. It has local invasive growth and is susceptible to recurrence, and thus, expanded resection of the jaw is often employed during surgery. However, the excess jaw defect may cause a burden to the life of patients, which is a major cause of poor prognosis in ameloblastoma patients.

In recent years, epithelial mesenchymal transition (EMT) has been found to play important role in the invasion and metastasis of tumors. The intercellular adhesion is essential for the maintenance of normal tissue function. It has been confirmed that a series of adhesion molecules are involved in the regulation of intercellular adhesion. EMT refers to the loss of stable structure and intrinsic polarity, the acquisition of phenotype of interstitial cells, transformation into interstitial cells with free migration in extracellular matrix in epithelium under special physiological or pathological conditions. To date, studies have confirmed that EMT is involved in a variety of biological processes including embryogenesis, tissue repair, and tumor metas-

tasis [3]. The invasion and metastasis of tumors are complex processes with involvement of multiple genes and multiple steps, in which the compromised intercellular adhesion and enhanced migration of tumor cells are the basis of these processes.

E-Cadherin is a calcium dependent transmembrane glycoprotein and expressed in a majority of epithelium, and it is able to maintain the integrity of epithelial tissues and polarity [4]. The intracellular domain of E-cadherin may bind to some members of catenin family, including β-catenin, to form intercellular junction complexes. The down-regulated expression or dysfunction of E-Cadherin in tumor cells may cause disruption of epithelium, and then tumor cells may migrate from the primary site and acquire migration capability, which may cause the invasiveness and metastasis of tumor cells [5, 6]. The expression defect of E-Cadherin and up-regulated expression of Vimentin are the key events in the EMT of epithelial cells [7].

Vimentin is an important cytoskeletal protein and type III intermediate fibrin. It is mainly expressed in interstitial cells, and under special conditions also expressed in migrating epithelial cells, such as during embryonic development and wound repair. Vimentin expression in oral tumor epithelial cells has been found to be closely and pathologically related to the invasion and metastasis of oral tumor [6, 8, 9].

β-catenin is a key molecule in the Wnt signaling pathway. Abnormal Wnt signaling pathway activation may cause the inability of β-catenin degradation, leading to its accumulation in the cytoplasm. Then, β-catenin translocates into the nucleus and then binds to T-cell factor/lymphoid enhancer-binding factor-1 (TCF/LEF-1), which serves as a transcription factor to regulate the expression of target genes. This process has been found to be involved in the development, invasion and metastasis. On the cell membrane, β-catenin binds to E-Cadherin to maintain the stability of intermolecular adhesion, and the abnormality of β-catenin-E-Cadherin complexes may cause the loss of cellular adhesion and epithelial cell interstitialization [4, 6, 8]. Moreover, the role of Wnt signaling pathway in the tooth development has been reported [10]. During the root development, knock out of β-catenin (CTNNB1) in odontoblasts and dentinoblast may cause incomplete root development in mice [11, 12]. Since β-catenin is an important molecule in Wnt signaling pathway and involved in the pathogenesis of EMT and tooth development, we speculate that abnormal β-catenin expression might be also related to the occurrence of ameloblastoma.

EMT is closely related to the metastasis and invasion of tumor cells. Studies on the EMT in oral tumors mainly focus on the oral squamous cell carcinoma and oral mucosal dysplasia [13-15]. Currently, the mechanisms underlying the focal invasion and high recurrence of ameloblastoma are still poorly understood. In the present study, the expression of E-Cadherin, Vimentin and β-catenin, proteins related to EMT, was detected in ameloblastoma, and their relationship with clinicopathological features was further evaluated, aiming to elucidate the role of EMT in the invasion of ameloblastoma.

Materials and methods

Collection of clinical samples

Paraffin embedded tissues of ameloblastoma (n=138) and oral squamous cell carcinoma (OSCC, n=18) used for immunohistochemistry were from the Department of Pathology, Affiliated Dental Hospital of China Medical University, and these tissues were collected between 2004 and 2014. Normal oral mucosa (NOM) was collected from 10 patients who received surgical removal of third mandibular molar in clinic. All the tissues were processed, and sections were evaluated by two experienced pathologists according to the WHO classification criteria 2005. Tissues were collected from 72 males (52.2%) and 66 females (47.8%) with the median age of 42 years (range: 8-76 years). In addition, 105 tissues were collected from the mandible (76.1%), 33 from the maxilla (23.9%). Clinical manifestation and pathological classification are shown in **Table 1**.

Processing of paraffin embedded tissues

Tissues were fixed in formaldehyde, then dehydrated in ethanol (60% for 2 h, 70% ethanol for 2 h, 80% ethanol for 3 h, 90% ethanol for 12 h, 95% ethanol for 2 h, 100% ethanol I for 2 h, 100% ethanol II for 2 h) and transparentized in xylene (xylene I for 5 min; xylene II for 20 min). Finally, tissues were embedded in paraffin (paraffin I for 30 min; paraffin II for 40 min; paraffin

Table 1. Correlation analysis of EMT related protein expression with clinical manifestation of ameloblastoma

Items		Case number (%)	E-cadherin (%)		X^2	P	Vimentin (%)		X^2	P	β-catenin (%)		X^2	P
			-	+			-	+			Normal expression	Abnormal expression		
Gender	Male	72 (52.2)	28 (38.9)	44 (61.1)	0.094	0.76	52 (72.2)	20 (27.7)	0.072	0.789	16 (22.2)	56 (77.8)	0.132	0.716
	Female	66 (47.8)	24 (36.4)	42 (63.6)			49 (74.2)	17 (25.8)			13 (19.7)	53 (80.3)		
Age (yrs)	≤30	22 (15.9)	9 (40.9)	13 (59.1)			17 (77.3)	5 (22.7)			5 (22.7)	17 (77.3)		
	30-60	76 (55.1)	28 (36.8)	48 (63.2)	0.121	0.941	56 (73.7)	20 (26.3)	0.404	0.817	17 (22.4)	59 (77.6)	0.105	0.949
	>60	40 (29.0)	15 (37.5)	25 (62.5)			28 (70.0)	12 (30.0)			10 (25.0)	30 (75.0)		
Location	Upper jaw	33 (23.9)	14 (42.4)	19 (57.6)	0.416	0.519	23 (69.7)	10 (30.3)	0.269	0.604	7 (21.2)	26 (78.8)	0.001	0.975
	Under jaw	105 (76.1)	38 (36.2)	67 (63.8)			78 (74.3)	27 (25.7)			22 (21.0)	83 (79.0)		
AB	Primary[1]	73 (52.9)	20 (27.4)	53 (72.6)			60 (82.2)	13 (17.8)			24 (32.9)	49 (67.1)		
	Recurrent[2]	48 (34.8)	23 (47.9)	25 (52.0)	7.115	0.029	34 (70.8)	14 (29.2)	12.029	0.002	4 (8.3)	44 (91.7)	13.184	0.001
	Malignat[3]	17 (12.3)	9 (52.9)	8 (47.1)			7 (41.2)	10 (58.8)			1 (5.9)	16 (94.1)		
	Total	138	52 (37.7)	86 (62.3)			101 (73.2)	37 (26.8)			29 (21.0)	109 (79.0)		

Notes: E-Cadherin: 1&2 P=0.018; 2&3 P=0.470; 1&3 P=0.043; Vimentin: 1&2 P=0.107; 2&3 P=0.031; 1&3 P=0.001; β-catenin: 1&2 P=0.001; 2&3 P=0.608; 1&3 P=0.019.

Table 2. Expression of EMT related proteins in NOM, ameloblastoma and oral squamous cell carcinoma

Items		Case number (%)	E-cadherin (%)		X^2	P	Vimentin (%)		X^2	P	β-catenin (%)		X^2	P
			-	+			-	+			Normal expression	Abnormal expression		
NOM		10	1 (10.0)	9 (90.0)			10 (100)	0 (0)			8 (80.0)	2 (20.0)		
AB		138	52 (37.7)	86 (62.3)	7.352	0.025	101 (73.2)	37 (26.8)	10.684	0.005	29 (21.0)	109 (79.0)	17.302	0.000
OSCC		18	11 (61.1)	7 (38.9)			8 (44.4)	10 (55.6)			-	-		
AB	Solid/multicystic	99 (71.7)	38 (38.4)	61 (61.6)			74 (74.7)	25 (25.3)			20 (20.2)	79 (79.8)		
	Unicystic	17 (12.3)	6 (35.3)	11 (64.7)	0.086	0.993	11 (64.7)	6 (35.3)	0.748	0.862	4 (23.5)	13 (76.5)	0.178	0.981
	Peripheral	8 (5.8)	3 (37.5)	5 (62.5)			6 (75)	2 (25.0)			2 (25.0)	6 (75.0)		
	Desmoplastic	14 (10.2)	5 (35.7)	9 (64.3)			10 (71.4)	4 (28.6)			3 (21.4)	11 (78.6)		
	Total	138	52	86			101	37			29	109		

Notes: There was significant difference of E-Cadherin, Vimentin and β-catenin in NOM, AB and OSCC, while no significant difference in different pathological types of ameloblastoma. AB: ameloblastoma; NOM: normal oral mucosa; OSCC: oral squamous cell carcinoma.

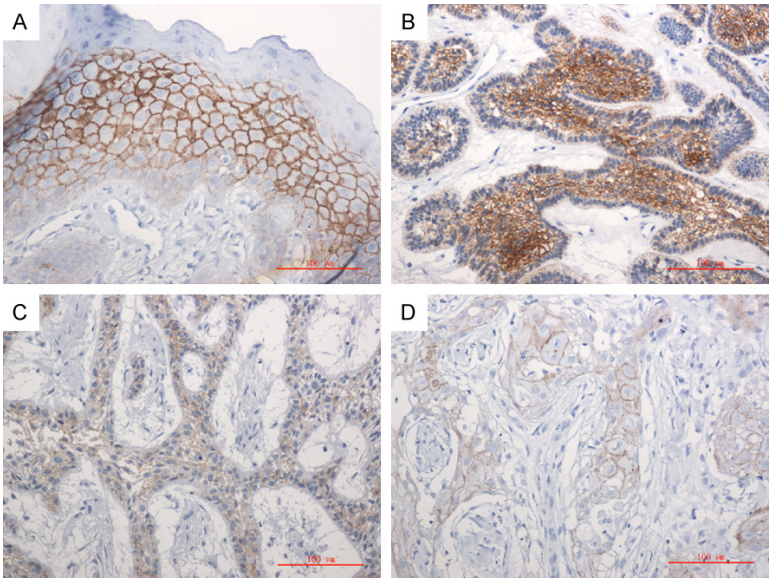

Figure 1. E-Cadherin expression (Immunohistochemistry; SP method). A: Normal oral mucosal epithelial cells membrane positive for E-Cadherin expression (×200); B: Central stellate epithelial reticular cell membrane strongly positive for E-cadherin in plexiform type of ameloblastoma, and reduced expression of E-cadherin in peripheral columnar or cubic epithelial cells (×200); C: Reduced expression of E-cadherin in epithelial cells of ameloblastoma, and positive expression in plasma (×200); D: Moderate positive expression of E-Cadherin in oral squamous cell carcinoma (×200).

III for 1 h). The paraffin embedded tissues were cut into sections (5 μm in thickness).

Immunohistochemistry

Sections were dried at 68°C for 20 min. Sections were de-paraffinized in xylene and the dehydrated in a series of ethanol solutions (xylene I for 20 min; xylene II for 20 min; 100% ethanol I for 10 min; 100% ethanol II for 10 min; 95% ethanol for 5 min; 80% ethanol for 5 min; 70% ethanol for 5 min in 0.01 M). Sections were treated with 3% H_2O_2 at 37°C for 10 min to inactivate endogenous peroxidase, followed by washing in PBS thrice (3 min for each). Antigen retrieval: Sections were boiled in 0.01 M citrate buffer (PH 6.0), and then allowed to cool for more than 20 min. After washing in water, the sections were allowed to cool to room temperature. These sections were washed in PBS thrice (5 min for each). Sections were blocked in normal goat serum at 37°C for 10 min, and then the solution was removed. Sections were treated with rabbit anti-human E-Cadherin monoclonal antibody, mouse anti-human Vimentin monoclonal antibody or mouse anti-human β-catenin monoclonal antibody in 1% PBS (1:100) at 4°C over night, and then washed in PBS thrice (5 min for each). In negative control, the primary antibody was replaced with PBS. Sections were incubated with biotin conjugated secondary antibody at 37°C for 30 min, followed by washing in PBS thrice (5 min for each). Sections were incubated with horseradish peroxidase label ed streptomycin at 37°C for 30 min, followed by washing in PBS thrice (5 min for each). Sections were treated with DAB/H_2O_2 for staining. After washing in flowing water, counterstaining was done with hematoxylin, followed by routine dehydration, transparentization, drying and mounting. Sections were observed under a light microscope, and representative photos were captured.

Determination of protein expression

The expression of three proteins was assessed by immunohistochemical semi-quantitative method. The final score was calculated as the product of score of proportion of positive epithelium (0, 0-20%; 1, 21-40%; 2, 41-60%; 3, 61-80%; 4, 80-100%) and score of staining intensity (0, negative; 1, weak; 2, moderate; 3, strong). The final score of E-Cadherin and Vimentin less than 4 points was defined as negative expression (-), 4-12 points as positive expression (+). Normally, β-catenin was expressed on cell membrane. There were more than 10% cells with strongly positive expression in cell plasma, cell plasma/cell nucleus, and cell membrane/cell nucleus, which was abnormal.

Statistical analysis

Statistical analysis was performed with SPSS version 17.0. Data were analyzed with chi square test. A value of $P<0.05$ was considered statistically significant.

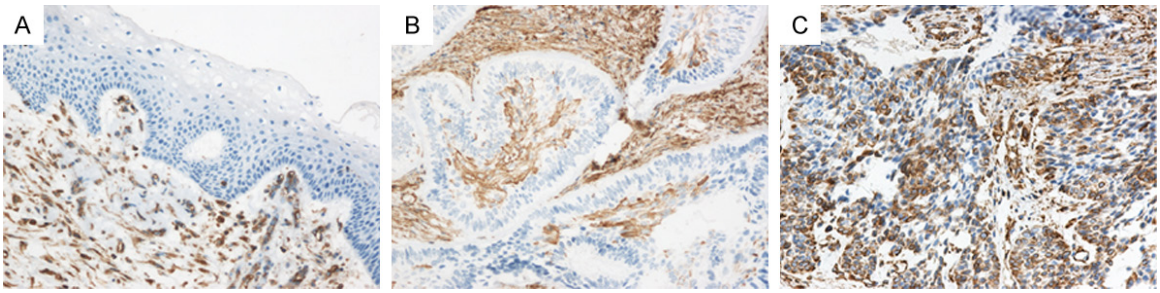

Figure 2. A: None expression of Vimentin in epithelia cells of normal oral mucosa; B: Positive Vimentin expression in interstitium of ameloblastoma; C: Positive Vimentin expression in epithelial cells of ameloblastoma (Immunohistochemistry; SP method; ×200).

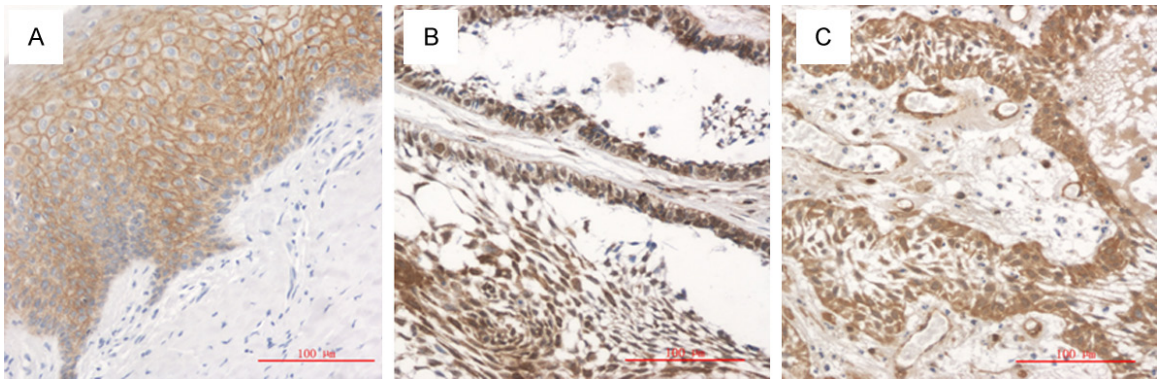

Figure 3. β-catenin expression. Immunohistochemistry (A) β-catenin was moderately expressed on the cell membrane of epithelial cells of normal oral mucosa; (B, C) β-catenin was expressed in peripheral cubic cells and stellate epithelial reticular cells in follicular type and plexiform type of ameloblastoma; the cytoplasm and nucleus were positive for β-catenin expression in some cells (Immunohistochemistry, SP method; ×200).

Results

Correlation of immunohistochemical results of E-cadherin, vimentin, and β-catenin with clinical manifestation of ameloblastoma

Epithelial cells of NOM were positive for E-Cadherin, which was expressed on cell membrane. In ameloblastoma, the E-Cadherin expression was observed on the membrane and in the plasma of stellate epithelial reticular cells, but its expression reduced in peripheral columnar or cubic epithelium. In OSCC epithelium, weakly positive expression of E-Cadherin was observed (**Figure 1**). The expression of E-Cadherin was moderate to strong in normal mucosa epithelium, decreased or disappeared in columnar or cuboidal epithelium in ameloblastoma, and was weak or disappeared in epithelium in squamous-cell carcinoma, which was with significant difference ($P<0.05$). Meanwhile, the expression of E-Cadherin was significant different among primary, recurrent and malignant ameloblastoma ($P<0.05$).

Vimentin was expressed in NOM mesenchyme, not in epithelium. However, the interstitium was strong positive for Vimentin expression, and tumor epithelial cells were also positive for Vimentin expression in ameloblastoma (**Figure 2**). β-catenin is moderately expressed on the epithelial cell membrane of normal mucose, but β-catenin expression reduced on the cell membrane of ameloblastoma and was mainly observed in the cytoplasm and/or nucleus (ectopic expression) (**Figure 3**). The expression of β-catenin in NOM, ameloblastoma, and OSCC tissues were significant different, as well as in primary, recurrent and malignant ameloblastoma (**Tables 1** and **2**). In malignant ameloblastoma, β-catenin expression reduced on the cell membrane, but cytoplasm and nucleus were positive for β-catenin expression, especially nucleus (**Figure 4**). There was no significant difference of E-Cadherin, Vimentin and

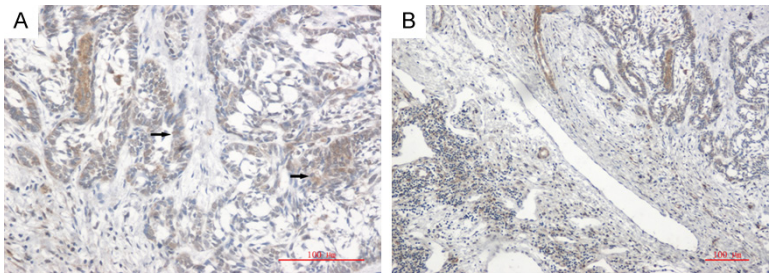

Figure 4. β-catenin expression in malignant ameloblastoma. A: Immunohistochemistry, SP method; ×200, arrow: positive nucleus; B: Immunohistochemistry, SP method; ×100.

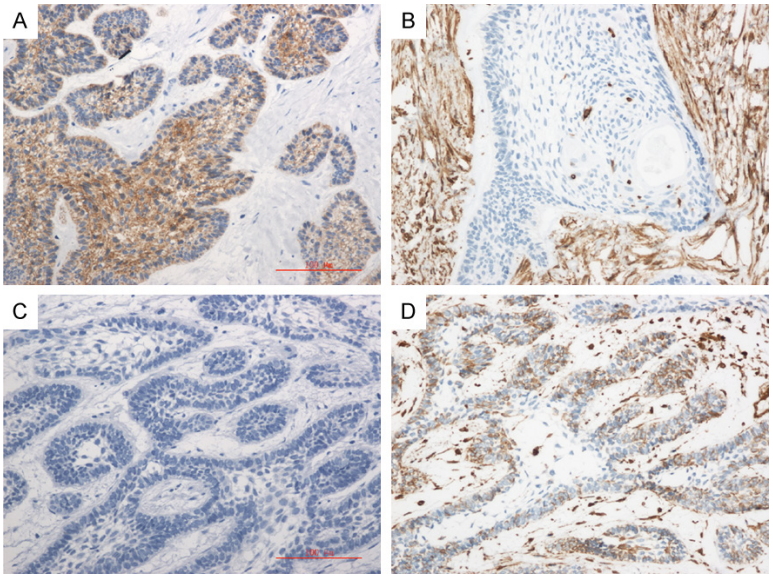

Figure 5. E-Cadherin and Vimentin expression in ameloblastoma. A: Strongly positive expression of E-Cadherin; B: None expression of Vimentin in epithelium cells; C: Disappeared expression of E-Cadherin in in epithelium cells; D: Strongly positive expression of Vimentin (SP method; ×200).

Table 3. Correlation analysis of E-Cadherin and Vimentin expression in ameloblastoma

		Vimentin	
		-	+
E-Cadherin	-	32	20
	+	69	17

Notes: r=-0.205, P=0.016.

β-catenin in different age groups, gender, and pathological types of ameloblastoma (P>0.05) (**Tables 1** and **2**).

Correlation analysis of E-cadherin and vimentin in ameloblastoma

Immunohistochemistry showed the E-cadherin expression was negatively related to Vimentin expression in ameloblastoma, and the Vimentin expression increased with the loss of E-Cadherin expression (**Figure 5**; **Table 3**).

Discussion

E-Cadherin belongs to type I calcium-dependent transmembrane glycoprotein. The intracellular structure of E-Cadherin may form complexes with α-catenin and β-catenin and then bind to actin cytoskeleton. The extracellular domain may bind to adjacent cells in a homology manner. The intracellular and extracellular actions may form the intercellular conjunction, which is essential for the maintenance of epithelial functions. Vimentin is a marker of interstitial cells, and up-regulated Vimentin expression is often accompanied by the reduction or defect of E-Cadherin, which is opposite to the epithelial cell phenotype [16]. To date, a variety of studies have conducted to investigate the expression of E-Cadherin and Catenin complexes in malignancies [17]. It has been confirmed that the reduction or defect of E-Cadherin is closely related to the dedifferentiation, invasive growth and metastasis of tumors, and has the potential as a prognostic factor of malignant tumor [18].

To elucidate the mechanism underlying the invasiveness of ameloblastoma, this study was undertaken to detect the expression of EMT related proteins (E-Cadherin, β-catenin and Vimentin) in ameloblastoma, normal oral mucosa and oral squamous cell carcinoma by immunohistochemistry. A large number of studies have shown that there are EMT and reduction or defect of E-Cadherin expression in the occurrence, development and metastasis of oral tumors [14, 16, 19]. Nevertheless, the E-Cadherin expression in immunohistochemistry in epithelial hyperplasia and benign tumor is

similar to that in normal tissues, suggesting the preservation of epithelial adhesion [20]. In our study, results showed the cell membrane of stellate epithelial reticular cells in ameloblastoma was strong positive for E-Cadherin expression, but E-Cadherin expression reduced in peripheral columnar cells. This indicates that the peripheral cells of ameloblastoma as a benign tumor display EMT and have the potential of focal invasion.

Vimentin is a marker of interstitial cells and involved in the formation of cytoskeleton. In some malignancies including prostate cancer, colon cancer, breast cancer, and bladder cancer, Vimentin expression increases [21-24], and the extent of increase of Vimentin expression is closely related to the invasiveness and poor prognosis. There is evidence showing that Vimentin expression increases in oral squamous cell carcinoma [8, 14, 25, 26]. Our results also showed Vimentin expression occurred in the epithelium with the recurrence and malignant transformation, which was accompanied by reduced E-Cadherin. In our previous study, results also confirmed the primary ameloblastoma cells were positive for keratin, a marker of epithelium and weakly positive for Vimentin, a marker of interstitium. Results at tissue and cell levels confirmed the presence of EMT in ameloblastoma, which might play an important role in the invasive growth of ameloblastoma.

In our previous study, the CTNNB1 exon3 mutation was screened in β-catenin gene of 30 samples, but mutation was found in only 1 sample, suggesting that the abnormal β-catenin expression is possibly not caused by gene mutation. In this study, it was found that the expression of glycogen synthase kinases 3β decreased in AB, suggesting that abnormal active of Wnt signaling pathway was involved in pathogenesis and progress of ameloblastoma. Thus, we speculate that the abnormal activation of Wnt signaling pathway might be one of mechanisms underlying the pathogenesis of ameloblastoma, and abnormal expression of β-catenin, a key factor of Wnt signaling pathway, was observed in ameloblastoma. Moreover, the reduced E-Cadherin expression may be another cause of abnormal expression of β-catenin. With the genesis, recurrence, and canceration of ameloblastoma, the positive expression of β-catenin increased in nucleus. The binding between β-catenin and E-Cadherin on cell membrane is essential for the maintenance of intercellular junction, which blocks the accumulation of β-catenin in the cytoplasm, its nuclear translocation and subsequent binding to nuclear TCF/LEF-1 to form transcription factor [27]. Increased Vimentin expression was related to reduced expression of β-catenin on cell membrane and increased expression of β-catenin in the cytoplasm and nucleus. Some investigators propose that there is a transcriptional target of β-catenin- TCF/LEF-1 complex in the promoter of Vimentin gene, and the abnormal activation of Wnt signaling pathway and reduced expression of E-Cadherin may induce the cytoplasmic accumulation of β-catenin and the subsequent nuclear translocation of β-catenin to form this complex [6, 28], which induces the Vimentin expression. In addition, in malignant adenoma, E-Cadherin/β-catenin complex may serve as an important factor in the intercellular adhesion, which is associated with tumorogenesis. In squamous cell carcinoma, β-catenin as an important molecule in Wnt signaling pathway is involved in the tumorogenesis [29].

Conclusion

Our results indicate that the expression of E-Cadherin and β-catenin in epithelium, but ectopic expression of β-catenin occurs in the cytoplasm and nucleus, which is accompanied by increased expression of Vimentin. The EMT related proteins E-Cadherin, β-catenin and Vimentin are involved in the occurrence and development of ameloblastoma and thus may serve as biomarkers of tumor invasiveness.

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Disclosure of conflict of interest

None.

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