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Inhibitory Effects of Targeted Regulatory LKB1 Gene on the Proliferation and Invasion of Osteosarcoma Cells

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KEYWORDS Inhibition. Osteosarcoma Cells. Proliferation and Invasion. Targeted Regulatory Genes. Osteosarcoma Cells. Proliferation and Invasion

ABSTRACT In this study, Immunodeficiency Nude Mouse Osteosarcoma Xenograft Model was subjected to the drug intervention to explore the effect of bexarotene on the proliferation and invasion of osteosarcoma cell lines in vitro. The inhibitory effects of targeted regulatory genes on the proliferation and invasion of osteosarcoma cells were studied through various in vitro experiments include bioinformatics combined with tissue microarray research, transcription factor prediction combined with co-expression analysis to predict the transcription factor of targeted regulatory genes in osteosarcoma. The RXR protein family, LKB1, AMPK pathway, and mTOR are closely related to the body's immune regulation. The oral administration of Bexarotene could inhibit the proliferation and able to up-regulate the expression of LKB1 gene in living osteosarcoma tissue. The xenograft model of immunodeficiency nude mice used in this study was reason for reduced the potential immunoregulatory effect of drug targeted LKB1 therapy to a certain extent. However, overexpression of LKB1 in vivo, and combined immunotherapy may become an important immunotherapy approach for osteosarcoma. LKB1 targeted therapy can potentially be used as one of the alternative treatments for mTOR inhibitors.

INTRODUCTION

Osteosarcoma brings a great social and economic burden, but its diagnosis and treatment progress has not been significantly improved in the past 30 years (Corre et al. 2020). Many new treatments have been applied in the basic and clinical research of osteosarcoma. Diagnosis and treatment of osteosarcoma, panoramic view of osteosarcoma genes, and the progress of osteosarcoma small molecule therapy and immunotherapy have been evolved in past years (Arif et al. 2019; Chang et al. 2020). Potential application value and significance of the less studied AMPK pathway and its upstream LKB1 gene in the treatment of osteosarcoma have been explored in recent research (Evangelisti et al. 2020; Huang et al. 2020). Gene chip and high-throughput sequencing technology provide a fast channel for tumour research. The high mutation of the focal gene sequence is closely related to the occurrence and development of osteosarcoma (Liang et al. 2020). At the same time, the larger multi-omics database TARGET provides a series of sequencing data sets including clinical information, transcription, methylation, CAN, miRNA, etc. Existing studies at the gene level indicate that p53 deletion and Rb gene mutation are always the most common and high-incidence genetic changes in osteosarcoma (Jin et al. 2019; Minami et al. 2020).

However, the recent whole-genome sequencing results showed that the unique chromosomal instability of osteosarcoma showed a new mutation pattern. There is evidence that about 33 percent of primary osteosarcoma have chromosomal mutation breakage and recombination, and all tumours. The incidence of chromosome mutation breakage and recombination is only 2-3 percent (Zhao et al. 2019). Besides, the changes in gene copy number usually affect larger regions of the genome, so it is usually impossible to use the amplified or missing data of these genes alone to identify specific driving factors in the pathogenesis of osteosarcoma (Wright et al. 2019). Also, some studies that attempt to use the data of gene copy number variation, mRNA expression, and methylation of human tumours to discover driver genes have identified a limited number of candidate genes, and there is almost no overlap between the results of these studies (Lemos et al.

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2020). These findings indicate that there may be many low-frequency driver gene changes in osteosarcoma. Genetic changes osteosarcoma high instability of the complexity of the pathogenesis aspect tips osteosarcoma, while allowing the driver to find the group osteosarcoma because more difficult, on the other hand, lead to a lack of suitable osteosarcoma biomarkers to predict and treat Prognosis, and lack of specific targeted therapy drugs (Corre et al. 2020). It is necessary to find the driving genes and signal pathways for the development and metastasis of osteosarcoma. The discovery of LKB1 established a direct relationship between tumourogenesis and energy metabolism. LKB1 also functions by forming heterogeneity with STARD protein and MO25 protein (Zhang et al. 2020). Animal experiments show that LKB1 is the most important AMPK pathway activator in most tissue cells. The other activator CAMKK2 also be prepared by the phosphorylation of AMPK of Thr172 whereas the activation site AMPK pathway. In LKB1 knockout cells, CAMKK2 can maintain a small amount of AMPK pathway activity. This finding explains why there is still a small amount of AMPK activity in a variety of tumours lacking LKB1. LKB1 was first reported in Peutz-Jeghers syndrome, LKB1 mutation and a deletion of a gene is PJS important cause of the disease, but worth noting. PJS patients have a higher incidence of tumours (Wang et al. 2019). Not only does the gene mutation of LKB1 exist in PJS, but there are also significant expression deletions or gene mutations in LKB1 in a series of tumours including non-small cell lung cancer, liver cancer, and prostate cancer (Yamaguchi and Murata 2020). The loss of LKB1 also led to a significant decrease in the responsiveness of tissue cells to AMPK activators. Animal studies also found that the loss of LKB1 was accompanied by a significant inhibition of the AMPK pathway (Wang et al. 2019).

Objectives

Based on the above reasons, this study intends to study expression of LKB1 gene in osteosarcoma and explored the feasibility of drug intervention targeting LKBI to inhibit the proliferation and invasion of osteosarcoma.

MATERIAL AND METHODS

Animal Model and Staining

This study used constructed LRP1-SNRNP25 xenograft animal model fusion gene and was observed in vivo LRP1-SNRNP25 fusion gene therapy on tumourogenicity and metastasis ability of osteosarcoma cells. The lentivirus-infected osteosarcoma cells were inoculated into the ventral side of the right hind leg of nude mice by subcutaneous implantation, and the changes in tumour volume were regularly observed and measured, and further detected by immunohistochemical analysis. The expression of LRP1-SNRNP25 and the expression of Ki-67 was used to evaluate the effect of LRP1-SNRNP25 on the proliferation activity of osteosarcoma cells in vivo. The method of analysis detects the expression of LRP1-SNRNP25 on the downstream target genes of osteosarcoma in the two groups of tumour tissues, verifies in vivo the downstream molecular pathways that the LRP1-SNRNP25 fusion gene plays a biological function in osteosarcoma, and uses specific Nude mice were treated with sex inhibitors to verify their therapeutic effects. On the other hand, lentivirus-infected osteosarcoma cells and empty vector were injected into nude mice through the tail vein, and then the nude mice were sacrificed uniformly. The nodules in the liver were counted and compared, and the lungs and liver were tissue-embedded and then hematoxylin-eosin (HE) staining was performed to confirm the nature of the nodules.

Sequencing Data

It takes the supernatant and marks the volume of the supernatant, and 1/10 time the volume of sodium acetate and 2 times the volume of precooled absolute ethanol, and gently shakes for 5 min. A white precipitate is seen at the bottom of the tube (D´Acunto et al. 2019). The researchers placed the EP tube upside down on sterile gauze and dry the excess water for about 2h and added an appropriate amount of TE solution to dissolve the white precipitate, mix well to obtain the DNA stock solution, and place it in a refrigerator at 4°C overnight. It used TE solution to dilute the sample fold, dissolve it for half an hour, measure the DNA concentration with an ultraviolet-visible

spectrophotometer, and place it in a refrigerator at -80°C for later use. According to the fluid whole-genome sequencing, specifically 150-200ng/L, the sequencing data were analysed.

Bioinformatics

This study used a marker pen to draw horizontal lines evenly along the ruler on the back of the good cell culture plate, approximately every 0.5-1cm. The horizontal lines traverse the position of the culture holes, and each hole passes through at least 5 lines. A good single-cell suspension is cultured in the culture well for 48 hours. After the culture time is up, the researchers scratch the cell culture plate to remove part of the cells. To make the traces regular and easy to observe, it used a ruler as a reference. The scribing line should be as straight as possible and perpendicular to the horizontal line on the back of the culture plate. After scribing, the cells at the line have fallen off the culture plate and can be washed repeatedly with phosphate buffer to wash away the marked cells. The researchers added a serum-free medium to the cell culture plate and incubate in a carbon dioxide incubator for 48 hours. It observed pictures under a MOTIC inverted microscope, calculated the distance between the cells on both sides of the scratch, and takes the average value. It used IPP software for data to perform analysis and processing.

Transfection

Upper chamber gel of the Transwell chamber was prepared for the preparation and mixing process at a low temperature of 4°C. The centrifuge tube for mixing needs to be pre-cooled. The prepared gel solution was injected into each transwell cell in turn, and the amount added to each cell was 100. At this time, you can suck out the remaining liquid outside the jelly in the cell, and then rinse the cell repeatedly with phosphate buffer to ensure that the residue is washed away the unregulated solution. After 48 hours of transfection of the cells with the previous experimental method, each well of the cell culture plate was added with 4% paraformaldehyde-containing PBS cell fixation solution, 50ul was added to each well and incubated at room temperature for 30 minutes. After the culture is completed, the fixative needs to be removed first, and then the cells are washed repeatedly with phosphate buffer. The final concentration of the staining solution is 20ìM. When filling the staining solution, it needs to pay attention to making the staining solution completely cover the sample. The staining solution was removed, and the cells were repeatedly washed with phosphate buffer. The washed cells were observed under a microscope, and the fluorescence excitation wavelength was set to 460nm. MG-63 cells were transformed with miR-363/NC in a 6- well plate as before. Before the flow cytometry test, the cells are prepared first. The cell suspension to be tested is centrifuged at 1000 pm, and the concentrated cell pellet is taken out. After staining with the staining solution, the cells are resuspended in the cell culture medium and beaten. After dispersing the cells, incubate for 15 minutes in the dark, and detect the apoptosis of the cells with a flow cytometer. In the well plates MG-63 fine cells transformed with miR-363 before the same, when the cell cycle, the cell concentration starts with the operation portion to detect apoptosis, except that cells were resuspended at -20C precooled 70 percent ethanol solution. The cells are stained with the staining solution. After 15 minutes of dark staining, the cell cycle is detected by flow cytometry.

Real Time PCR (qPCR)

qPCR experiment data were processed according to the Ct method. The researchers calculated the difference between the Ct value of the target gene and the Ct value of the internal control. The second step is to calculate the Ct of the target gene in the test sample and the target in the reference sample. In the case of no reference sample, the sample with the largest Ct value is selected as the reference sample for calculation. The relative sample is obtained the initial template amount average standard deviation. To reduce errors, when experimental operation, each the same experiments were performed in triplicate. The results obtained with the average of the three values standard deviation expressed using t-test, one-way analysis of variance, or two-way analysis of variance Compare the differences between two or more groups. All data were statistically processed with SPSS19.0 software. $P<0.05$ was considered statistically different.

RESULTS

Targeted Regulatory Gene Constructions and Identification Results Analysis

The recombinant plasmid undergoes a transformation, single-clonal selection, and plasmid extraction. The LRP1-SNRNP25 overexpression recombinant plasmid was sequenced. The sequencing results were compared with the gene sequences of LRP1 exon 1-8 and SNRNP25 exon 2-5 published by NCBI using Blast. The results showed that the sequence homology was 100 percent. This result proves that the overexpression stable line was successfully constructed and can be used for subsequent experiments. The vector used for the LRP1-SNRNP25 overexpression plasmid kept in the researchers' laboratory is pc DNA3.1, while the vector used for LRP1, SNRNP25, and the empty vector plasmid is pCMV6-Entry, which is convenient for fusion proteins to ensure that the vectors used in the experimental group. In the experiment, the researchers used cells in the logarithmic growth phase with good growth and then transfected with Lipo3000. As shown in Figure 1, the SNRNP25 protein levels showed each group stably expressing cell lines were appears unregulated corresponding protein levels. This part of the experiment uses the public data sets of three independent centres, such as E-MEXP-3628, GSE12865, and GSE42352. The R2 online analysis website is used to obtain the original data of LKB1 expression in the three data sets, and the t-test is used to calculate the significance of the difference. As shown in Figure 2, the gene expression of LKB1 in human osteosarcoma tissue was significantly lower than that of normal human bone tissue ($p<0.0001$), and the expression of human osteosarcoma LKB1 was lower than that of the HOB cell line which compared with the non-tumour origin. The hob cell lines, and osteosarcoma cell lines have significantly lower expression of LKB1 ($p=0.0001$).

To clarify whether the LKB1 protein is the same as the LKB1 mRNA in human osteosarcoma samples, the expression of LKB1 protein is as significant as that of LKB1 mRNA. In this experiment, tissue microarray Immunohistochemical staining was used to detect the expression of LKB1 protein in human osteosarcoma samples and healthy bone tissues. As shown in Figure 3, the results show that the expression of LKB1 protein in os-

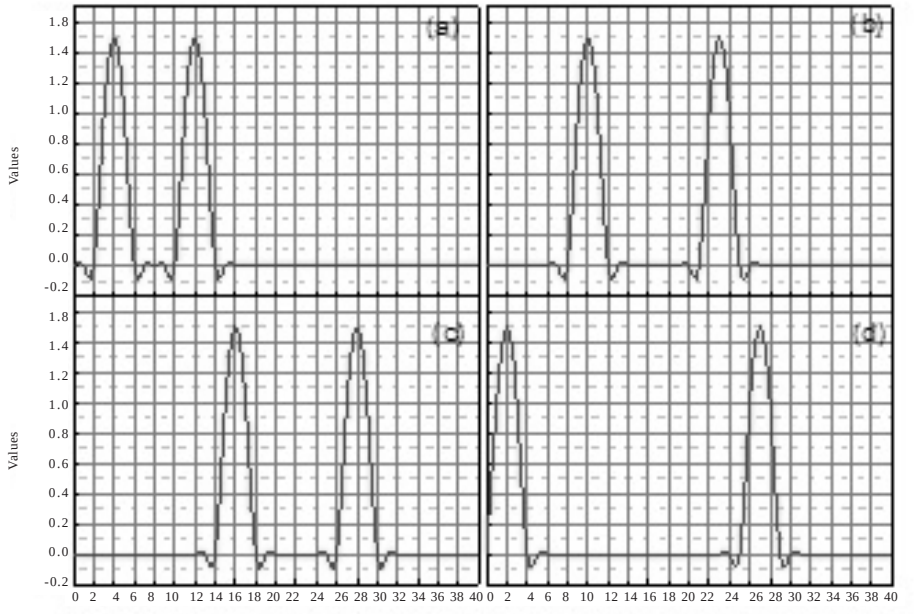

Fig. 1. Electrophoresis diagram of the ligation product of gene sequence after digestion

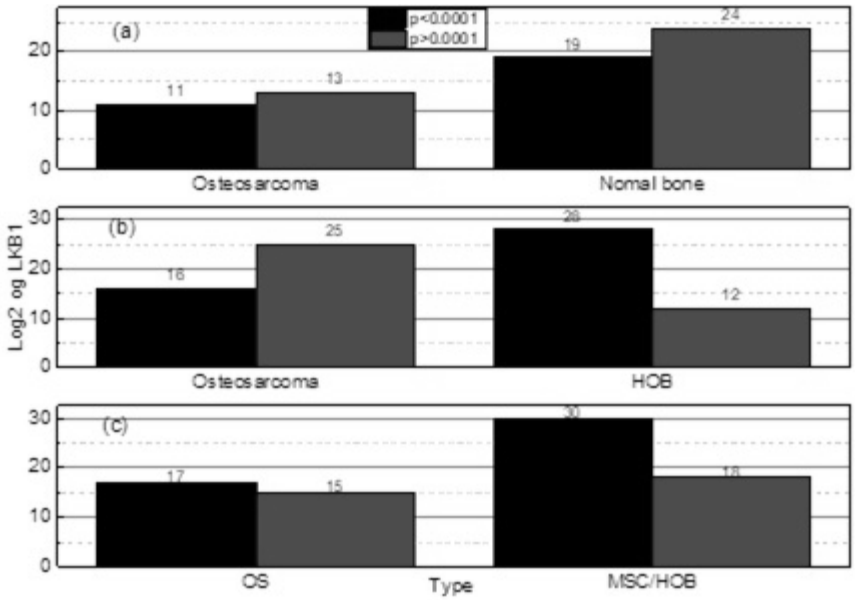

Fig. 2. Gene expression in human osteosarcoma and various cell lines

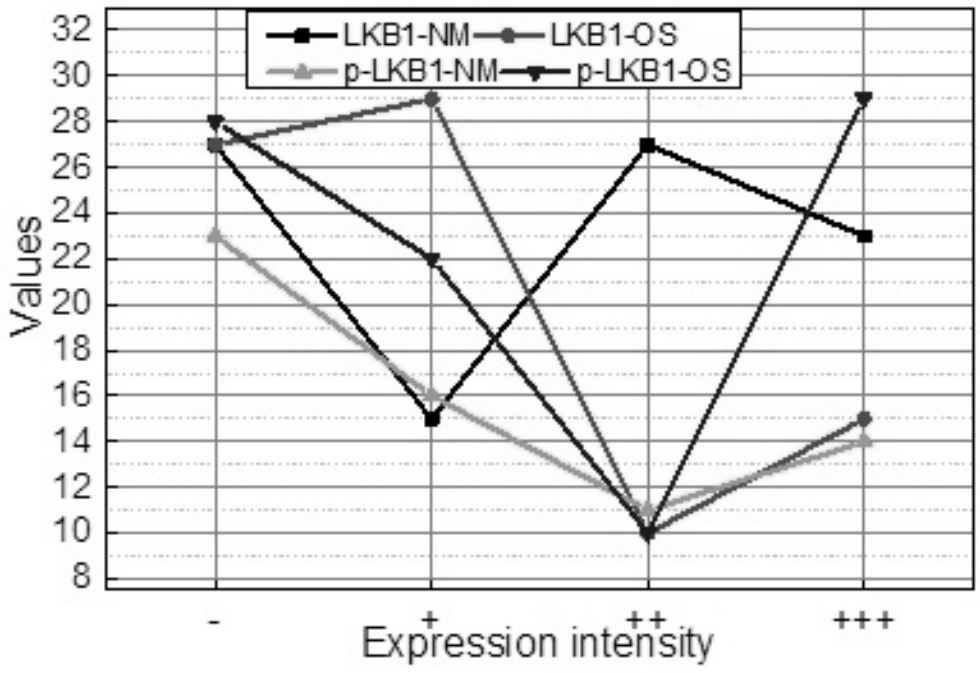

Fig. 3. Immunohistochemical staining sample

teosarcoma is lower than that of healthy bone tissue. The positive and strong positive expression rate of LKB1 in osteosarcoma tissues was 12.5 percent, of which no cases were strongly positive, 5 cases were positive, and 9 cases were weakly positive. The positive and strong positive expression rate in normal bone tissue was 63.7 percent. The difference in the positive expression of LKB1 protein between the two samples was statistically significant (P<0.01). To clarify the role of LKB1 gene overexpression in living osteosarcoma, we used lentivirus-packaged LKB1 overexpression plasmid LV-STK11 and the control plasmid CON335 to translate M-HOS cells. Western blot and immunofluorescence were used to detect the overexpression effect of the LKB1 gene. As shown in Figure 4, the results showed that the expression level of LKB1 protein in M-HOS cells was significantly increased, and the difference between the two groups was statistically significant (P<0.05).

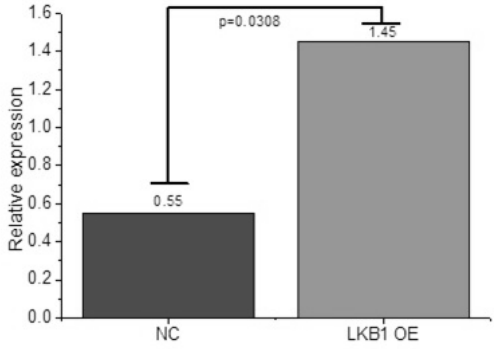

Fig. 4. Protein expression in the two groups

Analysis of the Results of Osteosarcoma Cell Proliferation and Invasion Inhibition

The studies showed that the stably transformed LKB1-OE and NC group M-HOS cell lines were successfully constructed. To clarify whether LKB1 affects the growth of osteosarcoma in vivo, this part of the experiment uses BALB/c to conduct tumour formation experiments in vivo xenotransplantation. The results show that the tumour model building was observed 6 weeks later. The results are shown in Figure 5. The tumour volume of the LKB1-OE group was significantly smaller than that of the NC group, and the tumour weight of the LKB1 group was lighter than that of the NC group (Xu et al. 2019). The difference between the two groups was statistically significant (P=0.02). There was no significant difference in weight between the two groups.

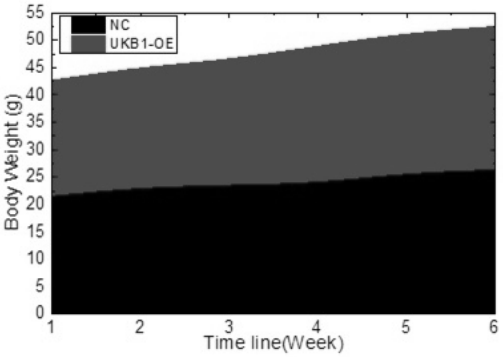

Fig. 5. Growth results of osteosarcoma

Combining the results of the previous part of the experiment, there is a positive correlation with the LKB1 gene. At the same time, transcription factors that can bind to the LKB1 promoter include RXR. Among them, RXRA not only exhibits the most significant co-expression association with STAT4 but also RXRA, as a resinous acid classic receptor, has a large number of clinically used specific teaching activators. As showed in Figure 6, through the NCBI database, the E-

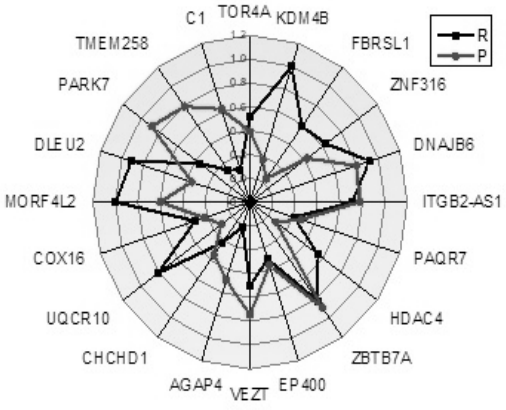

Fig. 6. Osteosarcoma dataset GSE12865 with LKB1 positively correlated with the expression

MEXP-3628, GSE12865, and GSE43252 data sets were used to verify the co-expression association between LKB1 and RXRA. The results are shown in Figure 7. In the three data sets, there is a co-expression association between LKB1 and RXRA. This EDU was detected by staining cells overexpressing MIR-363 changes osteosarcoma proliferation of neoplastic cells. As shown in Figure 8, the comparison of the qPCR detection of osteosarcoma tissue specimens and normal tissues also found that the expression of PDZD2 in tumour tissues increased. The expression of miR-363 showed a negative regulatory relationship, suggesting that miR-363 may be PDZD2 gene expression has an inhibitory effect, which provides clues for the study of the second part of the regulatory mechanism. This part of the experiment measures the function of miR-363 in osteosarcoma cells in vivo. The results showed that overexpression of transplanted miR-363 in the MG-63 grown significantly smaller tumour cells in mice, the growth rate is significantly slower. It shows that miR-363 also has an effective inhibitory effect on the growth of osteosarcoma tumours in animals and can inhibit the growth of tumour tissues. In tumours excised tumour nude mice, mRNA and protein levels detection confirmed that miR-363 was successfully unregulated and PDZD2 down-regulated, and MG-63 assay showed cell-induction. The study also used the cut tumour tissues to detect the apoptosis rate of tumour cells, and the results proved that miR-363 can also significantly induce osteosarcoma cell apoptosis in animals. These inhibitory effects are related to the reduction of PCNA expression in tumour tissues overexpressing miR-363.

DISCUSSION

The origin of osteosarcoma is still no definitive evidence of unity than other tumours. Nevertheless, the application of bioinformatics techniques to the research of osteosarcoma can greatly reduce the research cost and provide rich biological phenotype and genetic information. In tumour research, different tumours usually have completely different gene abnormal expression or abnormal activation of pathways. In osteosarcoma, its gene variation and pathway activity status are also significantly different from other tumours (Zhao et al. 2019).

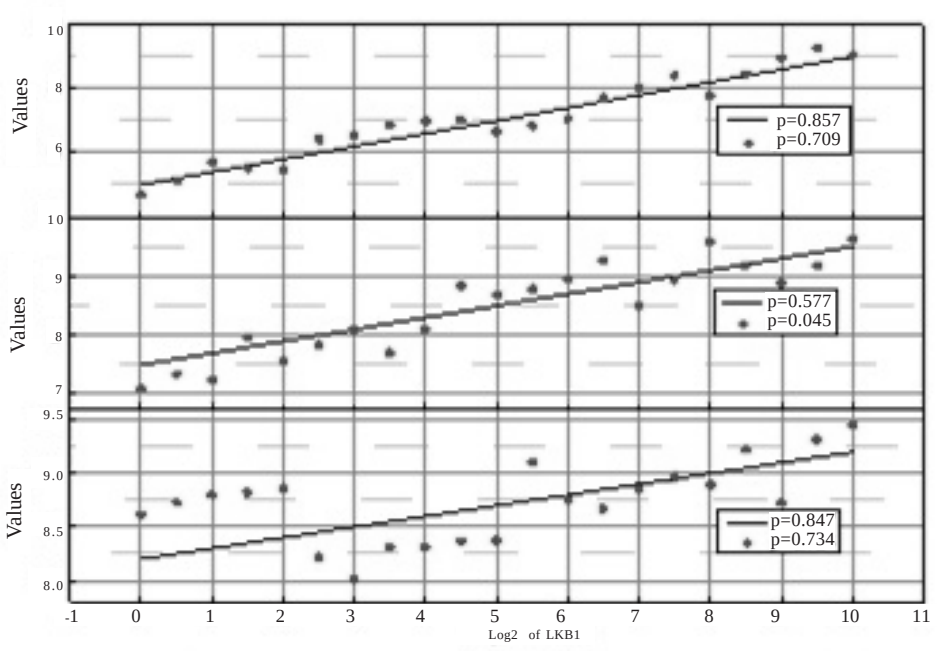

Fig. 7. The expression of RXRA and L KB1 is related in three independent data sets

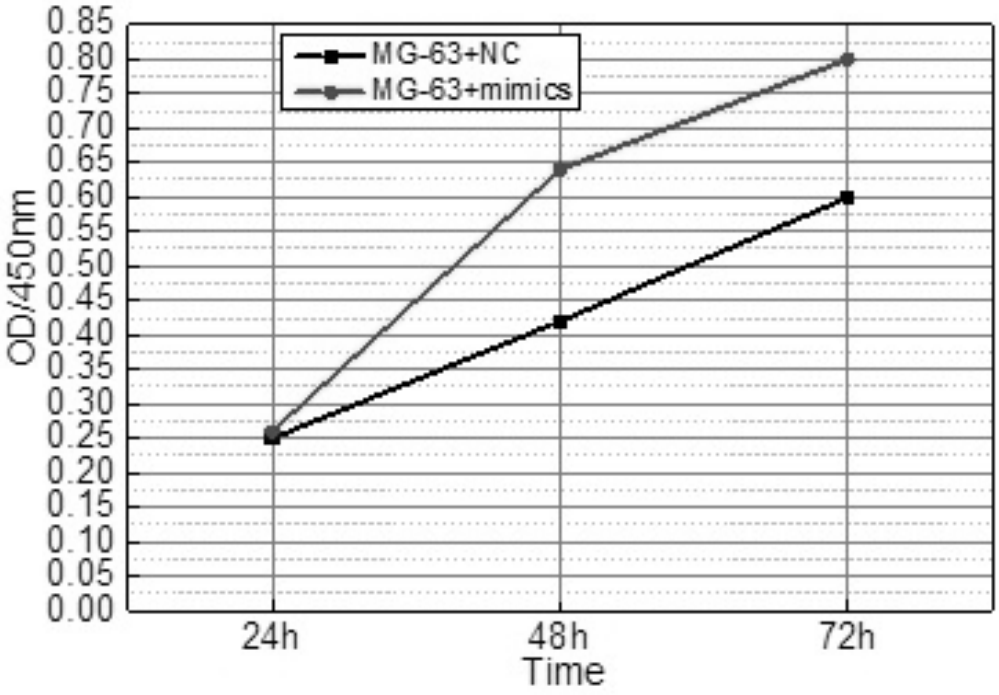

Fig. 8. CCK8 detects cell viability at different time points

Previous transcriptase sequencing results showed that the LRP1-SNRNP25 fusion gene (Yang et al. 2014), which is specific and frequently occurring in osteosarcoma, is caused by the fusion of exon 8 of the LRP1 gene and exon 2 of SNRNP25 gene. However, previous results and analysis came from transcriptase sequencing, and the cause of its occurrence and the structure at the DNA level is still unclear. The results showed that the LRP1-SNRNP25 had sequence homology up to 100 percent indicating overexpression stable line was successfully constructed and can be used for subsequent experiments. Then, according to the principle of the central principle, to interfere with the expression of LKB1, a more feasible method is to interfere with the specific transcription factor of LKB1 in osteosarcoma (Xu et al. 2020). In further research, the researchers plan to use biological information combined with in vitro experimental research methods to screen specific transcription factors of LKB1 in osteosarcoma and find transcription factors that can be targeted by drugs and conduct in vitro intervention studies on overexpression of LKB1 genes. It hopes to provide a theoretical and practical basis for non-invasive overexpression of LKB1 in vivo.

The expression of miR-363 showed a negative regulatory relationship, suggesting that miR-363 may be PDZD2 gene expression has an inhibitory effect, which provides clues for the study of the second part of the regulatory mechanism. This part of the experiment measures the function of miR-363 in osteosarcoma cells in vivo. MG-63 cells were transformed with miR-363 mimics and negative control and then injected into nude mice to produce a xenograft model of human MG-63 tumour (Ling et al. 2019; Wang et al. 2019a). The study also used the cut tumour tissues to detect the apoptosis rate of tumour cells, and the results proved that miR-363 can also significantly induce osteosarcoma cell apoptosis in animals. These inhibitory effects are related to the reduction of PCNA expression in tumour tissues overexpressing miR-363. By analysing the gene co-expres-

sion of the LKB1 gene in the osteosarcoma and normal tissue data set GSE12865 (Yamaguchi and Murata 2020), the results showed that a significant co-expression association was positively related genes. Preliminary gene pathway and function analysis showed that these 4061 genes are involved in development categories, cell differentiation categories, and kinase regulation categories. The gene ontology analysis showed that the 4061 genes are closely related to protein transmembrane transport, energy metabolism, immune regulation, and transcription and translation regulation (Jin et al. 2019). RXRA has a specific activator Bexarotene, and it has been used in the treatment of non-small cell lung cancer, cell lymphoma, and other tumours. In this experiment, Bexarotene was selected as the activator of RXRA for the next biological function study. Benefiting from the continuous development and progress of high-throughput sequencing technology and tumour big data, rapid progress has been made in the study of gene associations and potential signalling pathways in tumourogenesis and development. Upstream of the transcription control factor may also be downstream therefrom the mRNA, protein degradation regulation control. For this study, screening specific co-expression transcription factors for the LKB1 gene is the most economical and reasonable research method. Therefore, based on the existing database, this study analyzed gene co-expression of the LKB1 gene in osteosarcoma tissue and found that LKB1 gene has a significant co-expression association with multiple genes, especially genes related to proliferation and metabolism. At the same time, through transcription factor prediction, transcription factors that can bind to the LKB1 promoter sequence were found. The LKB1 showed a significant association of co-expression of the transcription factor. There is the potential regulation of LKB1 expression that can be a scientific hypothesis, the project lock and LKB1 was the most significant expression of transcription factors associated with RXRA conducted further research (Hou et al. 2019; Han et al. 2020).

CONCLUSION

Study concludes that RXR protein family, LKB1, AMPK pathway, and mTOR have been closely related to the body's immune regulation and reduces the potential immunoregulatory effect of drug targeted LKB1 therapy to a certain extent as shown in xenograft model of immunodeficiency nude mice used in this study also. The oral administration of Bexarotene can inhibit the proliferation of living osteosarcoma cancels and able to up-regulate the expression of LKB1 gene in living osteosarcoma tissue.

RECOMMENDATIONS

However, there is better drug formulation that can achieve overexpression of LKB1 in vivo, and combined immunotherapy may become an important immunotherapy approach for osteosarcoma. At the same time, LKB1 targeted therapy can potentially be used as one of the alternative treatments for mTOR inhibitors.

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