The Effects of IFN- γ and IL-4 Expression on PCNA Expression in Transplanted Bone Tumors

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Research Article

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Abstract

Objective: The rabbit VX2 bone tumor model is an ideal experimental animal model for studying malignant bone tumors, but few studies on cytokines and tumor proliferation. This paper is aimed to study the effect of interferon- γ (IFN- γ) and interleukin-4 (IL-4) on the expression of proliferating cell nuclear antigen (PCNA) in tumor tissue.

Methods: The experiment included 30 Japanese white rabbits divided into an experimental group (n=15) and a control group (n=15) according to the random number method. Peripheral blood and tumor tissue were collected at 1 and 2 weeks after tumor implantation. We used enzyme-linked immunosorbent assay to detect the expression levels of IFN- γ and IL-4 in peripheral blood and PCNA in tumor tissue. After the rabbits were euthanasia, the tissues were taken for pathological examination.

Results: After tumor implantation, the expression level of IFN- γ in the experimental group in the 2nd week was significantly lower than that in the 1st week and 2nd week in the control group (p -value<0.005). In contrast, the expression level of IL-4 in the experimental group in the 2nd week was significantly higher than that in the 1st week and 2nd week in the control group (p -value<0.005). The expression level of PCNA in the experimental group in the 2nd week was significantly higher than that in the 1st week (P <0.005), and the PCNA expression in the 1st and 2nd week in the experimental group was higher than that in the 1st and 2nd week in the control group (p -value<0.005). In the 1st and 2nd week of tumor implantation, the correlation between IFN- γ and PCNA was negatively correlated($r=-0.566$, p -value=0.028; $r=-0.604$, p -value=0.017), while the correlation between IL-4 and PCNA was positively correlated($r=0.583$, p -value=0.023; $r=0.884$, p -value<0.005).

Conclusion: In the VX2 bone tumor model of rabbit, with the extension of time after tumor implantation, there was a negative correlation between IFN- γ and PCNA and a positive correlation between IL-4 and PCNA.

Introduction

The VX2 tumor originated from squamous cell epithelial carcinoma derived from the vulva papilloma of rabbits induced by the Shope virus. The biological properties of VX2 tumors are stable and their proliferation ability is rapid. The VX2 tumor can lead to osteolytic changes, progressive destruction of bone marrow structure, destruction of bone cortex, and periosteum reaction in rabbit bone. The VX2 tumor is similar to the biological behavior of human malignant bone tumor. Therefore, there are significant value to study the growth characteristics of malignant bone tumor [1–3]. T Helper (Th) cells play an important role in tumor immune regulation. Under the action of different cytokines, they can be divided into two subsets: Th1 and Th2. IFN- γ and IL-4 are important factors in Th1 and Th2 cells, respectively. The changes of the levels of IFN- γ and IL-4 will affect the anti-tumor immune response and tumor proliferation [4–5]. PCNA protein, known as cycling, is highly expressed in many tumor tissues and can effectively reflect the proliferation activity of tumor cells. It is one of the recognized cell proliferation

genes [6–7]. However, there are few studies on the relationship between cytokines and tumor proliferation in the rabbit VX2 bone tumor model. In view of this, we conducted the enzyme linked immunosorbent assay (Elisa) quantitative analysis of IFN- γ , IL-4 in peripheral blood and PCNA expression levels in tumor tissue to explore the effect of changes in IFN- γ , IL-4 on PCNA expression.

1 Material And Methods

1.1 Laboratory animals, tumor lines

Thirty Japanese rabbits were divided into two groups by the random number method: the experimental group and the control group, 15 in each group, aged 3 months, male, weighing 2.0-2.5Kg. They were purchased from Beijing Changyang Xishan farm where animal license number was SCXK (Beijing) 2016-0007. According to the relevant regulations of Chengde Medical College, all the experimental animals were kept in cages and fed with standard rabbit feed and sanitary water. VX2 tumor tissue was purchased from Chongqing Mengbo Biotechnology incorporated company. The process of animal use in this experiment has been reviewed and approved by the the affiliated Hospital of Chengde Medical College committee on animal care and use, which follow the requirements of medical ethics (Approval batch number: LL047). The study was conducted in strict accordance with the recommendations of the National Institutes of Health's guidelines for the Care and use of Experimental Animals.

1.2 Main experimental equipment and reagents

X-ray machine (model: OEC9800, USA), optical microscope (Model: Olympus-BX53, Japan), microplate reader of the multi-wavelength measurement system (Model: Multiskan FC, USA), the self-designed and improved channel built-in bone tumor pathological tissue extraction device (Patent number: ZL20141065578.0, Affiliated Hospital of Chengde Medical College) which is made of titanium alloy, ELISA kit was purchased from Shanghai Yubo Biotechnology incorporated company. We used them to detect IFN- γ , IL-4 and PCNA. The other instruments were derived from our laboratory. For example, scissors, leather knives, cotton swabs, cotton balls, etc.

1.3 The establishment of VX2 bone tumor model in rabbit

1.3.1 The preparation of VX2 tumor tissue

Cryopreserved VX2 tumor tissue was resuscitated under -4°C conditions, and then minced tissue into 1 mm×1 mm pieces under aseptic condition, and implanted the tissue piece into the lateral femoral muscle of the posterior limb of the rabbit to prepare the tumor-bearing rabbit. The tumor growth diameter was about 1 cm every week. The tumor-bearing rabbits were successfully prepared after 3 weeks of inoculation. The tumor-bearing rabbits were anesthetized by intravenous injection of 3% pentobarbital (35

mg/Kg) at the ear margin. The rabbits were fixed on the sterile operating table in supine position. We disinfected the skin of the tumor area and laid the sterile hole sheets. We cut the skin and subcutaneous tissue and completely removed the tumor tissue, which was placed in phosphate buffer solution. We also carefully removed the surrounding fibrous connective tissue and necrotic tissue and left the fish-like tissue. Under sterile conditions, the tumor tissue was minced into 1mm×1mm×1mm pieces for spare.

1.3.2 The establishment of rabbit VX2 bone tumor model.

Thirty Japanese white rabbits were anesthetized by intravenous injection of 3% pentobarbital (35 mg/Kg) at the ear margin and then fixed on the operating table in supine position to remove the hair of the hind limbs of rabbits. After routine disinfection, we laid a sterile hole sheet. A 2cm long incision was made on the medial side of the upper tibia. The skin, subcutaneous tissue, and fascia were cut and separated layer by layer. The patellar ligament was split longitudinally to expose the medial tibial plateau. We used a 1.6 mm Kirschner wire to drill a hole parallel to the longitudinal axis of the tibia and perpendicular to the medial bone surface of the tibial plateau. The depth of the hole was about 2 cm. In the experimental group, we placed the prepared 2-3 pieces of tissue which size was 1mm×1mm×1mm, into the bone marrow cavity. In the control group, we took the above operation. But no tumor was implanted. We used bone wax to block the tumor graft hole to avoid tumor extravasation and medullary hemorrhage. We used distilled water to clean the surrounding tissue of the implant hole and reconstructed the patellar ligament. We used silk thread to suture the subcutaneous tissue and skin layer by layer. The incision was disinfected with Iodophor and bandaged with sterile gauze. The rabbits were sent back to the cage after complete recovery. The antibiotics were injected intramuscularly into rabbit three days after operation to prevent infection.

1.4 The device for removing pathological tissue of bone tumor

After the first week, all the instruments were sterilized. The suitable puncture needle was rotated into and through the tibial cortex of rabbit. We pulled out the core of the puncture needle. The hollow metal channel sleeve was screwed into the bone cortex with the channel implantation handle to keep the sleeve position still. The pathological needle was taken and rotated along the channel into the rabbit tibial bone marrow cavity to take out the bone tissue, and the plugging tail nail was screwed into the metal channel sleeve with a screwdriver to block the puncture hole. The puncture site was compressed for 5 minutes and then bandaged. After the completion of the puncture, we used tibial X-ray examination to evaluate the removal device (Figure 1A: hollow metal channel and blocking tailpiece; Figure 1B: adaptable piercing needle; Figure 1C: cross reamer and tailpiece; Figure 1D: channel implantation handle) .

1.5 The detection of peripheral blood and tissue supernatant

1.5.1 The specimen collection

The collection of peripheral blood: in the first and second weeks of tumor implantation, 3ml of ear vein blood was collected from the vacuum blood collection vessel containing coagulant. The collected blood was stood at room temperature for 1 hour, and then the serum was precipitated. After centrifugation at 2000r / min for 10min, the upper serum was collected at -80°C which was preserved in refrigerator at low temperature. The collection of tumor tissue: after the first week of tumor implantation, the tumor tissue and surrounding soft tissue were collected by using the self-designed channel built-in bone tumor pathological tissue removal device. After the second week of tumor implantation, intraperitoneal injection anesthesia (pentobarbital sodium, 40 mg/kg), and ear vein air embolism were performed to kill the rabbits, with the disappearance of palpable chest heartbeat as the death mark. The tumor tissue and surrounding soft tissue were collected by tibial anatomy. After weighing all the collected tumor tissue, a certain proportion of phosphate buffer solution (1mg: 9ml) was added, and the tumor tissue was fully homogenized. After centrifugation at 2000 r / min for 10 min, the supernatant serum was collected at -80°C which was preserved in refrigerator at low temperature.

1.5.2 The Elisa detection of collected specimens

The serum and tissue supernatant sample were take out of refrigerator and we put it in the refrigerator at 4 °C overnight. The kit, serum and supernatant sample were returned to room temperature 30 minutes before the experiment, and the test was carried out in strict accordance with the instructions of ELISA kit. We set up 7 standard solution tubes. Blank holes and sample holes to be tested were set on 96 well enzyme plate. 40ul of sample diluent was added to the sample hole to be tested, and then 10ul of sample to be tested was added. Two double holes were set for each standard and sample. After the enzyme plate label was mixed, the plate was sealed with plate sealing membrane and then incubated at 37 °C(The detection of IFN- γ and IL-4 was incubated for 30min, and the detection of PCNA was incubated for 60min). Then the 30 fold concentrated washing solution was diluted with distilled water for 30 times and then was used for standby. After the completion of incubation, the sealing plate membrane was removed and the liquid was discarded and dried. Each hole was filled with washing solution, and then discarded after standing for 30s. After repeated washing for 5 times, the enzyme labeled reagent was added to each hole(the detection of IFN- γ and IL-4 was added 50ul, and the detection of PCNA was added 100ul). The plate was sealed with plate sealing membrane and then incubated at 37 °C for 30min again. Then, we wash it repeatedly for 5 times, and then dry it. Then add A50ul of chromogenic agent and B50ul of chromogenic agent into each hole. After shaking and mixing, the color was developed at 37°C for 15 min,and then we add 50ul of termination solution into each hole and mix well. Finally, we measure the

absorbance value of each hole in 450nm wavelength and calculated the concentration of IFN- γ , IL-4 and PCNA.

1.6 The histopathological examination

The tumor tissue and surrounding soft tissue collected in two weeks were fixed with 10% formalin, dehydrated, embedded in paraffin, sectioned and stained with hematoxylin eosin(HE). The histopathological morphology was observed under microscope to understand the growth of tumor and verify the value of tumor biopsy. The results of microscopic examination were performed by the same senior pathologist with blind method.

1.7 Statistical data processing

The measurement data are expressed as mean $\pm$ standard deviation and t-test is adopted. The counting data were tested by χ^2 tests. Pearson correlation analysis was used to test the correlation between IFN- γ and PCNA, IL-4 and PCNA. Using SPSS 25.0 (SPSS Inc., Chicago,IL, USA) for statistical analysis, the difference was statistically significant when $P < 0.05$.

2 Results

2.1 The construction of rabbit bone tumor model

All tumor tissues were cut out by bone tumor pathological tissue removal device. And the VX2 tumor tissue was successfully transplanted in 15 experimental rabbits. There was no infection or death in the experiment.

2.2 X-ray of tibial

One week after the tumor was implanted, X-ray showed that the removal device was complete, in good position and no loosening and the bone cortex was complete. There was not obvious bone discontinuity and periosteal reaction. (Figure 2)

2.3 The expression results of IFN- γ , IL-4 and PCNA

In the second week after tumor implantation, in the experimental group the expression level of IFN- γ was significantly lower than that in the first week and the second week of the control group ($t=17.184$, $P < 0.001$; $t=-3.741$, $P=0.001$); In the second week after tumor implantation, in the experimental group the expression level of IL-4 was significantly higher than that in the first week and the second week of the control group ($t=-26.235$, $P < 0.001$; $t=6.279$, $P < 0.001$); There was no significant difference in the expression level

of IFN- γ and IL-4 between experimental group and control group($t=-1.280, P=0.213$; $t=0.952, P=0.349$); In control group, there was no significant difference in the expression level of IFN- γ and IL-4 between the first week and second week after tumor implantation($t=1.187, P=0.255$; $t=1.282, P=0.221$). In experimental group, the expression of PCNA in the second week after tumor implantation was significantly higher than in the first week($t=-24.619, P<0.001$) ; In the first and second week after tumor implantation, the expression of PCNA in experimental group was significantly higher than in control group($t=2.469, P=0.02$; $t=13.031, P<0.001$); In control group, there was no significant difference between the first week after tumor implantation and the second week after tumor implantation($t=0.499, P=0.626$);

Table 1

expression levels of IFN- γ IL-4 and PCNA $\bar{x} \pm s$

group	experimental group	control group
	1st week of implantation/2nd week of implantation	1st week of implantation/2nd week of implantation
IFN- γ $\bar{x}ng \cdot ml^{-1}$	728.9 $\pm$ 28.4 / 698.0 $\pm$ 32.9 ^{ac}	739.8 $\pm$ 16.9 / 736.9 $\pm$ 23.0
IL-4 $\bar{x}ng \cdot ml^{-1}$	169.3 $\pm$ 26.3 / 207.6 $\pm$ 24.7 ^{ac}	161.6 $\pm$ 16.8 / 160.2 $\pm$ 15.6
PCNA $\bar{x}ng \cdot L^{-1}$	73.5 $\pm$ 4.8 ^b / 85.8 $\pm$ 4.0 ^{ac}	69.8 $\pm$ 3.1 / 69.6 $\pm$ 2.6
Within-group 1st week of implantation compared with 2nd week , a: $P<0.05$; between-group 1st week of implantation compared, b: $P<0.05$; 2nd week of implantation compared, c: $P<0.05$.Within-group 1st week of implantation compared with 2nd week , a: $P<0.05$; between-group 1st week of implantation compared, b: $P<0.05$; 2nd week of implantation compared, c: $P<0.05$.		

2.4 The results of correlation analysis between IFN- γ and PCNA, IL-4 and PCNA after tumor implantation

The correlation between peripheral blood IFN- γ and PCNA was negative in the first and second week($r=-0.566, P=0.028$; $r=-0.604, P=0.017$); The correlation between peripheral blood IL-4 and PCNA was positive($r=0.583, P=0.023$; $r=0.884, P<0.001$);

2.5 The results of histopathological examinations

Pathology confirmed that the establishment of rabbit VX2 bone tumor model and one week puncture biopsy was successful. In the experimental group, tumor cells with large size, irregular cell morphology,

large nuclei and obvious nucleoli staining were observed in the puncture biopsy tissue at 1 week of implantation and in the bone cortex at 2 weeks of implantation. The same morphology of VX2 tumor cells was found around the puncture holes of 3 rabbits at 2 weeks of implantation, while no tumor cells were observed in the puncture holes of the rest of the experimental rabbits, which were normal soft tissues.(Figure 3)

A: the tissue within the tibial marrow cavity of the puncture biopsy at 1 week of the implant; B: the tibial bone at 2 weeks of the implant; C: the abnormal tissue around the puncture hole at 2 weeks of the implant; D: the abnormal soft tissue around the puncture hole at 2 weeks of the implant; E: the normal soft tissue around the puncture hole at 2 weeks of the implant.

Discussion

The rabbit VX2 bone tumor model is essentially a non-osteogenic allogeneic transplantation model, which possesses the feature of rapid growth, high tumor formation rate, osteolytic changes, and periosteal reaction similar to human metastatic bone tumor. All of them have been proved in relevant studies to be a large ideal experimental animal for the study of malignant bone tumors [8–9]. Combined with the literature reports and the our group's pre-experiment results, we found that the growth of rabbit VX2 bone tumor model was stable within 2 weeks, and distant metastasis could occur in the third week. Therefore, we choose the first week and second weeks after tumor implantation to avoid the influence of the accuracy of experimental data due to the occurrence of metastasis [10].

The abnormal high expression of PCNA in most tumor tissues with the increase of tumor malignancy and invasion ability indicates that PCNA can indicate the active proliferation of tumor cells and objectively reflect the biological characteristics and growth status of tumor [11–12]. PCNA changes in cells are periodic. The expression of PCNA was not discernible in the G0-G1 phase, but PCNA began to express in late G1 phase and reached the peak in S phase. It finally decreased significantly in G2 / M phase. Moreover, as the auxiliary protein of DNA polymerase δ and the necessary component of DNA replication, PCNA is an important regulator of DNA proliferation to participate in the synthesis of leading chain and post-chain in the early stage of cell proliferation [13–14]. In this study, pathological examination showed that the tumor had invaded the bone cortex in the second week. In the second week, the expression of PCNA in the experimental group was significantly higher than that in the first week and the control group. This is consistent with the relevant research results when VX2 tumor was used to make rabbit models of liver cancer, cervical cancer and breast cancer, which confirmed that PCNA can reflect the growth and infiltration of rabbit VX2 bone tumor tissue [15–17]. It also shows that VX2 tumor is a kind of squamous cell tumor with rapid proliferation, high malignancy and strong replication ability. With the extension of VX2 tumor implantation time in rabbits, the expression level of PCNA is increasing, and the abnormally high expression of PCNA will promote the enhancement of tumor proliferation ability.

IFN- γ is a critical factor in Th1 cytokines. It can regulate the expression level of p21 receptor and promote RB phosphorylation. It can induce G1 phase arrest and inhibit tumor cell proliferation. It also can induce

tumor cell apoptosis through death receptor mediated exogenous pathway and mitochondrial mediated endogenous pathway. And it play a synergistic role with calcineurin B subunit to promote the transformation of tumor associated macrophages into M1 phenotype, which can enhance the anti-tumor activity of macrophages and improve the tumor microenvironment [18–20]. Dighe et al. [21] have proved that when the IFN- γ receptor deficient mice were attacked by different chemicals, and the speed and type of tumorigenesis will increase. The results of this experiment have shown that the expression level of IFN decreased significantly in the second week and the expression level of PCNA increased significantly. In the first and second weeks after implantation, there was a negative correlation between IFN and PCNA- γ , which indicated that IFN- γ could inhibit the proliferation of tumor cells in the rabbit VX2 bone tumor model. However, with the prolongation of tumor implantation time, the body's anti-tumor immune response was inhibited, and the expression level of IFN- γ decreased, which could not inhibit the proliferation of tumor cells and induce apoptosis of tumor cells. And PCNA was inhibited by IFN at the G1 stage of beginning proliferation. In addition, the VX2 tumor has a strong ability of proliferation and replication, which means that when the expression level of PCNA gradually increases, the tumor tissue will continue to proliferate, grow and invade the surrounding tissue [22–23].

IL-4 is the most specific cytokine of Th2, which can inhibit the secretion of IFN- γ by Th1. It reduces the activation of natural killer cells (NK) and the ability of cytotoxic lymphocytes (CTL) to recognize tumor antigens. It makes tumor cells escape the killing effect of specific CTL and act directly on CD8 + T cells which can reduce their toxicity to tumor cells and increase regulatory CD4 + T cells and myeloid derived suppressor cells. It also plays an important role in the proliferation of tumor associated macrophages which can promote the production of M2 macrophages and other suppressive immune cells[24–26]. In related experiments, it was also found that with the increase of IL-4 concentration in the body, the host's anti-tumor immunity would decrease, resulting in the continuous growth of tumor and possible metastasis. When anti-IL-4 neutralizing antibody was injected into the transplanted tumor mice, it was found that reducing the concentration of IL-4 could enhance the anti-tumor immune ability, and inhibit the development of tumor, and prolong the survival time of mice [27–28]. The results of this study showed that IL-4 and PCNA were significantly increased after the second week of tumor implantation, and there was a significant positive correlation between them, which indicated that in the rabbit VX2 bone tumor model, with the extension of VX2 tumor implantation time in the rabbit tibia, after adapting to the body's immune regulation, tumor cells may produce a large amount of IL-4 in the form of autocrine and inhibit the secretion of IFN- γ . It can directly mediate the proliferation of tumor cells through the IL-4 receptor on tumor cells and promote the proliferation of tumor related macrophages. The macrophages infiltrate and phagocytize tumor cells which can protect them from apoptosis, increase the body's negative regulatory factors and constantly inhibit the immune system so that the tumor cells cannot be completely eliminated, the proliferation of tumor cells is no longer limited, and the expression level of PCNA in tumor tissue is increased, so IL-4 may play an auxiliary role in the abnormal expression of PCNA[29–31].

The channel built-in bone tumor tissue removal device used in this experiment is an improvement based on the previously self-designed puncture biopsy instrument and sealing plug removal device, which has obtained the Chinese practical patent [32]. In the course of the experiment, we consider that VX2 tumor

implanted in the tibia needs to adapt to the internal environment of medullary cavity before osteolytic changes which was similar to human metastases occur, so early implantation of the device may not be conducive to the proliferation of tumor cells and affect the accuracy of experimental data and we choose to implant the device after the first week of tumor implantation to reduce the impact on tumor growth as much as possible. Combined with the results of this experiment, the metal sleeve of the device can be stable in the puncture hole, and the position of the metal device is difficult to change. After entering the puncture hole, the surrounding of the puncture hole is isolated. After removing the tumor tissue, the puncture hole is sealed with metal screws. In the whole process, not only the same amount of tissue can be taken out for pathological examination, but also the trauma to the surrounding tissue is small. It can reduce the external infiltration of the tissue fluid in the puncture hole and can reduce the risk of surrounding implant metastasis caused by biopsy which is an effective physical plugging technology. However, there is no grouping comparative experiment in this experiment, and no further imaging and molecular biological detection on the value of the removal device, which needs to be improved in the future experiments.

In this experiment, we found that the expression level of PCNA in the experimental group was significantly higher than that in the control group in the first week of tumor implantation, while there was no difference between the expression level of IFN- γ and IL-4 in the experimental group in the first week compared with the control group in the first week. The correlation between IFN- γ , IL-4 and PCNA in the first week was less than that in the second week which indicates that the changes of early peripheral blood were not enough to effectively reflect the changes of early tumor cell proliferation. Referring to the relevant literature and the results of this study, we believe that VX2 tumor may be implanted in the early tibia of rabbits, and the body's anti-tumor immune response is not completely suppressed, so it can still play a compensatory role in tumor immune regulation. However, as a kind of squamous cell tumor with rapid proliferation and strong replication ability, VX2 tumor has strong adaptability and can proliferate a lot after implantation, so PCNA can be significantly increased in the early stage [10, 33].

In summary, this study showed that in rabbit VX2 bone tumor model, with the extension of tumor implantation time, the expression level of IFN- γ and PCNA was significantly negatively correlated, the expression level of IL-4 and PCNA was significantly positively correlated which indicate that IFN- γ may inhibit the proliferation of tumor cells and IL-4 may promote the proliferation of tumor cells. Therefore, the analysis of the relationship between the changes of the expression level of IFN- γ , IL-4 and PCNA is conducive to the analysis of the immune regulation, tumor proliferation and growth of the animal model, which can provide a basis for the future treatment of rabbit VX2 bone tumor model as the carrier.

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Hospital of Chengde Medical College. All animal experiments comply with the ARRIVE guidelines, and the care and use of

laboratory animals are carried out in accordance with the U.K. Animal (Scientific Procedure) Act, 1986 and associated guidelines, the European Union Directive 2010/63/EU animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH publication NO. 8023, revised in 1978). Informed consent obtained from each participant was written. All protocols are carried out in accordance with relevant guidelines and regulations.

Consent for publication

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor of this journal.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Readers can access the data and material supporting the conclusions of the study by contacting JiaXing Lv at 705057610@qq.com.

Competing interests

The authors declare that they have no conflict of interest.

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Not applicable.

Authors' contributions

JY designed the study. LJX, ZL wrote the main manuscript text. SZY revised manuscript. BLP, XY and LZH analyzed the data.

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Figures

Figure 1

Channel built-in device for pathological tissue extraction of bone tumor A: hollow metal channel and blocking tailpiece; B: adaptable piercing needle; C: cross reamer and tailpiece; D: channel implantation handle.

Figure 2

X-ray of tibia at 1 week of tumor implantation

Figure 3

Pathology of the experimental group (HE, $\times 40$) A: the tissue within the tibial marrow cavity of the puncture biopsy at 1 week of the implant; B: the tibial bone at 2 weeks of the implant; C: the abnormal tissue around the puncture hole at 2 weeks of the implant; D: the abnormal soft tissue around the puncture hole at 2 weeks of the implant; E: the normal soft tissue around the puncture hole at 2 weeks of the implant.