

Original Article

Association between clinicopathological characteristics and hTERT expression as well as telomere length in ameloblastoma

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Abstract: Objective: This study aimed to investigate the human telomerase reverse transcriptase (hTERT) expression and telomere length in ameloblastoma, and explore the role of hTERT in the invasiveness and recurrence of ameloblastoma. Methods: hTERT expression was detected by immunohistochemistry and Western blotting and telomere length by fluorescence in situ hybridization (FISH) in human ameloblastoma, normal mucosa, and oral squamous cell carcinoma (OSCC). Association between clinicopathological characteristics and hTERT expression as well as telomere length in ameloblastoma was analyzed. Results: hTERT expression in ameloblastoma was higher than that in normal oral mucosa, and the highest in OSCC ($P < 0.05$). The hTERT positive rate and expression increased with the recurrence and malignant transformation. hTERT expression was significantly different among different types of ameloblastoma. The telomere was the shortest in OSCC and the longest in normal oral mucosa. Conclusion: Ameloblastoma has shortened telomere, and is positive for hTERT expression which is related to the recurrence and malignancy of ameloblastoma. These findings indicate that telomerase is involved in the occurrence and development of ameloblastoma.

Keywords: Ameloblastoma, human telomerase reverse transcriptase, telomere

Introduction

A telomere is a region of repetitive nucleotide sequences ($[TTAGGG]_n$) at each end of a chromatid, and has two main functions: (1) it maintains the integrity of chromosome structure, prevents the nuclease induced degradation of chromosomes, and prevent chromosomes from fusion with neighboring chromosomes; (2) it prevents the chromosome from shortening during replications (with loss of genetic information), which resolves the end replication problem [1]. A telomere will be shortened by 50-200 bp in each mitosis, and then the telomere will become dysfunctional with the mitosis. Under this condition, it can not protect the chromosome terminal, leading to the initiation of response to DNA damage, which may induce the senescence and apoptosis of cells. This can avoid carcinogenesis in cells with gene mutation. In cell division, the telomere is abnormally lengthened, leading to the unlimited

cell proliferation, which is a cause of carcinogenesis. In some malignancies, the telomere is abnormally lengthened, which is mainly ascribed to the elevated telomerase activity in about 85% of cells and the alternative lengthening of telomeres (ALT) in remaining 15% of cells. In cell division, the excess lengthening of telomere may cause the acquisition of capability of unlimited proliferation. Telomerase can synthesize telomeres via reverse transcription with itself as a template to maintain the telomere length. As a rate limiting subunit in the telomerase catalyzing reaction, human telomerase reverse transcriptase (hTERT) plays a determinant role in the telomerase activity, and can regulate the telomerase activity at transcription level [2]. hTERT expression is up-regulated in germ cells, stem cells with differentiation potential, activated lymphocytes, some specific benign tumors, some precancerous lesions and all the malignancies, but normal tis-

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sues or some benign lesions have almost no hTERT expression [3].

Ameloblastoma is a common benign tumor of jaw, and accounts for about 1% of all oral tumors and 11%-18% of odontogenic tumors. The incidence of ameloblastoma follows the keratocystic odontogenic tumor (KCOT) and odontoma [4]. In China, ameloblastoma has an incidence of as high as 36.52%, following the KCOT, but its incidence is higher than that of odontoma [5]. Ameloblastoma is focally invasive, and shows local invasive growth. Some characteristics of ameloblastoma are similar to those of low-grade malignancies. In addition, ameloblastoma has a high post-operative recurrence rate and may become malignant or even metastasize [6]. Surgery is the main treatment for ameloblastoma, but extended excision is needed to acquire safe margin, which, however, brings difficulties to the post-operative repair and improvement of quality of life. Although extended excision has been performed, it still has a risk for recurrence [7, 8]. There is evidence showing that the telomerase activity increases in ameloblastoma, which implies that telomerase is related to the special biological behaviors of ameloblastoma [9].

In present study, immunohistochemistry was employed to detect the hTERT expression in ameloblastoma, normal oral mucosa, and oral squamous cell carcinoma (OSCC), and the telomere length was measured by quantitative fluorescence in situ hybridization (Q-FISH), aiming to explore the role of hTERT in the occurrence and development of ameloblastoma, and analyze the change in telomere length in ameloblastoma.

Materials and methods

General data

This study has been approved by the Ethics Committee of Affiliated Stomatological Hospital of China Medical University. A total of 89 patients who were hospitalized in the Affiliated Stomatological Hospital of China Medical University between 2005 and 2012 were recruited. They were pathologically diagnosed with ameloblastoma. In addition, 12 patients with OSCC and 10 healthy subjects were recruited as controls. Informed consent was obtained before study. There were 6 males and 4

females with a median age of 31 years (range: 18-47 years). Pathological assessment was done by two experienced pathologists according to the WHO classification criteria.

Immunohistochemistry for hTERT

Tissues were fixed in 10% formalin, embedded in paraffin and sectioned (3 μ m in thickness). These sections were deparaffinized in xylene and then treated with a series of concentrations of ethanol. After incubation with 0.3% H₂O₂ at room temperature for 30 min, sections received antigen retrieval in citric acid buffer for 10 min. Following blocking in 10% BSA-PBST at room temperature for 1 h, sections were incubated with rabbit anti-human hTERT monoclonal antibody in 1% BSA-PBST (Abcam, 1:100) at 4°C over night. After incubation with biotinylated goat anti-rabbit IgG at room temperature for 2 h, sections were treated with Streptavidin-HRP at room temperature for 1 h. Visualization was done with DAB, followed by counterstaining with hematoxylin and mounting with neutral gum. hTERT positive cells had brown granules in the nucleus and/or cytoplasm. hTERT expression was evaluated by two experienced pathologists. The positive cells were counted on representative photos. The staining intensity (SI) was evaluated and scored as 0 (negative), 1 (weakly positive), 2 (moderately positive) and 3 (strong positive). The labeling indices (LI) was calculated as the proportion of positive cells in 300 counted cells: 0-10%, 0; 11-25%, 1; 26-50%, 2; >50%, 3. The labeling scores (LS) was calculated as the product of LI and SI: 0-1, negative (-); 2, weakly positive (+); 3-4, moderately positive (++); 5-6, strong positive (+++).

Fluorescence in situ hybridization (FISH) to detect the telomere length

In brief, the paraffin-embedded sections were deparaffinized and hydrated (100% ethanol I for 10 min; 100% ethanol II for 10 min; 95% ethanol for 5 min; 75% ethanol for 5 min). Then, sections were washed in PBS thrice, followed by antigen retrieval in citric acid buffer (0.1 M citric acid [9 ml], 0.1 M sodium citrate [41 ml], pH value: 6.0) for 10 min. After digestion in protease K (1:10) at 37°C for 10 min, sections were washed in PBS, and then dehydrated in a series of concentrations of ethanol. Sections were subsequently incubated with (CCCTAA)₄-

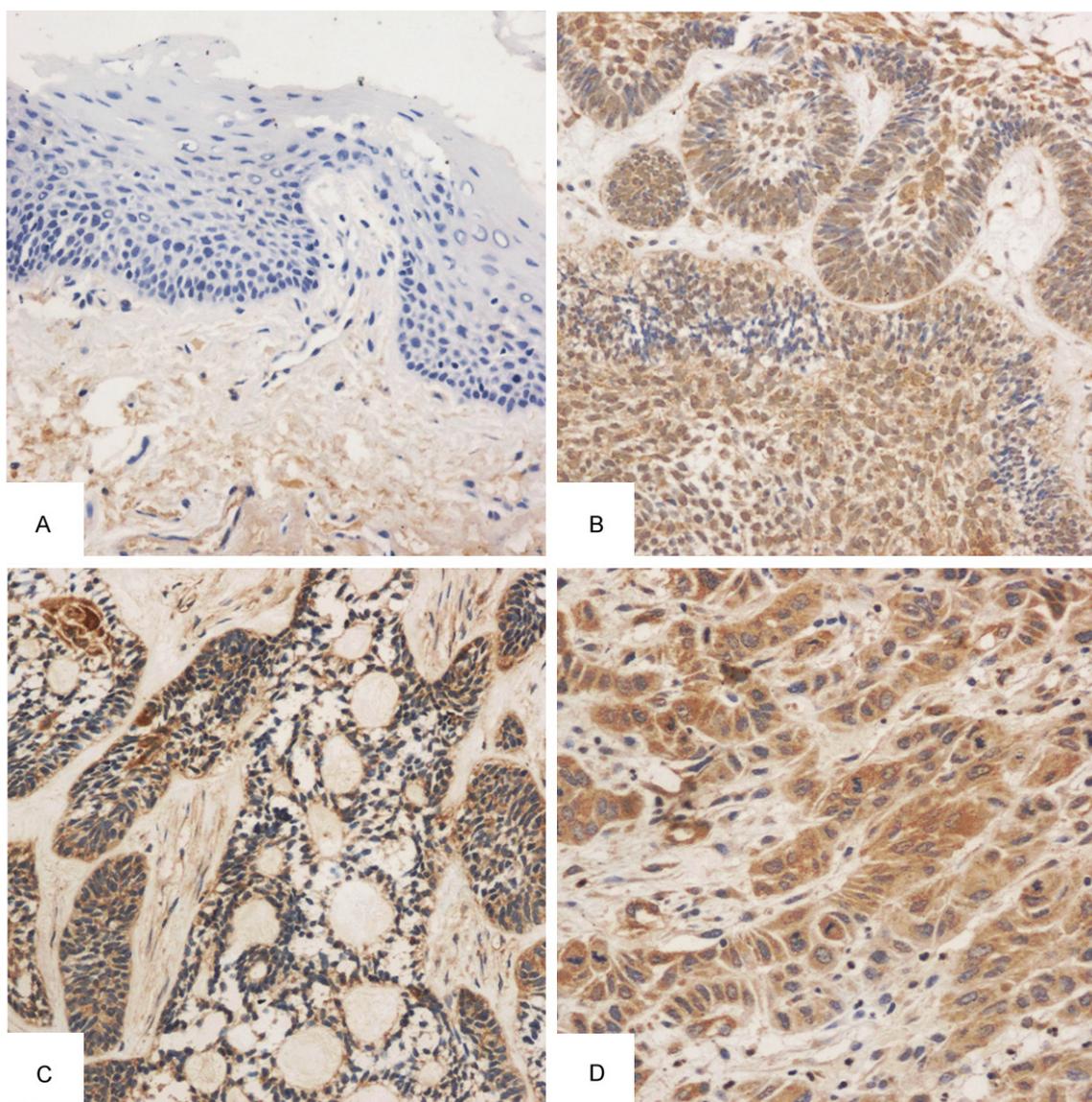


Figure 1. Immunohistochemistry for hTERT (A: NOM negative for hTERT, SP method, $\times 100$; B: Epithelial bud-like hyperplasia in plexiform ameloblastoma and hTERT expression in the cytoplasm and some nuclei of peripheral columnar cells and stellate reticulum-like cells, SP method, $\times 100$; C: hTERT expression in acanthomatous ameloblastoma and low expression in keratinized area, SP method, $\times 100$; D: hTERT expression in the cytoplasm [strong positive] of moderately differentiated OSCC, SP method, $\times 200$).

Cy3 (10 μ l) in hybridization solution (1:9) at 95°C for 5 min, followed by hybridization in dark at room temperature for 2 h in a humidified box with 50% formamide. Then, sections were washed in 2 \times SSC at 42°C, followed by counterstaining with DAPI. After washing in methanol for 10 min, mounting was done with glycerin. Sections were observed under a fluorescence microscope (Olympus, CX61, Japan) and representative images were captured. Images were analyzed for the fluorescence intensity with Motic Image Advanced 3.2. Ten cells were randomly selected, and the sum of

fluorescence intensity of each Cy-3 positive cells was calculated as the optical density (OD) of the telomeres and the intensity of DAPI as the OD of nuclei. The ratio of telomere OD to DAPI OD was calculated as the telomere length. The mean telomere length was calculated from 10 cells selected.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0. Data from immunohistochemistry were analyzed with chi square test. A value of

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Table 1. hTERT expression in ameloblastoma, NOM and OSCC

	n	-	+	++	+++	Positive rate (%)
NOM	10	8	2	0	0	20.0
Ameloblastomas	89	23	36	22	8	74.2
OSCC	12	1	4	2	5	91.7

Table 2. hTERT expression in primary, recurrent and malignant ameloblastoma

Group	n	-	+	++	+++	Positive rate (%)
Primary	51	16	18	15	2	68.6
Recurrent	33	7	18	5	3	78.8
Malignant	5	0	0	2	4	100

Table 3. Correlation between hTERT expression and pathological type of ameloblastoma

Pathological type	n	-	+	++	+++	Positive rate (%)
Plexiform	22	7	9	4	2	68.2
Follicular	32	7	13	9	3	78.1
Mixed	12	2	5	3	2	83.3
Acanthomatous	7	1	3	3	0	85.7
Basal cells	5	1	1	2	1	80
Unicystic	7	4	2	1	0	42.9

Note: desmoplastic ameloblastoma in 2 patients and peripheral ameloblastoma in 2 patients were not included in this table.

$P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of ameloblastoma patients

The primary ameloblastoma was found in 51 patients (57.3%) and recurrent ameloblastoma in 33 (37.1%). The interval to recurrence ranged from 4 months to 16 years. Malignant ameloblastoma was found in 5 patients (5.6%). There were 38 males (42.7%) and 51 females (57.3%). The age of ameloblastoma patients ranged from 14 to 72 years old median: 39 years old). Most patients (27%) were 31 to 40 years old. Ameloblastoma was noted in the mandible in 80 patients (89.89%), the maxilla in 7 (7.87%) and the periphery in 2 (gums; 2.24%). According to the WHO classification criteria for odonto-

genic tumors, solid/multicystic ameloblastoma was noted in 78 patients (87.64%), unicystic ameloblastoma in 7 (7.86%), desmoplastic ameloblastoma in 2 (2.25%) and peripheral ameloblastoma in 2 (2.25%). In addition, in 78 cases of solid multicystic ameloblastoma, follicular ameloblastoma was found in 32 patients (41.03%), plexiform ameloblastoma in 22 (28.21%), acanthomatous ameloblastoma (8.97%), basal cell ameloblastoma (6.41%) and follicular/plexiform mixed ameloblastoma in 12 (15.38%).

Expression and localization of hTERT in ameloblastoma

In 89 cases of ameloblastoma, 66 were positive for hTERT expression. hTERT expression was mainly found in the peripheral columnar and cubic epithelial cells as well as stellate reticulum-like cells. In addition, hTERT expression was observed in both cytoplasm and nucleus (**Figure 1**).

Positive rate of hTERT was 91.7% in OSCC cells and 74.2% in ameloblastoma cells. In NOM, being weakly positive for hTERT was noted in only 2 patients. The positive rate was significantly different among groups (**Table 1**). According to the clinically biological behaviors, the hTERT positive rate and expression increased with the recurrence and malignant transformation: 68.6% (35/51) of primary ameloblastoma and 78.8% (26/33) of recurrent ameloblastoma were positive for hTERT, but 100% (5/5) of malignant ameloblastoma was positive for hTERT, showing significant difference among groups ($P < 0.01$; **Table 2**). hTERT expression was significantly different among different types of ameloblastoma ($P < 0.05$; **Table 3**)

Telomere length

FISH showed the mean telomere length was 0.1260 in 89 cases of ameloblastoma, 0.0487 in 12 cases of OSCC and 0.2105 in 10 cases of NOM, showing significant difference among groups ($F = 8.965$, $P < 0.0001$) (**Figure 2** and **Table 4**).

Correlation between telomere length and hTERT expression in ameloblastoma

The telomere length showed an increased tendency with the increase in hTERT expression

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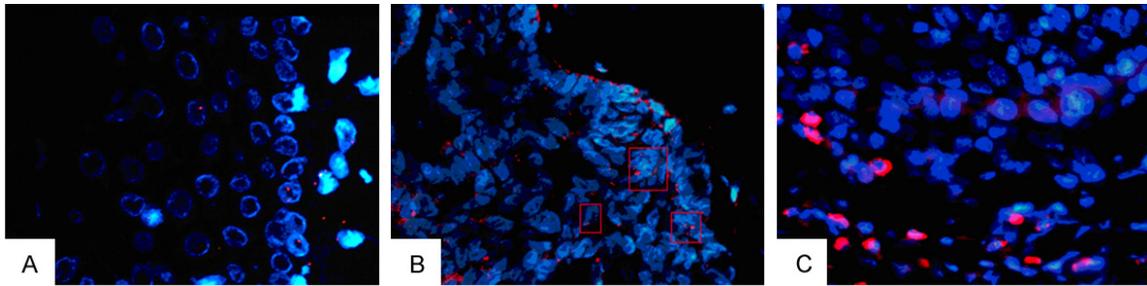


Figure 2. Telomere Cy-3 (A: NOM; B: Ameloblastoma; C: OSCC; $\times 400$).

Table 4. Telomere length in ameloblastoma, OSCC and NOM

Group	n	Telomere length
NOM	10	0.0487 \pm 0.0295
Ameloblastoma	89	0.1260 \pm 0.0886
OSCC	12	0.2105 \pm 0.1334

Table 5. Correlation between telomere length and hTERT expression in ameloblastoma

Group	n	Telomere length
hTERT (-)	23	0.0965 \pm 0.0747
hTERT (+)	36	0.1364 \pm 0.0749
hTERT (++)	22	0.1401 \pm 0.1227
hTERT (+++)	8	0.1487 \pm 0.0893

although significant difference was not observed ($F=1.984$, $P=0.144>0.05$) (Table 5).

In addition, patients were divided into 7 groups according to age. Results showed the telomere length in ameloblastoma had no relationship with the age at diagnosis ($F=0.756$, $P=0.606>0.05$). In addition, there was no marked correlation between telomere length and histological type in ameloblastoma ($F=1.454$, $P=0.233>0.05$).

Discussion

Early diagnosis before the invasion and metastasis is crucial for the prognosis and good therapeutic efficacy. Studies have shown that telomere and telomerase play important roles in the occurrence and development of malignancies, and determination of difference in the telomerase activity between malignancy and normal tissues is important. Generally, the telomerase expression in malignancies is higher than that in normal tissues, which renders telomerase a reliable biomarker in the early diag-

nosis of malignancies. A large number of studies confirmed that the telomerase activity increases in head and neck malignancies, including OSCC [10-12]. In oral precancerous lesions such as oral leukoplakia and oral submucosal fibrosis, the hTERT expression also increases [12-14]. Currently, only a few studies reported the hTERT expression in ameloblastoma [15]. Human ameloblastoma is a benign tumor derived from odontogenic epithelium, but shows focally invasive growth, susceptibility to recurrence and rapid proliferation of tumor cells, which are malignant characteristics [16].

Currently, some techniques can be employed to detect the activity and expression of telomerase. In situ hybridization (ISH) is able to specifically detect nucleic acid sequence [17]. ISH was employed to confirm the hTERT mRNA expression in ameloblastoma, and its expression increases with the recurrence and malignant transformation [18, 19]. In this study, immunohistochemistry was employed to detect hTERT protein expression in ameloblastoma and results showed human ameloblastoma was positive for hTERT. This may be related to the activate proliferation of ameloblastoma cells which leads to the invasiveness and recurrence. In this study, results showed several NOMs were also positive for hTERT (weakly or moderately positive). This implies that oral mucosal epithelium has rapid turnover and basal cells may compensate the shedding mucosal cells by rapid division and differentiation. Some investigators speculated that oral mucosal epithelium with rapid proliferation had resident stem cells with the potential of persistent proliferation and hTERT is expressed in these stem cells [12, 20]. Thus, in normal oral mucosal epithelium, there is low telomerase activity or low hTERT expression. In the present study, histo-

logical type had no relationship with hTERT expression. Krishna et al found MDM2 (a invasiveness related factor) expression in desmoplastic ameloblastoma was lower than in solid/polycystic and plexiform ameloblastoma and they speculated that the transformed granulocytes in follicular ameloblastoma had the most potent invasiveness and was susceptible to recurrence [21], but desmoplastic ameloblastoma possessed weak invasiveness. Kumamoto et al [22] found that the expression of Caspase3 and Fas in desmoplastic ameloblastoma was higher than that in solid/polycystic ameloblastoma and also confirmed that different types of ameloblastoma showed distinct capabilities of invasiveness and proliferation. In this study, desmoplastic ameloblastoma was found in only 2 patients, which might bias out results.

A telomere will be shortened by 50-200 kb after each normal cell division, and cell apoptosis will be initiated when the telomere is significantly shortened. In cancer cells, there are mechanisms for the elongation of telomeres which protect cells against senescence and death. Thus, telomere length has been regarded an important biomarker in the development of malignancies. Currently, there is still controversy on the change in telomere length in malignancies. Some studies on precancerous lesions and carcinoma in situ showed the telomere in early phase of carcinogenesis is shorter than in normal tissues [23, 24]. Thus, it is assumed that the telomere is aberrantly shortened which may cause the chromosomal instability; subsequently, multiple genes are involved in the carcinogenesis, the telomerase is activated and the telomere is elongated.

The present study for the first time investigated the telomere length in ameloblastoma, and these results showed the telomere length in ameloblastoma was shorter than in normal oral mucosa, which was accompanied by telomerase activation. This may be related to the invasive growth of ameloblastoma. In studies, the correlations of telomere length with age, smoking and other factors are not evaluated; other benign odontogenic tumors may serve as controls, which will be a direction in future studies on ameloblastoma. In head and neck tumors and precancerous lesions, the telomerase activity increases [25, 26], but the telomere is shortened in head and neck tumors, which is

related to the tumor grade [27]. There is evidence showing that the telomere length in the tumor surrounding normal tissues is related to the prognosis [28]. In future studies, it is necessary to detect the telomere length in tissues and blood, and further evaluate its relationship with the biological characteristics of ameloblastoma.

According to the findings, we speculate that telomere length and telomerase provide references for the differentiation and proliferation of ameloblastoma cells as well as the outcome of ameloblastoma.

Conclusion

The telomere is shortened in ameloblastoma and ameloblastoma is positive for hTERT. In addition, the telomere length and hTERT expression are related to the recurrence and malignant transformation. These findings indicate that telomerase is involved in the occurrence and development of ameloblastoma.

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Disclosure of conflict of interest

None.

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