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ORIGINAL ARTICLE

Clinical Value of Differential lncRNA Expressions in Diagnosis of Giant Cell Tumor of Bone and Tumor Recurrence

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SUMMARY

Background: **There is currently no clinical biomarker for the early diagnosis of giant cell tumor of bone. Long-chain non-coding RNAs (lncRNAs) have a wide range of important gene regulatory functions and play an important role in the occurrence and development of various malignant tumors. This study mainly screened the differential expression of lncRNAs in patients with giant cell tumor of bone by gene chip technology, verified the biological function. We tried to establish a reference basis for early diagnosis of tumor and prediction of tumor recurrence.**

Methods: **From September 2018 to September 2019, we randomly selected 20 cases of primary giant cell tumor of bone and 20 cases of recurrent giant cell tumor of bone, and 20 cases of bone trauma tissue. First, the differential expression of incRNAs in the bone tissue of the patients was evaluated via utilizing gene chip technology. The gene chip was Human LncRNA Array v 3.0 (8 x 60 K) was completed by Shanghai Kangcheng Biotechnology Co., Ltd. The DAVID online analysis platform was used to analyze the differentially expressed genes by GO and KEGG pathways. The target lncRNAs were screened; real-time quantitative PCR (qRT-PCR) was used to verify the relative expression levels of target lncRNAs in bone tissue and serum of three groups of patients.**

Results: **Using gene chip technology screening, fold-change (FC) value > 2.0 was used as standard. A total of 1,254 differentially expressed lncRNAs were detected of which 896 were up-regulated and 358 were down-regulated. Additionally, a total of 106 differentially expressed lncRNAs were detected with FC values > 10.0. Among these, 72 lncRNAs were upregulated and 34 lncRNAs were downregulated. We then selected two lncRNAs with up-regulation and down-regulation with the largest fold difference. qRT-PCR analytical results showcased that the expression of AK124776 in bone tissue and serum of patients in the recurrent group was significantly higher than that of the initial group and the normal group. Conversely, for RP11-160A10.2, the expression level in the recurrent group was significantly lower than that in the initial group, and the normal group was the highest; the difference was statistically significant ($p < 0.05$). Finally, we used the expression levels of AK124776 and RP11-160A10.2 in each group as the diagnostic indicators. According to the receiver operating curve (ROC), the accuracy of AK-124776 and RP11-160A10.2 in the diagnosis of giant cell tumor of bone (area under the curve), the AUC was 0.865 and 0.877, respectively; the accuracy of predicting recurrence of giant cell tumor of bone was 0.832 and 0.841, respectively.**

Conclusions: **The early detection of differential expression of lncRNAs in the serum of patients with giant cell tumor of bone is important for the diagnosis of disease and prediction of recurrence. The establishment of stable expression of lncRNAs and simple and easy detection methods are of great value for guiding clinical application.**
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KEY WORDS

giant cell tumor of bone, long-chain non-coding RNA, gene chip, recurrence, receiver operating curve

INTRODUCTION

Giant cell tumor of bone is a common primary bone tumor in clinical practice. Most are benign, but they also show early invasive and metastatic characteristics with poor prognosis [1]. In the early stage, there were no typical clinical symptoms and signs, and the imaging findings were lagging. Most of the patients were treated for pathological fractures and pains and of those 50 ~ 75% were diagnosed at the middle and advanced stage, and the chance of surgical resection was lost [2].Therefore, the search for biochemical markers closely related to tumorigenesis and development is a hot spot in clinical research. Long-chain non-coding RNAs (lncRNAs) have a wide range of important gene regulatory functions and play an important role in the development and progression of a variety of malignancies [3,4]. This study mainly screened the differential expression of lncRNAs in patients with giant cell tumor of bone by gene chip technology, verified the biological function, and tried to establish a reference basis for early diagnosis of tumor and prediction of tumor recurrence.

MATERIALS AND METHODS

Object information

Randomly selected patients from September 2018 to September 2019 included 20 pathologically confirmed cases of giant cell tumor of bone, 20 cases of recurrent giant cell tumor of bone, and 20 cases of bone trauma (normal group). Inclusion criteria: 1. Age: 18 to 75 years old; 2. Patients with giant cell tumor of bone who have not undergone radiotherapy and chemotherapy; 3. Able to obtain bone and peripheral serum without pollution which does not affect the technical analysis of gene chip; 4. Obtain informed consent and perfect clinical data. Exclusion criteria: 1. Combined with other types of malignant tumors; 2. Primary bone and joint diseases, such as rheumatoid arthritis, bone tuberculosis; 3. Autoimmune diseases, infectious diseases; 4. Severe heart, lung, liver, kidney and other organ functions abnormal.

There were 12 males and 8 females, 44 - 73 years old, with an average of 55.6 ± 10.3 years old. The tumor was located in the femur in 13 cases and in the tibia in 7 cases. The maximum diameter of the tumor was 1.5 - 4.5 cm, with an average of 2.6 ± 0.9 cm. There were 11 males and 9 females in the recurrent group, 48 - 74 years old, with a mean of 56.7 ± 12.2 years old. The tumor was located in the femur in 14 cases and in the tibia in 6 cases. The maximum diameter of the tumor was 1.3 - 4.2 cm, with an average of 2.4 ± 0.7 cm. There were 10 males and 10 females in the normal group, 43 - 72 years old, with a mean of 55.1 ± 11.4 years old, and included 13 fractures and 7 joint replacements. There were no differences in gender and age between the three groups. There was no difference in tumor location and maximum diameter between the initial and recurrent groups ($p > 0.05$).

Research methods

First, microarray technology was used to screen the differential expression of lncRNAs in the bone tissue of the three groups. The DAVID online analysis platform was used to analyze the differentially expressed genes for GO and KEGG pathway, and the target lncRNAs were screened. Then real-time quantitative PCR (qRT-PCR) was used. The relative expression levels of target lncRNAs in bone tissue and serum of three groups of patients were verified.

Gene chip analysis

The gene chip was provided and completed by Shanghai Kangcun Biotechnology Co., Ltd. using Human IncRNA Array v 3.0 (8 x 60 K). The main steps are as follows: (1) Purification of tissue specimen RNA: inactivation of RNase by high temperature sterilization, taking 50 mg of specimen and adding 500 μL Trizol extraction reagent (Invitrogen, USA), according to the instructions; centrifugation at 12,000 g for 5 minutes, transfer supernatant to new centrifuge tube add 200 μL of chloroform to the tube and let stand for 3 minutes. Centrifuge again at 12,000 g for 15 minutes. The liquid was divided into three layers, and the RNA in the uppermost aqueous phase was taken, and then, 500 μL of isopropanol was added and mixed for 10 minutes. After centrifugation at 12,000 g for 10 minutes, the supernatant was removed and 75% ethanol (500 μL) was added and centrifuged at 12,000 g for 10 minutes to obtain a white precipitate, i.e., RNA. The purity and concentration were measured and then it was stored at -80°C. (2) RNA labeling and chip hybridization: 5 μg of the RNA sample to be tested was added to the reverse transcription kit Prime Script™ RT Master Mix (Takara, Japan) to synthesize cDNA, which was then labeled with Cy3 fluorescent staining and precipitated with absolute ethanol. The probe is placed in the hybridization reagent, and after being denatured by heating, the hybridization solution is added to the spotting area of the chip, sealed in the hybridization chamber, and hybridized in a warm water bath at 42°C for 16 to 20 hours. (3) Image acquisition and data analysis using an Agilent Scanner biochip scanner (Agilent, USA). (4) Bioinformatics analysis, using the DAVID online analysis platform for GO and KEGG pathway analysis of differentially expressed genes screened by gene chip, wherein the results of GO analysis are represented by p-value, and the smaller the p-value, the difference gene is classified in GO. Chinese and Vietnamese enrichment, $p < 0.05$ is considered statistically significant; GO analysis can suggest that differential genes may be involved in which

gene function changes. KEGG pathway analysis focuses on functional pathways or metabolic pathways that may be involved in the target gene. The molecular composition was displayed in the form of a pathway map. It was calculated that the smaller the p-value, the more abundant the differentially expressed genes appeared in the pathway, and $p < 0.05$ was considered statistically significant.

qRT-PCR quantitative detection

Extraction of tissue RNA and reverse transcription synthesis of cDNA method as described above, using High Capacity cDNA Reverse Transcription kit (Invitrogen, USA); for design of target lncRNAs and internal reference β-actin sequences by Shanghai Kangcheng Biotechnology Co., Ltd., refer to GeneBank application Primer 5 software (Table 1). According to the Power SYBR Green PCR Master Mix Fluorescence Quantitation Kit (Invitrogen, USA), configure 20 μL of reaction system, including cDNA 5.0 μL + upstream and downstream primers 0.5 μL + 2 x SYBR Green PCR Master Mix 10 μL, adding sterile water to a total volume of 20 μL. The amplification reaction was carried out on an ABI-7300 real-time quantitative PCR instrument (Applied Biosystems, USA), and the reaction parameters were pre-denatured at 95°C for 5 minutes, (denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds). A total of 40 cycles were carried out at 72°C for 30 seconds. As a result, the relative expression amount of the target gene was calculated using a gel imaging system (Pharmacia Biotech, USA) and expressed by the $2^{-\Delta\Delta Ct}$ method.

Statistical methods

The measurement data were expressed as mean ± standard deviation using SPSS20.0 statistical software. The comparison between the three groups was performed by single factor ANOVA, and the comparison between the two groups was performed by LSD *t*-test. The count data was expressed by the number of cases (%), and contrasted with χ^2 test. Receiver operating curve (ROC) analysis of serum AK124776 and RP11-160A10.2 expression levels was used for the diagnosis of giant cell tumor of bone and prediction of tumor recurrence accuracy. The results are expressed as the area under the curve (AUC value). $p < 0.05$ was considered statistically significant.

RESULTS

Quality control results of tissue samples

The RNA extracted from all tissue samples was detected by UV spectrophotometer. The ratio of OD260/OD280 was in the range of 1.8 ~ 2.1, and the total concentration was over 0.2 μg/μL. The purity and concentration of RNA were all qualified. After degeneration of RNA, the clear bands were obtained through electrophoresis assay, suggesting that RNA has not undergone significant degradation, meeting the chip detection requirements (Figure 1).

Differential expression profiles of lncRNAs

Screening was done by gene chip technology. A fold-change (FC) value of > 2.0 as set as the standard. A total of 1,254 differentially expressed lncRNAs were detected, of which 896 were up-regulated and 358 were down-regulated. FC values > 10.0 detected a total of 106 differentially expressed lncRNAs, 72 upregulated and 34 downregulated. The differential expression profile (Figure 2), the cluster heat map (Figure 3), the scatter plot (Figure 4), and the volcano map (Figure 5).

Comparison of expression of lncRNAs in three groups

We selected two lncRNAs for each up-regulation and down-regulation with the largest fold difference. The expression of AK124776 in bone tissue and serum of patients in the recurrent group was significantly higher than that of the initial group and the normal group by reverse qRT-PCR. Conversely, for RP11-160A10.2 the expression level in the recurrent group was significantly lower than that in the initial group, and the normal group was the highest. The difference was statistically significant ($p < 0.05$) (Table 2).

ROC analysis to diagnose tumors and predict the accuracy of recurrence

According to the expression levels of serum ak124776 and rp11-160a10.2 in the normal group and the initial group, the accuracy of the diagnosis of giant cell tumor by ROC analysis was 0.865 and 0.877, respectively. According to the serum ak124776 of the patients in the initial and recurrent groups, the accuracy of rp11-160a-10.2 expression as a predictor of recurrence of giant cell tumor of bone was 0.832 and 0.841, respectively (Figure 6, 7) (Table 3).

DISCUSSION

IncRNA is a kind of RNA molecule with a length of more than 200 nt and no protein encoding. It regulates the expression of genes such as epigenetics, gene transcription, and post-transcriptional regulation at various levels and affects cell proliferation, differentiation, and metabolism [5,6]. IncRNA can be highly expressed in a variety of tumor cells, and is easily detected in normal tissues such as brain, heart, lung, stomach, liver, kidney, and malignant tumor cells [7,8].

It is now believed that mononuclear stromal cells are the true tumor cell component of bone giant cell tumor [9,10]. Mononuclear stromal cells are capable of secreting large amounts of cellular chemokines, inducing the recruitment of mononuclear cells or macrophages to differentiate into giant cells. IncRNA plays an important role in the development of bone and cartilage. It can abnormally activate the epidermal growth factor receptor

Table 1. Target lncRNAs and internal reference β-actin sequence and size.

Name	Forward sequence	Negative sequence	Size (bp)
AK124776	5'-ACCTTCTGTAGTTAGTTA-3'	5'-GATAGGCGGAGCGGAAGCT-3'	118
RP11-160A10.2	5'-AGCCTCGTGGAACTTTAGGT-3'	5'-GCAATGTAGCTTCGATTGGA-3'	115
β-actin	5'-GAAATCGTGCGTGACATTAA-3'	5'-AAGGAAGGCTGGAAGAGTG-3'	238

Table 2. Comparison of expression of lncRNAs in three groups.

Group	Number of cases	AK124776		RP11-160A10.2	
		Bone tissue	Serum	Bone tissue	Serum
Initial group	20	0.3265 ± 0.0985 [a]	0.2456 ± 0.0653 [a]	0.4123 ± 0.1036 [a]	0.3362 ± 0.0865 [a]
Recurrent group	20	0.4215 ± 0.1124 [ab]	0.3125 ± 0.1012 [ab]	0.3126 ± 0.0768 [ab]	0.2421 ± 0.0656 [ab]
Normal group	20	0.1235 ± 0.0356	0.0658 ± 0.0124	0.5253 ± 0.1462	0.4321 ± 0.1132
F		56.235	42.538	36.639	25.523
P		0.000	0.000	0.000	0.000

Note: [a] compared with the normal group, $p < 0.05$; [b] compared with the initial group, $p < 0.05$.

Table 3. ROC analysis to diagnose tumors and predict the accuracy of recurrence.

	Index	Accuracy	95% CI	p	Sensitivity	Specificity	Threshold
Diagnose tumor	Serum ak124776	0.865	0.823 ~ 0.912	0.009	85.6%	76.5%	0.1523
	Serum rp11-160a10.2	0.877	0.844 ~ 0.956	0.005	83.2%	72.4%	0.3564
Predict recurr	Serum ak124776	0.832	0.802 ~ 0.897	0.012	82.9%	70.5%	0.2628
	Serum rp11-160a10.2	0.841	0.812 ~ 0.903	0.014	83.6%	67.3%	0.2703

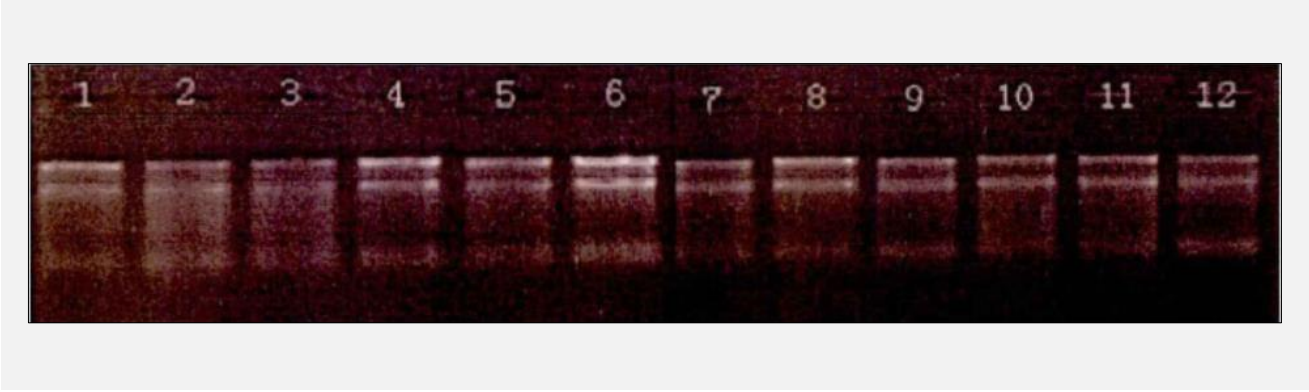


Figure 1. RNA electrophoresis band diagram of three groups of bone tissue (1 ~ 4 is the initial group, 5 ~ 8 is the recurrence group, and 9 ~ 12 is the normal group).

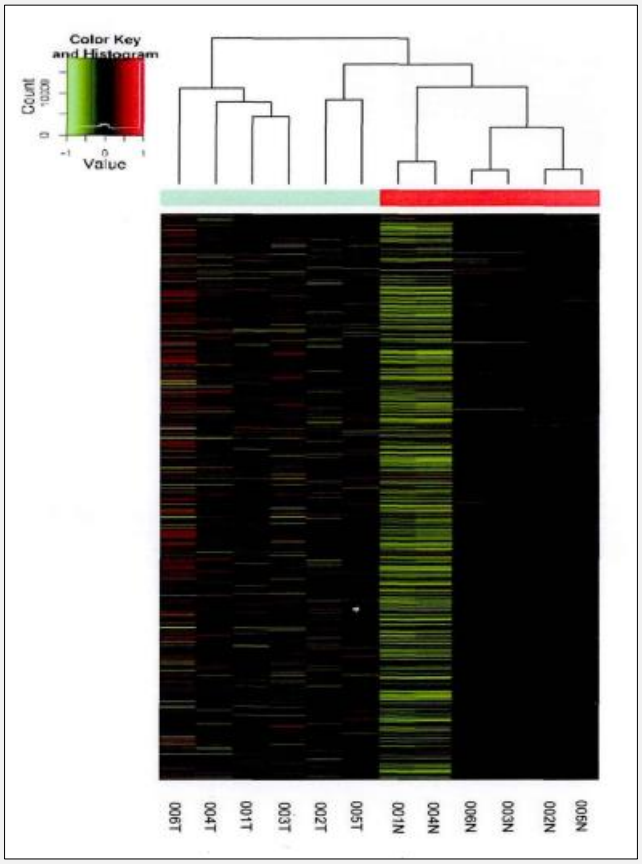


Figure 2. Differential expression profiles of three groups of lncRNAs.

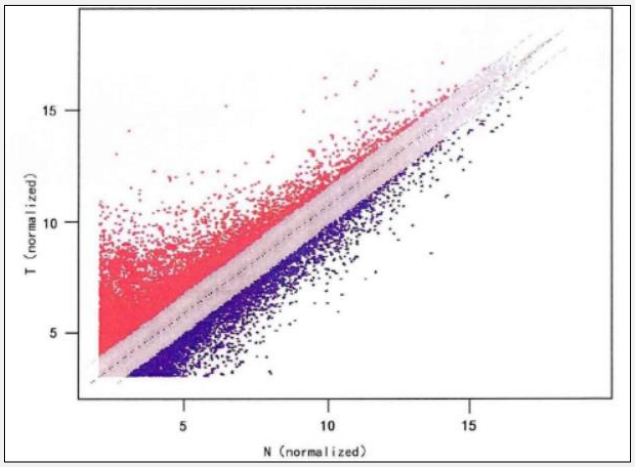


Figure 3. Cluster heat map (The abscissa is the sample name, which is ranked according to the calculated distance value; the ordinate is the probe number or gene name; the greater the difference in the expression of lncRNAs, the further the distance between the positions.)

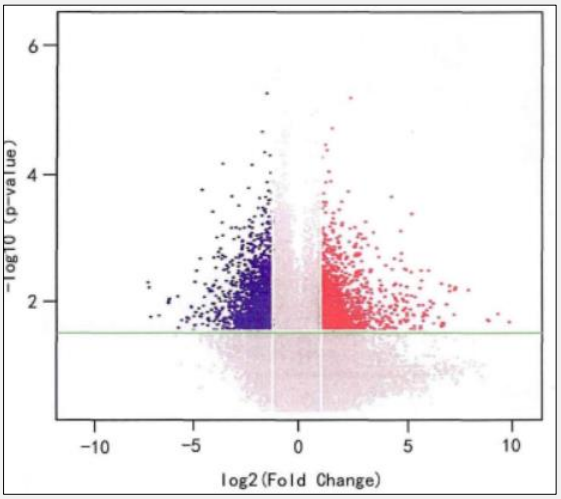
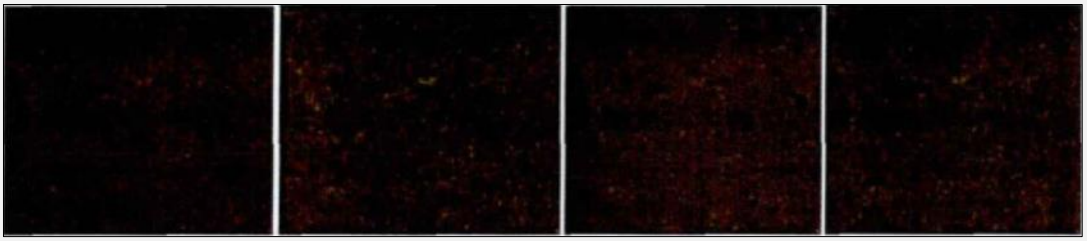
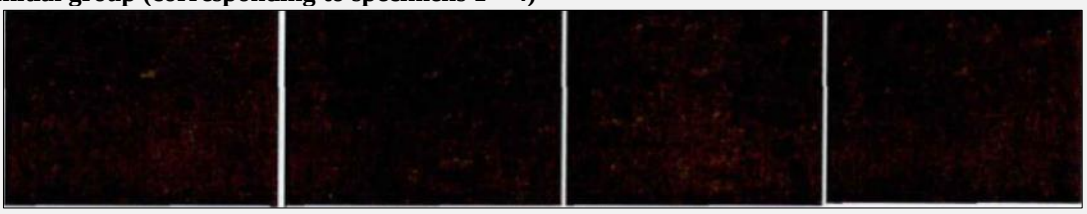


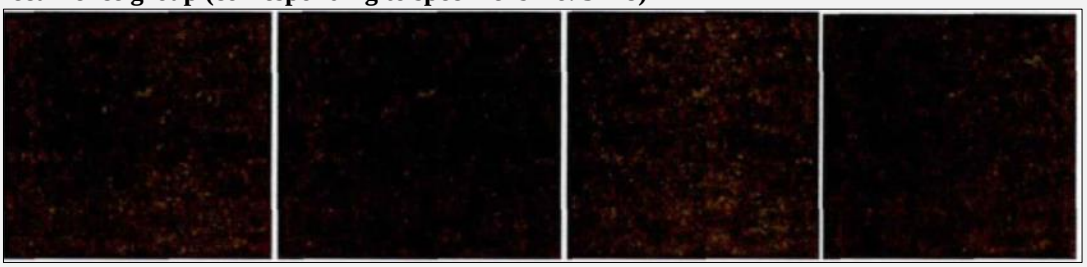
Figure 4. Scatter plot (each point represents a probe point on the chip, determined by the X-axis coordinates (normalized signal values in sample 1) and the Y-axis coordinates (normalized signal values in sample 2); the more scattered the pattern indicates that the difference in lncRNAs is greater.)



Initial group (corresponding to specimens 1 ~ 4)



Recurrence group (corresponding to specimens No. 5 ~ 8)



Normal group (corresponding to specimen 9 ~ 12)

Figure 5. Volcano map (the abscissa is log2, which is the fold-change value; the ordinate is -log10, which is the P value; the up-expressed or down-expressed gene level is signed by "±" symbol.)

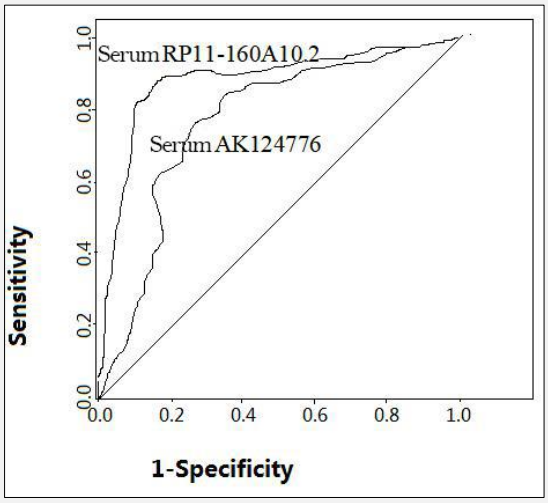


Figure 6. ROC analysis to diagnose the accuracy of the tumor.

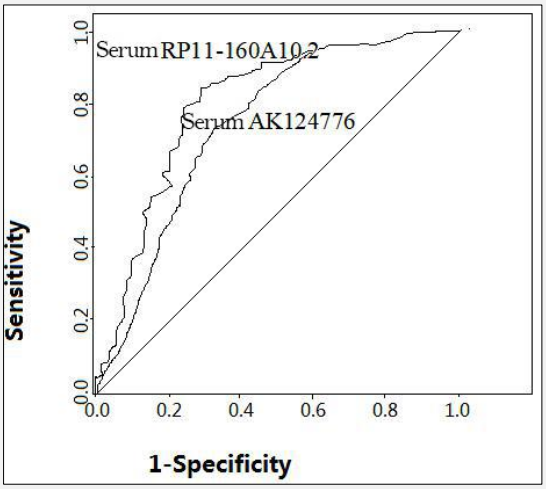


Figure 7. ROC analysis predicts the accuracy of recurrence.

(EGFR) signaling pathway in monocyte stromal cells and participate in the malignant biological functions of giant cell tumor, such as proliferation, primary, invasion, metastasis, and relapse processes [11-13].

The Agilent chip platform has a comprehensive, professional lncRNA database that simultaneously detects differential expression of lncRNA and mRNA. It contains the latest mRNA information in the NCBI RefSeq gene database, enabling simultaneous detection of lncRNA and mRNA on a single chip, exploring the relevance between lncRNA and mRNA. Meanwhile, the lncRNA data information from the authoritative public database and lncRNA classic research literature was excavated on the basis of the chip results [14,15]. The study was

screened by gene chip technology, and a total of 1,254 differentially expressed lncRNAs were detected by fold-change (FC) value > 2.0, of which 896 were up-regulated and 358 were down-regulated; FC values > 10.0 detected 106 differential expressions of lncRNAs, 72 were upregulated and 34 downregulated. A growing number of studies have shown that lncRNA regulates multiple signaling pathways in osteoclast formation, proliferation, differentiation, maturation, and apoptosis, including the RANK/RANKL/OPG pathway, the MAPK pathway, and the PPAR-γ pathway. A large number of new blood vessels and highly expressed angiogenic factors in giant cell tumor of bone induce and recruit mononuclear cells derived from monocytes or macrophages and are important chemotactic factors for fusion and differentiation to form multinucleated giant cells [16,17]. Angiogenesis is an important pathophysiological process in the development and progression of giant cell tumor of bone. LncRNA-MEG3 may block the malignant progression of tumor by inhibiting angiogenesis [18,19].

We selected two lncRNAs for both up-regulation and down-regulation from the largest fold difference. The expression of AK124776 in bone tissue and serum of patients in the recurrent group was significantly higher than that of the initial group and the normal group by reverse qRT-PCR. Conversely, RP11-160A10.2 the expression level in the recurrent group was significantly lower than that in the initial group, and the normal group was the highest. The difference was statistically significant ($p < 0.05$). The most significant energy metabolism abnormality of tumor cells is aerobic glycolysis (Warburg effect relationship), which is closely related to the malignant phenotypes such as tumor cell survival, proliferation, angiogenesis, and invasion [20,21]. AK124776 and RP11-160A10.2 are important substances regulating tumor cell material-energy metabolism. PI3K-Akt signaling pathway is an important in the EGFR signaling pathway, which plays an important role in the malignant transformation of cells and can reduce tumor resistance. The PI3K-Akt signaling pathway can be activated by AK124776 and RP11-160A10.2, which promotes osteoblast proliferation and attenuates its activity to promote osteoblast differentiation [22,23].

Finally, we used the expression levels of AK124776 and RP11-160A10.2 in each group as the diagnostic indicators. According to ROC analysis, AK124776 and RP11-160A10.2 diagnosed giant cell tumor of bone and predicted the recurrence of osteomyeloma. Sexuality, once again confirmed that detecting differentially expressed lncRNAs is important for diagnosing disease and predicting recurrence.

In summary, early detection of differential expression of lncRNAs in patients with giant cell tumor of bone is important for diagnosing disease and predicting recurrence. Establishing stable expression of lncRNAs and simple and easy detection methods are of great value for guiding clinical application. The next step is to investigate the effects of AK124776 and RP11-160A10.2 on the cell growth characteristics of bone giant cell tumor and the regulation of PI3K-Akt signaling pathway from the level of cell or animal model, and provide evidence for the efficacy of monitoring and prognosis of giant cell tumor of bone.

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Declaration of Interest:
None.

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