High-Expression of PTEN and an Absence of PCNA in Osteoclast-Like Multinucleated Giant Cells of Giant Cell Tumors in Bone

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Abstract: Giant cell tumors (GCTs) found in bone are so named for the conspicuous presence of numerous osteoclast-like multinucleated giant cells (OLMGCs). Although GCT studies have revealed that the OLMGCs are the cells responsible for tumor formation, these cells continue to receive a good deal of research attention. The tumor – suppressor gene, PTEN, is known to be involved in various malignancies. Recently, however, PTEN has been reported to be important for neuron enlargement and cardiomyocyte hypertrophy. Given the role of PTEN in both carcinomas as well as cell hypertrophy, we sought to elucidate the relationship between PTEN and OLMGCs. In this study, we confirmed the existence of PTEN in GCTs in bone using PCR. In particular, exons-3,4 and 5 of the PTEN gene was detected. Exons-3,4,5 of PTEN gene were found by PCR in all of 8 cases. Single cells microdissection was used to isolate OLMGCs from GCTs and verify the existence of the PTEN gene in the osteoclast-like multinucleated giant cells through PCR amplication of PTEN exon-3. Exon-3 of PTEN were detected by PCR in 5 of the 10 microdissected samples. PTEN mRNA expression was detected by *in situ* hybridization and the expressions of PTEN protein and proliferating cell nuclear antigen (PCNA) in GCTs were detected by immunohistochemistry. High expression levels of PTEN mRNA was detected only in OLMGCs in 23 of 27 GCT cases. Likewise, high expression of PTEN protein was also found only in OLMGCs in 21 of the 27 GCT cases and the giant cells did not express PCNA. In contrast, the neoplastic stromal cells with high PCNA labeling were almost always PTEN-negative by immunohistochemical staining. These results suggested that high-expression of PTEN in OLMGCs may involve in the formation size of GCTs.

Keywords: PTEN, giant cell tumor, bone, large cell.

INTRODUCTION

GCTs are usually regarded as benign. In most patients, giant cell tumors have an indolent course, but tumors recur locally in as many as 50% of cases. Metastasis to the lungs may occur, regional lymph nodes, mediastinum, skin, scalp and even to the pelvis may ocurr [1]. These mysterious features and the unpredictable behaviors of GCTs stimulate a great deal of interest from those who study bone diseases.

GCTs are primarily comprised of two cell types: OLMGCs and mononuclear stromal cells [2]. Although the primary proliferating cell in bone is the mononuclear stromal cell, many uniformly distributed OLMGCs are also present in osseous tissue. The origin of the mononuclear stromal cells is not fully known, but they are thought to be derived from primitive mesenchymal stem cells [3]. Likewise, the derivation of the OLMGCs is not fully understood. However, gaining an understanding of relationship between OLMGCs and mononuclear stromal cells as well as the derivation of OLMGCs is crucial to our understanding of size formation of GCTs.

The tumor suppressor gene PTEN (named for its homology to phosphatases and tensin and the deletion of its gene on chromosome 10 in human cancers), also called MMAC1 and TEP1 [4], was discovered by three independent research teams virtually simultaneously. PTEN inactivation by a somatic mutation is being identified in an increasing variety of malignant tumors [5-9] at both advanced and early stages of cancer, including prostate carcinoma, glioblastoma, hepatocarcinoma, kidney carcinoma, endometrial carcinoma and gastric carcinoma. More recently, it has been found that PTEN expression is an important factor in determining cell size, and is particularly involved in neuron enlargement and cardiomyocyte hypertrophy [10-17].

Cell size can be affected by DNA content, cellular environment as well as by temperature. Despite a long-standing interest in the mechanism of cell-size control, the underlying genetic networks are still poorly understood. An OLMGC is 15-30 mm in diameter and has traditionally been considered to be a type of large tumor cells in the human body. Although researchers had reported that the osteoclast-like multinucleated giant cells are not the actual tumor cells, a good deal of research continues to focus on their presence in and contribution to GCTs. The relationship between PTEN and OLMGCs is particularly intriguing given the role of PTEN in both carcinomas as well as in cell growth.

Initially, we determined whether PTEN is expressed in OLMGCs. Toward this end, we conducted PCR to detect the expression of PTEN gene in GCTs, indeed

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exons-3, 4 and 5 of PTEN were found in all 8 GCT tissues. High PTEN expression was also detected in OLMGCs by *in situ* hybridization and immunohistochemistry. The findings from the present study suggest, for the first time, that high-expression of PTEN, is closely related to the giant cell formation of OLMGCs.

MATERIALS AND METHODS

GCT Biopsy Samples and Raji Cell Line

Twenty-seven GCTs were obtained from the Department of Pathology, Xijing Hospital, Fourth Military Medical University, Xi'an, China. The mean age of the 27 patients was 30.4 years old (range: 14-65); 16 were males and 11 were females; 88.8% of them were 20-40 years old. Two male and one female patients were under 18 years old, while one male patient was over 60 (Table **1**). Three fresh GCTs were frozen in liquid nitrogen after surgery immediately and stored at –70°C. Twenty-seven GCTs including the three frozen fresh samples were fixed in buffered formalin and embedded in paraffin.

The Raji cell line (ATCC, USA) to be used for PTEN positive control, was cultured at 37˚C in medium 1640 (Gibco, Grand Island, New York, USA) containing 10% fetal bovine serum (FBS)(Hyclone, Logan, Utah, USA) and incubated at 37˚C in humidified atmosphere of 5% CO_2 in air.

Single Cell Microdissection Subsequently

Formalin-fixed, paraffin-embedded tissue was sectioned (5μm) with a microtome (Leica 2135,Germany), and subsequently mounted on slides

Table 1: Clinical Data of the 27 Analyzed GCTs

Case No.	Age/Sex	Grade of GCT	Location
1	21/M	I-II	Left distal tibia epiphysis
2	40/M	II	Right distal femur epiphysis
3	28/F	II-III	Right distal radius epiphysis
4	38/F	I-II	Right distal radius epiphysis
5	18/M	I-II	Os sacrum
6	53/M	I-II	Left distal femur epiphysis
7	46/M	I-II	Os sacrum(T11)
8	45/M	I-II	Right distal shoulder bone epiphysis
9	19/M	I-II	Os sacrum
10	44/M	II	Left distal femur epiphysis
11	17/M	I	Right proximal tibia epiphysis
12	36/M	II-III	Left proximal tibia epiphysis
13	19/F	I	Right proximal fibula epiphysis
14	30/F	II	Right distal femur epiphysis
15	16/M	I-II	Os sacrum
16	28/M	I-II	Left distal shoulder bone epiphysis
17	14/M	II	Right proximal tibia epiphysis
18	65/M	II	Right distal shoulder bone epiphysis
19	29/F	I-II	Left distal radius epiphysis
20	20/M	I-II	Right proximal fibula epiphysis
21	29/M	I-II	Left distal femur epiphysis
22	27/M	II	Left distal shoulder bone epiphysis
23	11/M	I-II	Right proximal fibula epiphysis
24	52/F	II-III	Left distal shoulder bone epiphysis
25	15/M	I	Right proximal tibia epiphysis
26	43/M	I-II	Left distal fibula epiphysis
27	14/F	I-II	Left distal femur epiphysis

with aluminium frames and containing a 2μm thick, clear and sticky membrane. Sections were then stained with haematoxylin and eosin (H&E). The frame slide was turned upside down on a regular glass slide and placed on the microdissection microscope (SL, μCUT, Molecular Machines & Industries, Switzerland). This system uses cold UV laser technology, thus the samples are not exposed to additional heat or radiation that may damage cells. After adjusting light intensity, aperture, and cutting velocity, the laser beam was carefully directed along the borders of the osteoclast-like multinucleated giant cells layer. Using a 20× objective, settings were as follows: aperture 1.2, intensity 45, speed 6. After dissection, the cap was lifted off the slide, the isolated area of tissue (in this case, the OLMGCs)remained adhered to the plastic and lifts off with the cap (Figure **1**). Subsequently, DNA was extracted from all dissected tissue samples using QIAamp DNA Micro kit (Qiagen, Germany) as detailed in the manufacturers instructions, and the resulting DNA was eluted in 50 μl of sterile distilled water and stored at -20°C.

Polymerase Chain Reaction (PCR)

Total genomic DNA was extracted from 5 formalin-fixed, paraffin-embedded GCT tissues, three frozen GCT tissues and the Raji cells as described previously [18], then eluted in 20 μl of sterile distilled water and stored at -20°C. Sequences of the exon3, 4 and 5 primers and PC03/PC04 were shown in Table **1**.

PCR was carried out in a 25μl of reaction mixture containing 10 mM Tris-HCL(pH8.3), 50 mM KCL, 1.5mM $MgCl_2$, 200μM dNTP, 2μM of each primer, 0.5U of Taq polymerase (TaKaRa, Japan), and approximately 100ng of the obtained genomic DNA. The PCR amplification of PTEN exons-3,4, and 5 was carried out in a Eppendorf Mastercycler gradient

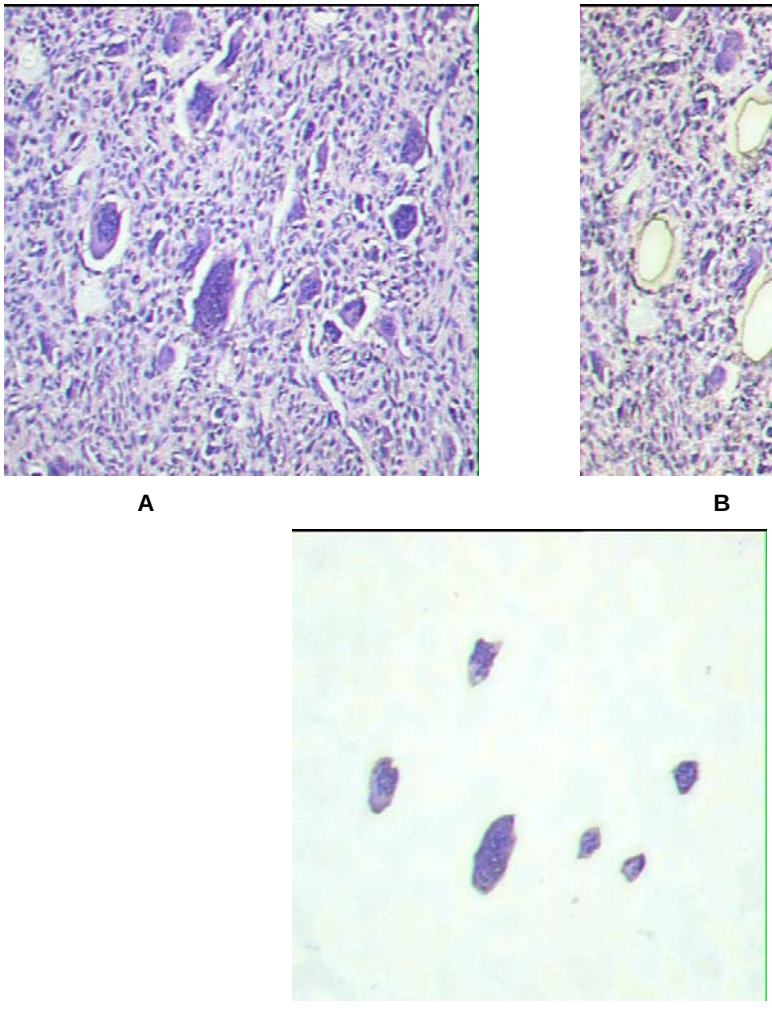

Figure 1: The process of laser captured microdissection in GCTs.
A. Before microdissection; **B.** Following microdissection; **C.** Captured giant cells.

thermocycler (Germany) using the following cycling conditions : initial denaturation at 95˚C for 5 min; 40 cycles with denaturation at 95˚C for 1 min; annealing at 54˚C for 1 min; primer extension at 72˚C for 1min and final extension at 72˚C for 10 min. PTEN exon-3 of microdissected tissues was amplified using the same cycling described above. However, an additional round of 25 cycles (for a total 65 cycles) with annealing at 54 ˚C was founded to be necessary to reliably am plify the small fragment from microdissected samples. The PCR amplification of PC03/PC04 was carried out with the following cycling conditions: initial denaturation at 95˚C for 3 min; 35 cycles with denaturation at 94˚C for 1 min; annealing at 60˚C for 1 min; primer extension at 72˚C for 1min and final extension at 72˚C for 10 min. Distilled water in the place of samples was used as a negative PCR control. The products were analyzed on a 1.5% agarose gels and visualized with ethidium bromide by image analysis software (Gene Genius Image Capture, SynGene, United Kingdom).

In Situ Hybridization

In order to improve the sensibility, 3 oligoprobes were used to detect PTEN mRNA by *in situ* hybridization. The sequences of PTENmRNA–specific oligoprobe are: 5'—ATTGC TATGG GATTT CCTGC AGAAA GACTT—3'; 5'—GGGAG TAACT ATTCC CAGTC AGAGG CGCTA—3'; 5'—TTCTG ACACC ACTGA CTCTG ATCCA GAGAA—3'. The oligoprobes were labelled with digoxin according to the Dig DNA Labeling and Detection Kit protocol (Roche, Germany). *In situ* hybridization was performed following the procedures described by Kriegsmann *et al.* [19]. Briefly, 5μm paraffin-embedded sections were deparaffinized, dehydrated, digested with pepsin for 30 min at 37℃, prehybridized, and hybridized overnight at a concentration of 2 ng/μl probe at 37℃. Tissues were washed in 0.02M PBS(pH7.2-7.6), the PTEN detection was performed by using avidin-biotin-peroxidase conjugated anti-digoxin antibody followed by developing the color with DAB staining kit(Dako, Denmark). A brown-yellow color in the cytoplasm indicated the positive reaction. The digoxin labeled control pBR328 DNA (Roche, Germany) was used as negative control and the pre-hybridization solutions without probe was used as a blank control.

Immunohistochemistry

PTEN and PCNA immunohistochemistry were carried out in all 27 GCTs. The sections were deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval was performed in pressure cooker for 1.5 min at its working pressure in 0.01M sodium citrate buffer (pH6.4) [20]. After blocking, the sections were incubated with anti-PTEN monoclonal antibody (17.A, MS-1601-S1, Neomarkers, USA; dilution 1:100) and anti-PCNA monoclonal antibody(M0437, Antibody Diagnostica Inc,USA; dilution 1:100) overnight at 4℃. After washing in 0.01M PBS (pH7.4), the sections were incubated with EnVision kit (Dako, Denmark) at 37˚C for 1 hour, followed by DAB staining kit (Dako, Denmark).Sections were then counterstained with hematoxylin. Prostate epithelium was used as an external positive control and endothelial cells were used as internal controls for PTEN. PTEN's negative control (X0942, Dako, Denmark) and PCNA's negative control (X0910, mouse normal serum, Dako, Denmark) were used in the place of the primary antibody. PBS served as blank control.

RESULTS

PCR of PTEN-3,4,5 exons and PC03/PC04

PTEN is highly expressed in the Raji cell line, according to Abbott *et al.* [1], thus genomic DNA of Raji cells was used as the positive control for PCR amplification of PTEN. From the crystal structure of PTEN protein, it is understood that a vital domain of the PTEN protein is phosphatases domain which is highly preserved in all metazoans. The majority of this domain is encoded by the exons-3,4 and 5 of PTEN gene, so in the current study the presence of these three exons indicated the existence of PTEN gene very well. Isolation of DNA was confirmed by β-globin PCR by

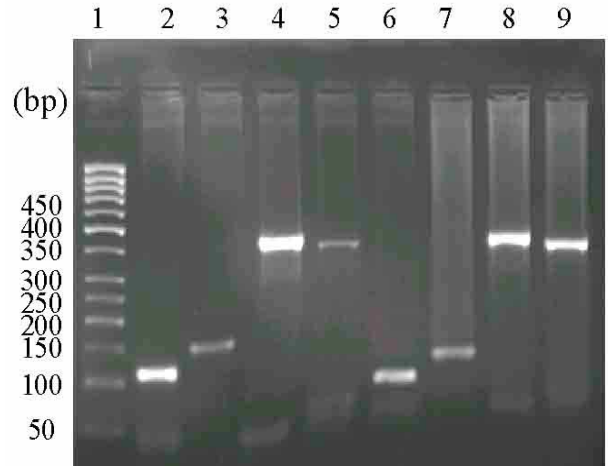

Figure 2: Representative PCR products of the PTEN-3, 4, and 5 exons in GCTs and Raji cells.

1: DNA Marker; **2**: Raji's DNA inner control PC03/PC04; **3-5**: Raji's PTEN-3,4,5 exons; **6**: Sample's DNA inner control PC03/PC04; **7-9**: Sample's PTEN-3, 4,and 5 exons

means of primers PC03/PC04. The patient characteristics of all 8 GCT cases were demonstrated in Table **1**. The 5 GCT samples (No1,2,5,15 and 21) were paraffin-embedded tissues. They were selected for PCR analysis because OLMGCs were abundant within the tissues. The 3 fresh GCT samples frozen in –70℃(No 25,26 and 27) were aslo detected for PCR analysis. Exons-3,4, and 5 of PTEN gene and PC03/PC04 were found by PCR in Raji cell line, the 3 fresh GCT tissues and the 5 paraffin-embedded tissues (Figure **2**). No DNA fragment was amplified from negative control.

PCR of PTEN-3 Exon of Microdissection Samples

It has been suggested that DNA amplification fragment of longer sequences (>250 bp) is inconsistent in formalin-fixed, paraffin-embedded tissue. Furthermore, the quantity of obtained DNA from microdissection cells is much smaller than the amount obtained from whole GCT samples, thus the amplification of small target sequences from formalin fixed tissue was more reliable. Because the resulting PTEN exon-3 is 150bp, in our experiments, only the exon-3 of PTEN gene was chosen for the PCR detection, product of PTEN exon-3. Exon-3 of PTEN gene was detected by PCR in 5 of all 10 microdissected samples (Figure **3**).

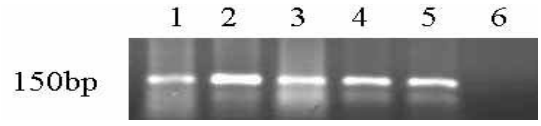

Figure 3: PCR products of PTEN-3 exon of capured giant cells after microdissection.
1--5: PTEN-3 exon of microdissected cells; 6: negative control.

In Situ Hybridization

Strong PTEN mRNA expression was detected in OLMGCs in 23 out of 27 GCT cases, and the positive reaction was demonstrated in cytoplasm of the giant cells, whereas the neoplastic stromal cells contained PTEN weak or no mRNA expression (Figure **4A**). The negative control and blank control showed no positive reactions.

Immunohistochemistry

PTEN protein expression is generally detected in the cytoplasm and nuclei whereas PCNA staining occurs within nuclei. High levels of PTEN protein expression were found in OLMGCs in 21 of the 27 GCT cases, whereas PCNA expression was absent. In contrast, neoplastic stromal cells expressed little or no PTEN expression but high PCNA labeling in all 27 GCTs (Figure **4B**, Figure **4C**). These findings suggest that the stromal cells are capable of proliferation whereas OLMGCs are not. The negative controls and blank control showed no positive staining.

DISCUSSION

GCTs are characterized by an abundance of OLMGCs. Historically, the giant cells were believed to be osteoclasts and the tumor was thus termed osteoclatoma. However, is now understood that the stromal cells induce neoplastic proliferation resulting in the giant cell tumor of bone, and the mechanisms underlying the formation of OLMGCs are not fully understood. The OLMGCs and neoplastic stromal cells in GCTs have similar nuclear morphology [21], which implies that OLMGCs may derive from neoplastic stromal cells.

PTEN has been the subject of a good deal of research in recent years because of its function as a tumor suppressor in various advanced cancers. The PTEN gene is expressed in all eukaryotic cells and is highly preserved in all mammals. Because of it's role as a tumor suppressor, inactivity of PTEN is believed to result in the development of various tumors. The underlying mechanisms may include mutational inactivation of the PTEN gene, a loss of PTEN expression, and/or methylation of the CpG islands of the PTEN promoter [22, 23]. In addition to its role in tumor suppressor, PTEN is also involved in cells apoptosis, proliferation, immigration and signal network [24-26]. PTEN gene encodes a 403-amino acid cytoplasmic protein with homology to the catalytic domain of tyrosine phosphatase and to the cytoskeletal protein tensin and auxilin. Perhaps the most important function of PTEN protein is to dephosphorylate PIP3 and PIP2, encountering the phosphatidylinositol 3-kinase (PI3K) efforts through the PI3K-PKB/Akt signaling pathway.

A novel function of PTEN is its ability to influence cell size. In addition to its function in neuron enlargement and cardiomyocyte hypertrophy, PTEN may also mediate tissue growth, such as the growth of Drosophila's eye cells and wings during development, and may even be involved in the growth of CNS cells in mice [10-13]. The number of OLMGCs in GCTs is very high, and each cell is 15-30 mm in diameter. Thus, it was of particular interesting to determine whether these giant cells express PTEN. In the current study, four

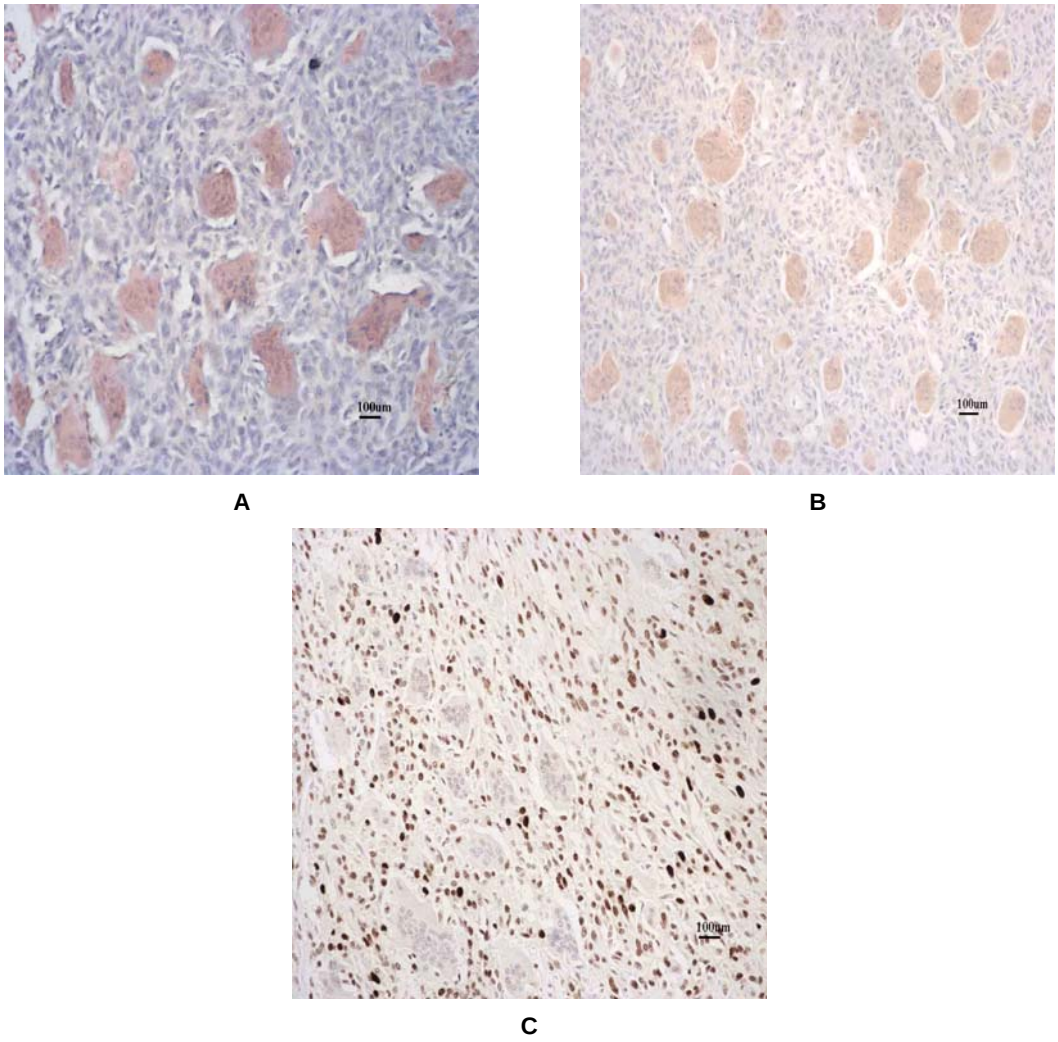

Figure 4: PTEN mRNA (**A**) and protein (**B**) expression in osteoclast-like multinucleated giant cells, PTEN- expression was weak or negative in neoplastic stromal cells of GCTs. Strong PCNA expression (**C**) was observed in neoplastic stromal cells, while osteoclast-like multinucleated giant cells' nuclei in GCTs were negative for PCNA expression.

Table 2: Sequence Specific Primers

Target sequence	Primers
PTEN gene-3 exon 150 bp	5'-ATTTC AAATG TTAGC TCATT TTG-3' 5'-TTTAG AAGAT ATTTC AAGCA TAC-3'
PTEN gene-4 exon 350 bp	5'-GGTGG TGATA ACAGT ATCTA-3' 5'-CTTTA TGCAA TACTT TTTCC TA-3'
PTEN gene-5 exon 310 bp	5'-ACCTG TTAAG TTTGT ATGCA AC-3' 5'-TCCAG GAAGA GGAAA GGAAA-3'
PC03/PC04 110 bp	5'-ACACA ACTGT GTTCA CTAGC-3' 5'-CAACT TCATC CACGT TCACC-3'

independent methods were used to evaluate the expression of PTEN in GCTs. We initially detected the existence of PTEN gene via. Accordingly, Exons-3,4 and 5 of PTEN gene were detected in all 8 GCT cases evaluated by PCR.

However, it remained unknown whether the PTEN-3,4,5 exons were amplified from the OLMGCs or the surrounding background cells, because the multinucleated giant cells comprised only a small percentage of all cells within GCTs and were intermingled with the other cells. Laser microdissection

is a quick and effective method to isolate single cells from complex tissue specimens for molecular analysis. However, most microdissection techniques are rather time consuming and require a high degree of manual dexterity, which limits their practical use. In this study, we used laser microdissection(MMI) to isolate the multinucleated giant cells from the other two cell types present in GCTs and demonstrated the existence of PTEN gene specially within the OLMGCs. Following H&E staining, the multinucleated giant cells were easily identified and harvested. It has been suggested that if the microdissection cells totals fewer than 50, the amplification efficiency will be greatly decreased. In order to ensure the quantity of extracted cellular DNA and to maximize amplification efficiency, we dissected nearly 350 multinucleated giant cells from each GCT sample, equallying nearly 600 thousands μM^2 of dissected tissue. Exon-3 of the PTEN gene was also detected by PCR in 5 of 10 microdissected samples.

Subsequently, in combination with *in situ* hybridization and immunohistochemical methods, we have confirmed not only that PTEN gene is present, but also that it is highly expressed in OLMGCs. Indeed, we believe this is the first report to identify the existence and exprerssion in OLMGCs of GCTs. By contrast, the stromal cells expressed little or no PTEN. The findings suggest that an high-expression of PTEN may involve in the formation and excessive growth of OLMGCs.

PCNA is a 36 kDa molecular protein that is synthesized in early G1 and S phases of the cell cycle. In late S phase, PCNA is prominent in the nucleoli and is also strongly associated with the nuclear regions where DNA synthesis is occurring. It is widely known that PCNA is a useful marker for cell proliferation. In the current experiments, the stromal cells were PCNA positive cells whereas OLMGCs were not. The results were similar to those found by others [2, 27-29]. In cell culture, the stromal cells survived after several passages whereas OLMGCs died off, suggesting that OLMGCs can not proliferate. Backman *et al.* [13] reported that enhanced proliferation could be detected in PTEN-null neural stem cells but not in fully differentiated granule neurons. Furthermore, differentiated neurons lacking PTEN exhibited abnormal cell growth. These authors concluded that the influence of PTEN on cellular proliferation is restricted to mitotic, undifferentiated cells, whereas in post-mitotic, differentiated cells, PTEN maintains the ability to influence cell growth. The hypothesis has since been supported by subsequent experiments. For example, PTEN-null embryonic stem cells displayed an accelerated G1/S progression in their cell cycle, and also they lack ectopic proliferation, ectopic growth was detected in differentiated cells that had withdrawn from the cell cycle [30]. In addition, following transfection with the PTEN-containing plasmid, mutant PTEN glioma cell lines have a G1 arrest during their cell cycle [31]. Based on our findings as well as previous studies, it is clear that OLMGCs are post-mitotic, differentiated cells, allowing for the possibility that PTEN may affect cell growth in this post-mitotic cell type and enlarge the cells.

The theory that PTEN is involved in neuronal enlargement and cardiomyocyte hypertrophy had been previously reported [2, 27, 28]. According to recent data, the possible mechanism may involve PTEN controls cell size *via* PTEN-AKT-PI3K cell signaling cascade. The PI3K-null cell's size decreases and over-expression or activation of PI3K induces excessive cell growth. Because PTEN can counteract the actions of PI3K directly through the dephosphorylation of PIP3, PTEN-null cells lack this regulatory influence of PTEN and ultimately grow excessively large. However, the relationship between PTEN and cell size is particularly complex. Gao *et al.* [32] reported that PTEN can regulate cell growth and proliferation through both PI3K-dependent and PI3K -independent pathways. It indicates that PTEN may control cell growth through other pathways. In our experiment, the PTEN expression level in the OLMGCs were much greater than they were in the surrounding small stromal cells. It remains unclear through which pathway(s) PTEN mediates cell growth in GCTs: the identification of such pathways will elucidate possible therapeutic targets to arrest cell hypertrophy.

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