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Anti-tumor effect of PTEN and its effect on inhibition of the bone tumor through the CD47-SIRPα signaling pathway

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ABSTRACT

This study aimed to explore the correlation between the expression of phosphatase and tensin homolog (PTEN), a tumor suppressor gene, and CD47-SIRPα signaling pathway, and clarify the underlying mechanisms of the bone tumor inhibition effect of PTEN. In this study, August x Copenhagen Irish (ACI) male rats were used, and 100µl of UMR-106 cell suspension (1×10^6 cells) was injected subcutaneously to induce the bone tumor model. The gene expression of PTEN, CD47 and SIRPα of both groups (control and bone tumor model) were analyzed by RT-PCR. For *in vitro* experiments, pEGFP-N1-PTEN plasmid was used to transfect the murine bone tumor UMR-106 and the human bone tumor KRIB cells. Also, we detected the invasiveness of the UMR-106 cells and KRIB cells after transfection. In this study, we observed that the gene expression of PTEN and SIRPα were significantly decreased in the ACI rats with bone tumors in comparison to the control group, however, the expression level of CD47 has been significantly increased. The tumor cells transfected with the pEGFP-N1-PTEN plasmid showed significantly higher levels of PTEN expression, however, the expression level of the CD47 gene has been decreased. Also, the invasion ability of tumor cells has been down-regulated. Also, we observed a negative correlation between the gene expression of the tumor suppressor gene PTEN and the CD47 and SIRPα genes. In summary, based on the anti-tumor effect of PTEN and its effect on inhibition of the bone tumor, it could be hypothesized that this phenomenon might be related to the phosphorylation of the CD47 and SIRPα gene.

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Introduction

Malignant bone tumors, including commonly seen tumors like chordoma, chondrosarcoma, osteosarcoma and Ewing's sarcoma could often form a highly heterogeneous group, it may confer an extremely poor prognosis and lead to a higher rate of metastasis (1-3).

PTEN is a gene-coding sequence in length of about 200 kb that is located on chromosome 10q23 (4), where any mutation or loss of heterozygosity would result in the development of a variety of diseases (5,6). Meanwhile, PTEN has various roles in the development and progression of various tumors, such as breast cancer, gastric cancer, and colorectal cancer (7-9). The loss of function of PTEN in the bone system would bring about the loss of tumor suppressor, which has been found to be relevant to bone malignant tumors, including bone metastatic tumors, multiple myeloma, chondrosarcoma and osteosarcoma (10-12).

When the CD47 (integrin-associated protein, CD47) was first identified, we associated it with integrin αvβ3. Therefore, CD47 is also known as integrin-associated protein (13). As a member of the Ig superfamily, CD47 has an extracellular domain similar to the v-type Ig, five putative transmembrane segments, and a short cytoplasmic tail (14). For instance, CD47 acts as a "self-labeled" marker in murine erythrocytes. The red blood cells that lack the expression of CD47 are rapidly cleared by spleen red marrow macrophages (15). As a transmembrane glycoprotein, signaling protein alpha (SIRPα) is an intracellular signaling protein and accessory factor. It is also an important surface receptor for CD47 (16,17). The binding of CD47 to SIRPα can induce the signal in the phagocytes, including the monocytes, macrophages, dendritic cells and neutrophils (18). The cell cycle axis of CD47-SIRP has been regarded as a negative checkpoint for innate immunity and adaptive immunity, and thus, considered as one of the most promising targets in immuno-oncology (18). Expression of PTEN in the macrophages can activate the macrophages, thereby modulating the innate and adaptive immunity (19,20), but whether this pathway could affect the activity of the CD47-SIRPα signal pathway remains unknown.

In this study, we investigated the correlation between PTEN gene expression and the expression of CD47 and SIRPα and revealed a potential regulatory role of the PTEN gene in the signaling pathway of CD47-SIRPα. Therefore, we investigated the pathological significance of PTEN gene expression and signaling pathway of CD47-SIRPα in bone tumors and peripheral blood as well as its application as a potential biomarker for the treatment or overall prognosis of bone tumors.

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Materials and Methods

Cell culture

Cancerous cells of murine bone, namely the UMR-106 cells, in a mixture with 10% fetal bovine serum were cultured in DMEM medium (American Life Technology). Similarly, the osteosarcoma cell line of humans, namely the KRIB cells, in a mixture with 10% fetal bovine serum were cultured in RPMI-1640 medium. These cells were obtained from the Institute of Biochemistry and Cell Biology, Shanghai, China. The cells were cultured at 37 ° C in 5% CO2.

Model development for the rat bone tumor

In this study, twenty ACI male rats (provided by Laboratory Animal Resources, Chinese Academy of Sciences) aged between 6 and 7 weeks, weighed at 200 ±20 g were housed in an SPF environment, from which 10 rats were randomly selected for establishment of a rat bone tumor model. The bone tumor model was constructed with the migration features of UMR-106 and the tumor susceptibility of ACI rats. Also, it has a very high metastasis rate. In this study, the rats were purchased from the Experimental Animal Center of Shanghai, Chinese Academy of Sciences. The UMR-106 was derived from mouse bone tumor cells. Then 100μl of UMR-106 cell suspension (1×10^6 cells) was subcutaneously injected into the back of the rats.

PEGFP-N1-PTEN plasmid preparation and transfection

The pEGFP-N1-PTEN plasmid was prepared from Huada Genetics Company. In total, 50μl of E. coli containing mixed cells was introduced into 1.5 ml of EP tube for ice bath. Then, 5μL pEGFP-N1-PTEN plasmid mixture (comprising of kanamycin and ampicillin co-expressing gene) was added and incubated on ice for 15 min. Then, 10 ml LB was added to the medium (comprising of kanamycin and ampicillin). The tube was oscillated at 37 °C for 60 minutes. Then, 200μL of the medium was added to the LB plate comprising kanamycin and ampicillin. After smearing, cell culture was performed using a humidity incubator at 37 ° C for 18 hours. Then we selected colonies for monoclonal culture and extracted the plasmid with a QIAGEN plasmid extraction kit. Then we mixed 5μg of the extracted pEGFP-N1-PTEN plasmid with 200μL of the optimized medium opti-mem and 10μL of the transfection solution and added it into the bone tumor cell culture medium for transfection. Plasmid transfection for ACI rats harboring the bone tumors was carried out as follows: The well-prepared plasmids were extracted by using the Tiangen Plasmid Extraction Kit (Tiangen Biotech) and then delivered to Tsingke Biotech (Nanjing, China) for sequencing and preparing the complex of plasmid and lipofectamine. Then, 5 ACI rats harboring tumor tumors were selected randomly to receive the injection of a 2 μL mixture of vector and lipofectamine by using a micro-syringe, while the remaining rats received the injection of normal saline in equal volume.

Real-time quantitative PCR

Isolation of total RNA (RNA was extracted from the bone tumor (tumor-bearing) and normal bone tissues (control) using the Trizol reagent (Bioneer, Korea) according to the manufacturer's instructions. RNA quality was assessed with a NanoDrop 1000 spectrophotometer. Subsequently, an Evolution 201 ultra-differential spectrophotometer was utilized to determine the RNA mass and concentration. Reverse transcription was performed with an Invitrogen reverse transcription kit. Aspirated 1μL of RNA and genomic DNA was removed using 4 x gDNA wiper Mix. To generate cDNA, 4Ml of 5 x qRT SuperMix II was added at 50°C for 15 minutes using the SYBR Green Real-Time qPCR Master Mix kit. The melting curve was analyzed after the end of the reaction to assess the quality of the final PCR product. The base fluorescence was fixed at 0.05 units for calculating the value of the threshold cycle, C(t). The experiment was conducted in triplicates. The average value was calculated and considered as the C(t) value. The value of ΔC(t) was calculated as C(t) - C(t) GAPDH. The n-th increase or decrease ΔΔCt value was referred to as the C(t) GAPDH value. The primers used in this trial were as follows: PTEN upstream and downstream primers were 5'-CAGCCAAGTCTGTGACTFGCCGTAC-3' and 5'-12CGCTCGAGCAGTCGCTGCAACCATCCA-3', respectively. CD47 upstream and downstream primers were 5'-GGCAATGACGAAGGAGGTTA-3' and 5'-ATCCGGTGGTATGGATGAGA- 3', respectively. SIRPα upstream and downstream primers were 5'-GTTTAAGTCTGGAGCAGGCACT-3' and 5'-GCAGATGACTTGAGAGTGAACG-3', respectively.

Detection of bone tumor proliferation with MTT assay

The inhibitory effect of PTEN on bone tumors was verified with an MTT assay. The UMR-106 cells, KRIB cells and those transfected with pEGFP-N1-PTEN plasmid were re-plated onto 96-well plate. In addition, 20ug /ml of MTT (Promega) was added after 24, 48, and 72 hours of incubation under 37℃, respectively, to test the cell viability. After incubation with MTT for 4 hours, aspirate the medium and add 30μL of MTT solubilizing solution to each well. Then after another incubation for 15 min at 37 ° C, 5% CO2, 200μL DMSO was added into each well. Then the cells were oscillated at low speed for 10 min. After calculating well absorbance under the spectrophotometry of 590 nm, the growth curve of each was plotted with reference to the spectrophotometer of 620 nm.

Flow cytometry to evaluate cell apoptosis

Cell suspension from each group was resuspended in 100 μL binding buffer of Annexin and then incubated in the presence of 5 μL of each Annexin V FITC and PI at room temperature for 20 minutes in the dark. Then, 400 μL Annexin binding buffer was added, and the mixture was loaded for the detection of fluorescent signals of PI and Annexin V using the flow cytometer (BD, USA).

Statistical methods

In this study, GraphPad Prism 8.0 software (La Jolla, CA, USA) was used to perform statistical analysis, and the appropriate methods were indicated in the figure legends. A P-value lower than 0.05 was considered to be statistically significant.

Results

The CD47 and SIRPα gene expression were negatively correlated with the PTEN gene

In the ACI rat models of bone tumors, the expression

levels of CD47, SIRPα and PTEN genes were detected by RT-PCR. Also, the same experiment was conducted on the control group. We found that PTEN gene expression was significantly reduced in the ACI rat models of bone tumors in comparison with the control, but CD47 gene expression increased significantly (***P<0.001, Figure 1A, B). In addition, the SIRPα gene level of the bone tumor group significantly decreased in comparison to control animals (****P*<0.001, Figure 1C).

CD47 expression was reduced by the transfection of pEGFP-N1-PTEN plasmid

The bone tumor cells were cultured in vitro and pEGFP-N1-PTEN plasmid was used to transfect the cells. The gene expression of PTEN, CD47 and SIRPα were detected by RT-PCR and compared with that of the control group. PTEN gene expression in the UMR-106 cells was significantly increased after transfection with pEGFP-N1-PTEN plasmid, but CD47 gene expression decreased significantly (***P<0.001, Figure 2A, B). However, no significant difference was observed in the expression of SIRPα gene. These results indicated that the PTEN gene inhibits CD47 expression in cancer cells, while SIRPα expression remained unchanged. To rule out some uncertainties, we performed Sanger-PCR sequencing on the above samples. We found that there were no other DNA mutations affecting the CD47 gene promoter. In addition, no abnormal changes in the methylation of the CD47 gene were observed. Therefore, these results showed that it is possible for CD47 to be up-regulated at the transcriptional level, and that CD47 up-regulation may be correlated with the formation and metastasis of bone tumors.

mRNA expression of CD47 and SIRPα were downregulated by the transfection of pEGFP-N1-PTEN plasmid

To find out whether the bone tumor-bearing rat was physiologically different from the healthy one, we dissected the bone tumor rats to observe the organ morphology, in an attempt to find out whether there are any potential pathogenic mechanisms. In addition to the abnormal tibial morphology, we found abnormal splenomegaly in the bone tumor rats. The abnormal edema of the spleen may result from the destruction of the immune system as well as the abnormalities observed in lymphocytes and macrophages in the spleen. In addition, we observed a significant difference in the spleen size was observed between the bone tumor-bearing rats and the control group (*P*<0.05).

To validate whether PTEN could regulate the development and progression of bone tumors via CD47-SIRPα axis, we transfected the ACI rats harboring bone tumors with pEGFP-N1-PTEN plasmid. Additionally, we found that the transfection could reduce the mRNA expression of CD47 and SIRPα (***P<0.001, Figure 3A, B).

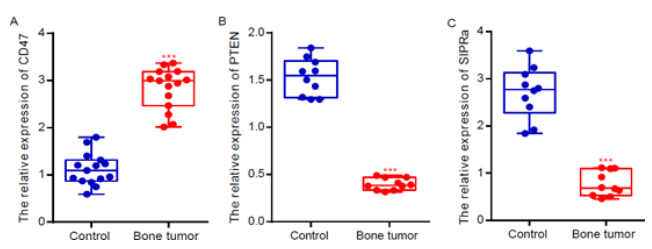

Figure 1. The results of the gene expression of CD47, PTEN and SIRPα by using RE-PCR in tumor-bearing tissues. The gene expression of CD47 and SIRPα were significantly higher in tumor-bearing tissues of ACI rats with bone tumors in comparison to control animals; PTEN mRNA levels were significantly lower in tumor-bearing tissues of ACI rats with bone tumors in comparison to control animals (Unpaired Student's test A-C, ****P*<0.001).

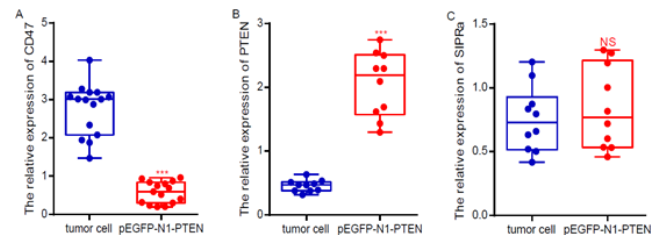

Figure 2. Evaluation of the gene expression of CD47, PTEN and SIRPα after transfectioning with pEGFP-N1-PTEN. The PTEN, CD47 and SIRPα mRNA levels in tumor cells after pEGFP-N1-PTEN plasmid transfection (Mann-Whitney U test A-C, ****P*<0.001).

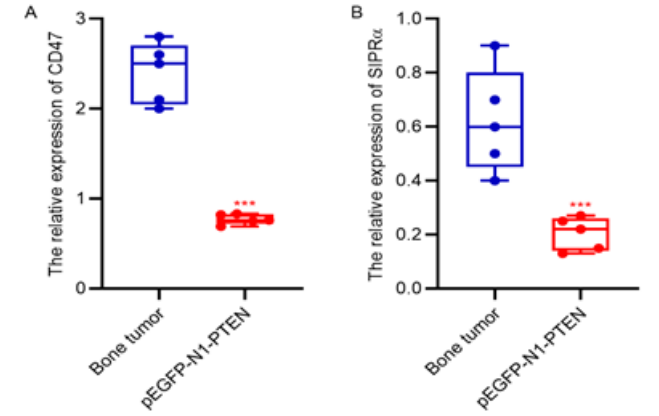

Figure 3. Evaluation of the gene expression of CD47 and SIRPα after transfection with pEGFP-N1-PTEN plasmid. pEGFP-N1-PTEN plasmid transfection downregulated the mRNA expression of CD47 and SIRPα (Mann-whitney U test A-B, *** *P*<0.001).

pEGFP-N1-PTEN plasmid transfection curbs the invasion of UMR-106 cells

To further explore the role of the PTEN gene in tumor inhibition, we examined the invasion ability of tumor cells after transfection with pEGFP-N1-PTEN plasmid. Both murine bone tumor (UMR-106) and human bone tumor (KRIB) cells were divided into two groups, and transfection was performed by pEGFP-N1-PTEN plasmid. The cells were plated onto a cell invasion assay vessel. After 22 hours, we performed cell counting. Results showed that the invasion abilities of UMR-106 cells and KRIB cells were significantly reduced after pEGFP-N1-PTEN plasmid transfection (****P*<0.001 for UMR-106 in Figure 4A, B; ****P*<0.001 for KRIB in Figure 4B). According to these results, the PTEN gene may be involved in the regulation of the invasion ability of bone tumor cells.

Transfection of the PTEN gene suppresses tumor cells and promotes apoptosis

Apart from the inhibitory effect on CD47 gene expression in bone tumors, the tumor suppressor gene PTEN could also inhibit the proliferation of UMR-106 cells and KRIB cells, as assessed with MTT assay (Figure 5A). The time required to reach a certain value of OD was longer in UMR-106 and KRIB transfected cells by pEGFP-N1-

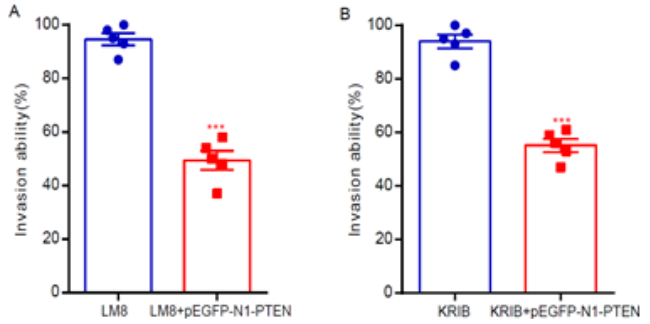

Figure 4. Evaluation of the invasion ability in UMR-106 and KRIB cells. pEGFP-N1-PTEN plasmid transfection reduced the invasion ability of bone tumor cells in vitro in both UMR-106 (A) and KRIB (B) cells (Mann-Whitney U test A-B, *** P<0.001).

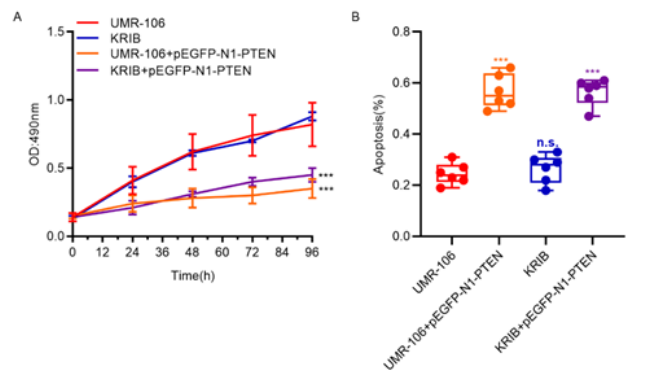

Figure 5. Evaluation the the tumor cells growth rate by using density (OD), measured at 490nm (A) and apoptosis rate (B). The growth of tumor cells was inhibited after pEGFP-N1-PTEN plasmid transfection (A), with an increase in the proportion of apoptotic cells (Mann-Whitney U test，***P<0.001，n.s., no significant differences).

PTEN plasmid in comparison with the control group (Figure 5, *** P<0.001). In addition, we observed a higher apoptosis rate in UMR-106 transfected with pEGFP-N1-PTEN in comparison to UMR-106. On the other hand, our results failed to show any significant difference between KRIB cells in comparison to UMR-106 cells. Also, we observed that KRIB-transfected cells by pEGFP-N1-PTEN have a higher apoptosis rate in comparison to KRIB cells.

Discussion

The expression of the CD47 gene has been well established on the surface of numerous cells, including keratinocytes, hematopoietics, and brain cells (21). CD47 is often combined with SIRPα and thereby involved in multiple cellular functions, including migration of cells (22). In addition, high levels of CD47 are often found in various cancers, which can serve as a cancerous cell marker. Higher levels of CD47 expression on bone tumor cells indicate a self-defense mechanism of these cells, which helps them avoid recognition by the host's immune system. The cellular environment is a more important factor in CD47 expression than the cancer cell numbers. Overall, it could be hypothesized that when the bone tumor cells proliferate, it causes changes in the local microenvironment, which thereby reduces the expression of the PTEN and the SIRPα genes, however, the recently mentioned hypothesis needs further investigation. It not only increases the migration rate of bone tumor cells but also reduces the expression of tumor suppressor genes in patients, favoring the growth of bone tumor cells.

The interaction of CD47-SIRPα is of great importance for the homeostatic regulation of bone marrow cell function, especially the phagocytosis of macrophages (23). Although the role of the CD47 and SIRPα genes in tumors has been elucidated (24), the relationship between the CD47-SIRPα signaling pathway and PTEN tumor suppressor gene still awaits clarification, and little is known about the function of the CD47-SIRPα signaling pathway in tumor patients (25).

In our study, the expression level of CD47 in bone tumor rats was 3-5 times higher than that in the healthy ones. We believe that CD47 was specifically expressed in bone tumors, suggesting that it may be an important biomarker for bone tumors. The decrease of SIRPα gene expression is closely related to the immune system destruction by tumors and the inhibition of the PTEN tumor suppressor gene. *In vitro*, plasmid transfection demonstrated the inhibitory function of PTEN on CD47. These results may indicate that the overexpressing PTEN gene might have a regulatory effect on the CD47 expression. That is, the metastasis and proliferation characteristics of tumor cells are regulated. The more the PTEN expression in tumor cells, the stronger the apoptotic activity in tumor cells in vitro. Therefore, the importance of the PTEN gene in bone tumor suppression is established. On the other hand, evaluation of the physiological status evaluation including exercise capacity, organ status, and tumor development might help us to have better insight into bone tumor models in animal models. However, due to limitations, we were not able to evaluate the physiological status in the current manuscript.

In conclusion, the PTEN gene has a certain relationship with the CD47-SIRPα signaling pathway, especially in the microenvironment where tumor cells are abnormally proliferating. The effects of PTEN inhibition on the CD47-SIRPα signaling pathway tend to favor tumor cell proliferation.

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Authors' contributions
SZ, ZL, QYX, BS, FH, ZCW, and XZ designed the study, performed the experiments and analyzed the data and wrote the draft. All authors read and approved the final manuscript.

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Availability of data and materials
The data supporting the conclusions of this article are available from the corresponding author upon reasonable request.

Ethics declarations
The animal experiment protocol complied with the international guidelines and was also approved by the Ethics Committees of Xiangyang Central Hospital (approval no. 20190235306, date: 2019.7.16).

Consent for publication
Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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