Investigation of PTEN promoter methylation in ameloblastoma

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Abstract

Background: Phosphatase and tensin homolog (PTEN) acts as a tumor suppressor gene. Inactivation of PTEN has been reported in various types of cancers. PTEN promoter methylation possibly underlies PTEN inactivation, which results in tumorigenesis. The aim of this study was to investigate whether PTEN promoter methylation contributes to PTEN inactivation in ameloblastoma and its associated protein expression.

Material and Methods: In total, 20 fresh-frozen ameloblastoma samples were evaluated for PTEN promoter methylation using methylation-specific polymerase chain reaction (MS-PCR). A subset of 10 paraffin-embedded ameloblastoma samples was examined for PTEN expression through immunohistochemistry. Four primary cultured ameloblastoma cells were investigated for PTEN promoter methylation and PTEN transcriptional expression via reverse transcription PCR.

Results: PTEN promoter methylation was detected in 65% (13/20) of the ameloblastoma samples. Of 10 ameloblastoma samples, 4 exhibited reduced PTEN expression. Of 5 samples with methylated PTEN, 3 (60%) were associated with loss of PTEN expression. However, PTEN expression was detected in 4 (80%) of 5 samples with unmethylated PTEN. In addition, 3 (75%) of 4 primary ameloblastoma cell cultures exhibited an inverse correlation between PTEN promoter methylation and PTEN transcription level.

Conclusions: PTEN promoter methylation is found in a number of ameloblastomas but not significantly correlated with loss of PTEN expression. Genetic or epigenetic mechanisms other than PTEN promoter methylation may contribute to PTEN inactivation in ameloblastoma tumor cells.

Key words: PTEN, promoter methylation, ameloblastoma.
Introduction

Ameloblastoma is the most frequently encountered neoplasm arising from the epithelium of the tooth-forming apparatus. Although this tumor is benign, it exhibits locally invasive behavior and has a high risk of recurrence. Its macroscopic features range from completely solid to multicystic appearance. Its histopathological subtypes include follicular, plexiform, acanthomatus, granular cell, basal cell, and desmoplastic ameloblastomas. In rare cases, ameloblastoma may metastasize despite its benign histology; this type of ameloblastoma is termed as metastasizing ameloblastoma (1). Ameloblastic carcinoma, a malignant counterpart of ameloblastoma, is markedly rare, with only 100 cases reported to date; this tumor exhibits cytological features of malignancy and may or may not metastasize (2).

Phosphatase and tensin homolog (PTEN) is located on chromosome 10q23.3 and has been implicated in many familial and sporadic cancers (3,4). Deletions or somatic mutations in PTEN have been detected in many types of cancers, including prostate, breast, and brain cancer (3). Apart from genetic mutation, the epigenetic regulation of PTEN via differential methylation may contribute to its inactivation (5). Methylation of the PTEN promoter region has been reported in some types of cancers and has been suggested to be involved in tumorigenesis (6-8). In ameloblastic tumors, PTEN displayed high frequent allelic loss (62%) (9). In addition, PTEN has been reported to be completely absent in 33.3% of ameloblastoma cases (10). We hypothesized that PTEN promoter methylation results in decreased PTEN expression in this odontogenic tumor. The aim of this study was to examine PTEN expression and investigate whether PTEN promoter methylation contributes to PTEN inactivation in ameloblastoma.

Material and Methods

- Sample recruitment

Fresh-frozen samples were obtained from 20 patients with ameloblastoma from the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Mahidol University between January 2018 and January 2019. Some parts of the specimens were fixed in 10% buffered formalin for hematoxylin and eosin staining. Histopathological diagnosis of solid/multicystic ameloblastoma was performed by two oral pathologists (PL and NK). Furthermore, a cohort study was performed wherein 4 fresh solid/multicystic ameloblastoma tissue samples were harvested to form a primary cell culture. Table 1 displays the detailed demographic data.

| No | Gender | Age at diagnosis (yrs.) | Location | Histological appearance | PTEN promoter methylation | PTEN expression (Immunohistochemistry) |
|----|--------|-------------------------|----------|-------------------------|---------------------------|---------------------------------------|
| AM1 | Female | 30                      | Mandible | Plexiform               | Unmet                     | Positive                              |
| AM2 | Male   | 63                      | Mandible | Plexiform               | Met                       | Positive                              |
| AM3 | Female | 47                      | Mandible | Plexiform               | Met                       | Negative                              |
| AM4 | Male   | 11                      | Maxilla  | Plexiform               | Unmet                     | Positive                              |
| AM5 | Female | 60                      | Mandible | Follicular              | Unmet                     | Positive                              |
| AM6 | Male   | 66                      | Mandible | Plexiform               | Met                       | Positive                              |
| AM7 | Male   | 23                      | Mandible | Follicular              | Met                       | Negative                              |
| AM8 | Male   | 51                      | Mandible | Follicular              | Unmet                     | Negative                              |
| AM9 | Male   | 51                      | Mandible | Follicular              | Met                       | Negative                              |
| AM10| Female | 25                      | Mandible | Follicular              | Unmet                     | Positive                              |
| AM11| Male   | 59                      | Mandible | Plexiform               | Met                       | Not available                         |
| AM12| Male   | 44                      | Maxilla  | Plexiform               | Met                       | Not available                         |
| AM13| Female | 24                      | Maxilla  | Plexiform               | Unmet                     | Not available                         |
| AM14| Male   | 54                      | Mandible | Follicular              | Unmet                     | Not available                         |
| AM15| Female | 54                      | Mandible | Plexiform               | Met                       | Not available                         |
| AM16| Male   | 73                      | Mandible | Plexiform               | Met                       | Not available                         |
| AM17| Female | 8                       | Mandible | Plexiform               | Met                       | Not available                         |
| AM18| Male   | 66                      | Mandible | Follicular              | Met                       | Not available                         |
| AM19| Male   | 50                      | Mandible | Follicular              | Met                       | Not available                         |
| AM20| Male   | 50                      | Maxilla  | Follicular              | Met                       | Not available                         |

Table 1: Detailed data of phosphatase and tensin homolog (PTEN) promoter methylation, PTEN expression in ameloblastoma and demographic data.
The cultures were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and maintained for 3–4 passages prior to DNA extraction.

- DNA extraction, bisulfite modification, and methylation-specific polymerase chain reaction (MS-PCR)

Genomic DNA was extracted using 10% sodium dodecyl sulfate buffer and proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation. Subsequently, the obtained DNA was air-dried, dissolved in distilled water, and quantified using a NanoDrop spectrophotometer (ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). An optical density (260/280 ratio) of >1.8 is acceptable for DNA purity and PCR. Next, the DNA samples were converted by sodium bisulfite treatment using the EZ DNA Methylation-Gold™ kit (Zymo Research, Irvine, CA, USA), as per the manufacturer’s instructions.

The bisulfite-treated DNA samples were then subjected to MS-PCR using primers specific for either the methylated or unmethylated forms of PTEN: 1) PTEN methylated sequence, forward 5’-GTTTGGGGATTTTTTTTTC-3’ and reverse 5’-AACCCCTTCTACGCCGC-3’ and 2) PTEN unmethylated sequence, forward 5’-TATTAGTTGGGATTTCATTTTCGC-3’ and reverse 5’-CCCAACCTTCTACACCACA-3’ (9). Both forms of PTEN were amplified with HotStarTaq (Qiagen, Tokyo, Japan) in 40 cycles at an annealing temperature of 55°C using the forward primer 5’-GGACGAACTG-GTGTAATGATATG-3’ and reverse primer 5’-TC-TACTGTGTGTGAAATGACG-3’ (12). To investigate the relative expression of a candidate gene, glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous DNA control, with the sequences of the forward and reverse primers being 5’-CTCAGA-CACCATGGGGAAGGTGA-3’ and 5’-ATGATCTT-GAGGCTGTGTTCATA-3’, respectively. Both PCR mixtures contained PCR buffer (1×) (Qiagen, Tokyo, Japan), deoxynucleotide triphosphates (0.2 mM), the two primers (final concentration: 0.4 μM), HotStarTaq (1 U) (Qiagen, Tokyo, Japan), and template DNA (50 ng). The PCR products (PTEN: 671 bp and GADPH: 450 bp) were separated via gel electrophoresis on an 8% nondenaturing acrylamide gel and stained with SYBR green nucleic acid gel stain (Gelstar, Lonza, Allendale, NJ, USA).

- Immunohistochemical staining of PTEN

In this experiment, the paraffin tissues were available in only 10 cases. Formalin-fixed paraffin embedded blocks were cut into 3-μm thick sections. The histological sections of the relative samples were confirmed by a pathologist. Immunohistochemistry was performed with an antihuman monoclonal antibody against PTEN Clone 6H2.1 (dilution 1:100, Dako, Glostrup, Denmark) in Tris-HCl buffer and a commercial rabbit antibody diluent (Dako, Glostrup, Denmark) on the Ventana® Benchmark XT (Ventana-Roche Diagnostics, Meylan, France) automated slide strainer in combination with the Ventana UltraView DAB IHC Detection Kit®. Before mounting, the sections were counterstained with Hematoxylin II® for 8 min, bluing reagent® for 4 min, Hematoxylin II for 4 min, and bluing reagent for 4 min. To support the validity of staining and identify experimental artifacts, negative (omitting the primary antibody) and positive controls (normal breast tissue) were included in each run. Nuclear and cytoplasmic immunostaining of PTEN in ameloblastoma tumor cells were graded based on the presence or absence of protein staining.

- Statistical analysis

SPSS software for Windows version 22 (SPSS Inc., Chicago, IL) was used to analyze all data. The effects of age and sex of the patients as well as the histological appearance of ameloblastoma on the PTEN methylation status and PTEN expression were investigated using the Pearson’s correlation coefficient test, chi-square test, and Fisher’s exact test. \( P < 0.05 \) was considered statistically significant.
Results
- MS-PCR and immunohistochemistry of ameloblastoma tissues
We examined PTEN promoter methylation and whether it affects PTEN expression in ameloblastomas. PTEN promoter methylation was observed in 65% (13/20) of ameloblastoma samples (Table 1). The exemplified gel electrophoresis is demonstrated in Fig. 1. Ten samples of these ameloblastoma cases were investigated for immunohistochemical staining of PTEN. We found loss of PTEN expression in 3 of 5 (60%) ameloblastoma samples with PTEN promoter methylation while PTEN expression was present in 4 of 5 (80%) ameloblastoma samples with no PTEN promoter methylation (Table 1). Representative samples showing positive and negative immunostaining of PTEN were shown in Fig. 2.
- Association among PTEN promoter methylation, PTEN expression, and clinicopathological parameters
Table 1 shows the association between PTEN promoter methylation and PTEN expression in the ameloblastoma samples. No significant correlation was found between PTEN promoter methylation and PTEN expression (P = 0.52). Furthermore, no correlation between PTEN promoter methylation and age (P = 0.49), gender (P = 0.40), location (P = 0.62) and the histological appearance of ameloblastoma (P = 0.41) was demonstrated. Similarly, no correlation was observed between PTEN expression and age (P = 0.25), gender (P = 1.00), location (P = 0.51), and the histological appearance of ameloblastoma (P = 1.00).
- MS-PCR and RT-PCR of primary ameloblastoma cell cultures
PTEN promoter methylation and PTEN expression were examined in four primary ameloblastoma cell cultures using RT-PCR (Fig. 3). Only 1 of 4 samples exhibited PTEN promoter methylation and showed no PTEN transcription. PTEN promoter methylation was inversely correlated with PTEN transcription level in the remaining ameloblastoma samples (75%) (Table 1).
PTEN promoter methylation in ameloblastoma

Discussion

Ameloblastoma is considered the most common benign neoplasm of the jaw (1). To prevent local recurrence, patients with ameloblastoma are mostly treated with radical surgery. Understanding the molecular mechanisms that underlie the formation of this tumor may help in developing an alternative and novel treatment for its cure with minimal tissue or bone removal.

PTEN, a putative tumor suppressor gene, is commonly mutated in many types of human neoplasms (3). The protein product of PTEN, a lipid phosphatase, negatively regulates the Akt signaling pathway, thereby stimulating cell cycle arrest and apoptosis (13). Kumamoto and Ooya first reported that the PTEN level is significantly lower in ameloblastic tumors than in tooth germs (14). The absence of PTEN in 33.3% of ameloblastoma samples was subsequently reported (10). These results suggest that the inactivation of PTEN may be involved in the molecular pathogenesis of ameloblastoma. In the present study, we investigated the possible role of PTEN promoter methylation and the associated loss of PTEN expression in a subset of ameloblastoma samples.

To the best of our knowledge, there have been no studies on PTEN promoter in ameloblastoma. Careful analysis of the PTEN promoter has been recommended because it shares a strong homology with the PTEN pseudogene (5). The genomic sequence of the highly conserved and processed PTEN pseudogene (GenBank accession number: AF040103, PTEN pseudogene; AF029308, Homo sapiens chromosome 9 duplication of the T-cell receptor β locus and trypsinogen gene families) is 98% identical to that of PTEN, and this identical sequence is composed of an 841-bp region in the promoter region (15). In the present study, PTEN promoter methylation was performed using methylation-specific primers that do not amplify the highly homologous PTEN pseudogene because these primers lie outside the sequence homology of the PTEN pseudogene.

Promoter methylation is reportedly one of the epigenetic mechanisms underlying the aberrant expression of tumor suppressor genes and contributing to the development of various types of cancers. For example, the methylation of adenomatous polyposis coli promoter is reportedly associated with tumor in the colon and breasts (16). PTEN promoter methylation is also observed in various types of cancers, including gastric, breast, colorectal, and lung cancer (7,8,17-20).

In the present study, PTEN promoter methylation was found in 65% (13/20) of the ameloblastoma samples. However, immunohistochemical staining of PTEN expression was performed in only 10 samples. Of these samples, 3 (60%) of 5 samples with PTEN promoter methylation were associated with loss of PTEN expression, whereas 4 (80%) of 5 samples without PTEN promoter methylation showed PTEN expression. PTEN promoter methylation and decreased PTEN expression were not significantly correlated, indicating that other genetic or epigenetic mechanisms possibly regulate PTEN expression, for example, genetic alterations, transcriptional silencing, post-transcriptional regulation, and modification (21). Previously, PTEN exhibited high frequency of allelic losses (62%) in ameloblastic...
In conclusion, PTEN promoter methylation, contributing to a decrease in protein expression, depends on the specific tumor type. Previous studies on lung and ovarian cancers did not see a correlation between PTEN promoter methylation and loss of protein expression (18,24). Notably, in the present study, 2 (40%) of 5 samples with PTEN promoter methylation showed PTEN expression. This may be attributed to the partial methylation of PTEN at the promoter region. It has been proposed that translational inactivation involves a series of events requiring a sufficient DNA methylation level. The silencing process is then maintained by the spread of methylation (25). This is also supported by the presence of unmethylated bands in several samples following MS-PCR. However, these unmethylated bands also represent normal fibrous tissue stroma in the ameloblastoma samples. Regarding the in vitro experiment, only 1 (25%) of 4 primary ameloblastoma cell cultures showed promoter methylation and loss of PTEN transcription. This result is consistent with a previous study on breast cancer; none of the breast cancer cell lines exhibited PTEN promoter methylation (17). Lastly, the limitation of the present study is the small sample size that may not represent ameloblastoma cases and cell lines in general; thus, further studies with a larger sample size are required to confirm our findings.

In conclusion, PTEN promoter methylation was detected in a subset (58.3%) of ameloblastoma samples; however, it did not significantly contribute to decreased PTEN expression. Other genetic mechanisms possibly underlie the loss of PTEN expression in ameloblastomas.

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Conflict of interest
All authors declare that they have no conflict of interest.

Ethics
This study was conducted in accordance with the approved human subject research guidelines and was approved by the Institutional Review Board of Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Thailand (COA. No. MU-DT/PY-IRB 2018/05.1101 and 2018/049.0409). Informed consent was obtained from all patients before the collection of specimens. Data were analyzed anonymously.