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Original research paper

Relaxin inhibits ^{177}Lu-EDTMP associated cell death in osteosarcoma cells through notch-1 pathway

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ABSTRACT

^{177}Lu-EDTMP (Ethylenediamine tetramethylene phosphonic acid) is the most used radioactive agent for pain palliation in bone cancer patients. The present study aims to study the impact of relaxin-2 on the ^{177}Lu-EDTMP associated cell toxicity and death in osteosarcoma cells. MG63 and Saos-2 cells were cultured with ^{177}Lu-EDTMP (37 MBq) for 24 h with and without pretreatment of recombinant relaxin 2 (RLXH2) for 12 and 24 h. ^{177}Lu-EDTMP associated cellular deterioration and death was determined by LDH, MTT, and trypan blue dye assays. ELISA-based kit was used to determine apoptotic DNA fragmentation. Western blotting was used to determine expression levels of apoptotic-related signalling pathway proteins like bcl2, poly(ADP-ribose) polymerase (PARP), and MAPK (mitogen-activated protein kinase). Our results found that RLXH2 counters ^{177}Lu-EDTMP associated cellular toxicity. Similarly, RLXH2 was able to counter ^{177}Lu--EDTMP induced cell death in a concentration and time--dependent manner. Furthermore, it was found that RLXH2 treatment prevents apoptosis in ^{177}Lu-EDTMP challenged cells through activation of the notch-1 pathway in a concentration- and time-dependent manner. We reported that RLXH2 significantly declined cellular toxicity and apoptosis associated with ^{177}Lu-EDTMP in MG63 and Saos-2 cells through the notch-1 pathway.

Keywords: osteosarcoma, relaxin-2, cell death, apoptosis, notch-1 pathway

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The most common malignant form of bone cancer is osteosarcoma (OS) and accounts for 60 % of all bone cancers. OS affects both children and adults ranging from 10 to 20 years (1). Various genetic factors have been linked with OS, however, its exact cause is still unknown. One of the main and frequent symptoms of OS includes pain (2). The treatment strategy of OS includes chemotherapy, followed by surgery and then post-operative inter-

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ventions. Despite tremendous advances in the medical field, the clinical use of most of the anti-osteosarcoma chemotherapeutics is limited due to toxicity towards normal cells, lack of sensitivity and selectivity to tumor cells, poor pharmacokinetics, multidrug resistance (MDR) *etc.* (3–5). Furthermore, the blood-bone marrow barrier prevents the efficient delivery of antitumor agents to the bone (6). Therefore, there is an urgent need to look for new anti-tumor targets for treating OS successfully.

Relaxin is a peptide hormone that was initially linked with pregnancy (7). With the passage of time, other functions performed by relaxin in different diseases like cancer, diabetes, heart failure, *etc*. were unearthed (8–11). Humans express three types of relaxins (relaxin 1, relaxin 2, and relaxin 3), and each performs a unique role (12, 13). It has been reported that relaxin 2 (RLXH2) gets overexpressed in different cancers and aids in cell proliferation, metastasis and invasiveness (14, 15). Relaxin has been found to induce the differentiation of peripheral blood mononuclear cells (PBMCs) into mature osteoclasts, suggesting its impact on bone metabolism and differentiation (16, 17). The role of relaxin in stimulating osteoclastogenesis and regulating mature osteoclasts' activity has opened new functions of this peptide hormone in bone physiology and cancer.

Currently, several β^- emitting radionuclides labelled phosphonates, such as EDTMP labelled with lutetium-177 (^{177}Lu-EDTMP), are used for bone pain palliation. A radionuclide therapy and combination with chemotherapy produce higher efficacy for bone pain palliation (18). ^{177}Lu-EDTMP has emerged as a new and inexpensive radioactive therapy agent and can be helpful for therapy in metastatic bone cancer patients (19, 20). It has been reported that ^{177}Lu-EDTMP treatment induces cell toxicity and apoptosis in osteosarcoma tumor cells (21, 22). The present study aims to investigate the role of RLXH2 in inhibiting ^{177}Lu-EDTMP linked cellular toxicities and death in osteosarcoma cells.

EXPERIMENTAL

Chemicals and antibodies

Recombinant human relaxin-2 protein (RLXH2) was purchased from Sigma-Aldrich, USA (SRP3147, expressed in *E. coli*, HPLC ≥ 98 %). Antibodies against, Cleaved PARP, Bcl2, Phospho-p38, p38, Notch ICD and GAPDH were purchased from Cell Signaling Technology (USA). Lactate Dehydrogenase (LDH) Assay Kit (ab65393) was obtained from Abcam (UK). The enzyme-linked immunosorbent assay (ELISA) based cell death assay kit and Cell Proliferation Kit I (MTT) was purchased from Roche Molecular Biochemicals. All other chemicals used were acquired from Sigma-Aldrich.

Radiolabeling preparation of ^{177}Lu-EDTMP

Radiolabeling of EDTMP with ^{177}Lu was accomplished using the modified protocol of Kumar *et al.* (21). Briefly, the ^{177}Lu-EDTMP complex was prepared by mixing 100 μL of EDTMP stock solution (2 mg mL^{-1}) with 100 μL of $^{177}LuCl_3$ (20 mCi, 740 MBq) and a pH of 7 was adjusted. The solution was then incubated at 50 °C for 30 minutes and was then allowed to attain room temperature. Radioactive check purity of ^{177}Lu-EDTMP was carried out using paper chromatography.

Cell culture, treatments and assays

MG63 and Saos-2 cells (Human osteosarcoma cells) were acquired from American Type Culture Collection (ATCC). Cells were grown in culture for 24 h and either left untreated or treated with ^{177}Lu-EDTMP alone or together with different concentrations of RLXH2 for 12 or 24 h in a 12-well plate (Corning, USA).

For MTT assay purposes, MG63 and Saos-2 cells (1×10^3 cells/well) were cultured in 96-well plates for 24 h and treated with different amounts of RLXH2 (5–50 nmol L^{-1}) for 12 or 24 h. It was followed by MTT assay using Cell Proliferation Kit I (MTT) following the manufacturer protocol. The percent of cell proliferation was calculated as a ratio of optical density (OD) of treated to control cells multiplied by 100. The IC_{50} value was taken from the results of triplicate reactions. The IC_{50} value of RLXH2 was calculated from the straight-line graph.

For LDH leakage assay purposes, MG63 and Saos-2 cells (3×10^5 cells/2 mL medium/well) were cultured in a 12-well plate for 24 h. Cells were treated as: ^{177}Lu- EDTMP only (24 h) or ^{177}Lu- EDTMP with RLXH2 (20, 30 and 40 nmol L^{-1}) for 12 h. For LDH activity analysis, 300 μL of the medium was taken out and analysed using LDH Assay Kit by following manufactures guidelines.

For trypan blue assay purposes, MG63 and Saos-2 cells (3×10^5 cells/2 mL medium/well) were cultured in a 12-well plate for 24 h. Cells were treated as: ^{177}Lu- EDTMP only (24 h) or ^{177}Lu- EDTMP with different concentration of RLXH2 (20, 30 and 40 nmol L^{-1}) for 12 h. For Trypan blue assay analysis, cells were collected and their viability was determined by using trypan blue dye (0.4 %). Dead cells take up the dye and are counted using a haemocytometer, whereas viable cells exclude the dye.

To carry out the apoptotic assay, MG63 and Saos-2 cells (1×10^3 cells/well) were cultured in 96-well plates for 24 h. Cells were kept untreated or treated as: ^{177}Lu- EDTMP only or ^{177}Lu- EDTMP with different concentrations of RLXH2 (20, 30 and 40 nmol L^{-1}). A cell death assay kit (ELISA based) was used to determine apoptosis (manufacturer's instructions were followed). Inhibition of Notch-1 signalling pathway was carried out by treating cells with 5 μmol L^{-1} of 3,5-difluorophenylacetamide (DAPT): a specific inhibitor of Notch receptor cleavage for 12 h followed by downstream processing.

Protein extraction

Preparation of MG63 and Saos-2 cell lysates was achieved using lysis buffer (NP-40). To prevent proteolysis of cell lysate, Halt Protease Inhibitor Cocktail (Thermo Scientific) was used. Centrifugation (3000 rpm for 10 min) of cell lysate was performed to obtain supernatant. Bradford protein assay was used to determine protein concentration.

Western blotting

Protein samples were prepared as described by Waza *et al.* (23). Detection of target proteins was determined using specific antibodies; anti-PARP, anti-bcl2, anti-p38, anti-phospho-p38, anti-ICD and anti-β-actin. LI-COR system was used for secondary detection.

Immunoblot quantification

LI-COR scanner was used to quantify protein bands. Fluorescently labelled secondary antibodies (Licor) were used to prepare the standards. The fluorescent spots from different

concentrations of fluorescently labelled secondary antibodies were measured to prepare a standard plot. Fluorescence of the individual blot bands was measured and compared to a standard plot to quantitate protein bands on immunoblots.

Statistical analysis

SPSS software was used to carry out statistical analysis. Experimental values were given as mean and standard error of the mean ($p < 0.05$ as statistically significant).

RESULTS AND DISCUSSION

Half-maximal inhibitory concentration of RLXH2 determination

Calculation of the half-maximal inhibitory concentration (IC_{50}) is measured to get information about the efficacy of a particular drug. The obtained information can be utilised to inhibit a biological process by half using a particular drug concentration in pharmacological research. The IC_{50} value of RLXH2 was measured by calculating the percent of proliferating cells using MTT assay and it was found to be 20 ± 0.87 nmol L^{-1} for MG63 and Saos-2 cells (Fig. 1). To study the inhibitory effect of RLXH2 on cells treated with ^{177}Lu-EDTMP, 20 nmol L^{-1} of RLXH2 was used to carry out further experiments.

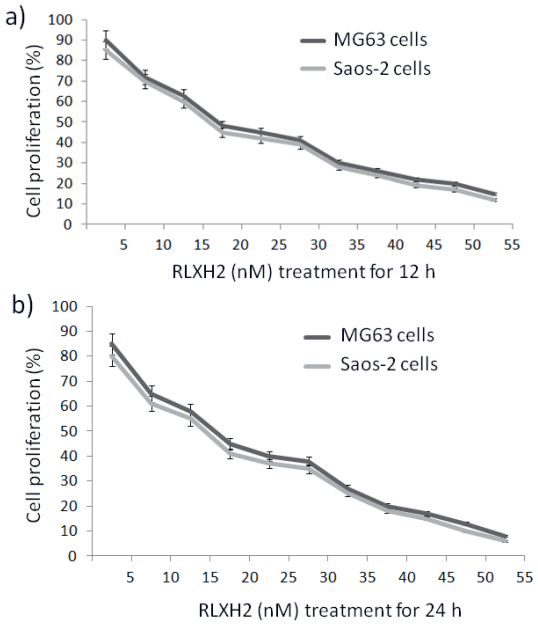

Fig. 1. The percent of proliferating cells determined with MTT assay was used to calculate the IC_{50} value of RLXH2 for MG63 and Saos-2 cells after RLXH2 treatment for: a) 12 h and b) 24 h. Data were shown as mean ± SD.

Effect of ^{177}Lu-EDTMP and RLXH2 on the toxicity of MG63 and Saos-2 cells

Earlier it has been reported that ^{177}Lu-EDTMP treatment induces cell toxicity by inducing apoptosis in bone cells. Here we try to look for the protective role of RLXH2 treatment to counter toxicities induces by ^{177}Lu-EDTMP in MG63 and Saos-2 cells. In the present study, cellular toxicities were studied by Lactate Dehydrogenase Assay, trypan blue dye uptake and MTT assay.

LDH is found within the cells and is responsible for cellular respiration. Breakage of the plasma membrane may cause LDH release from the cells. In the cell culture experiments, the presence of LDH in the culture medium is taken as a death call for the cultured cells. Treatment of ^{177}Lu-EDTMP resulted in membrane damage in MG63 and Saos-2 cells as determined by LDH release. However, RLXH2 treatment significantly prevented LDH leakage from ^{177}Lu-EDTMP-treated MG63 and Saos-2 cells, as shown in (Fig. 2a). Furthermore, it was observed that MG63 and Saos-2 cells treated with ^{177}Lu-EDTMP showed decreased cell viability. However, RLXH2 treatment significantly ($p < 0.05$) increased cell viability in ^{177}Lu-EDTMP treated MG63 and Saos-2 cells (Fig. 2b). Similarly, it was found that MG63 and Saos-2 cells treated with ^{177}Lu-EDTMP showed decreased cell proliferation. However, RLXH2 treatment significantly ($p < 0.05$) increased cellular proliferation in ^{177}Lu-EDTMP treated MG63 and Saos-2 cells (Fig. 2c).

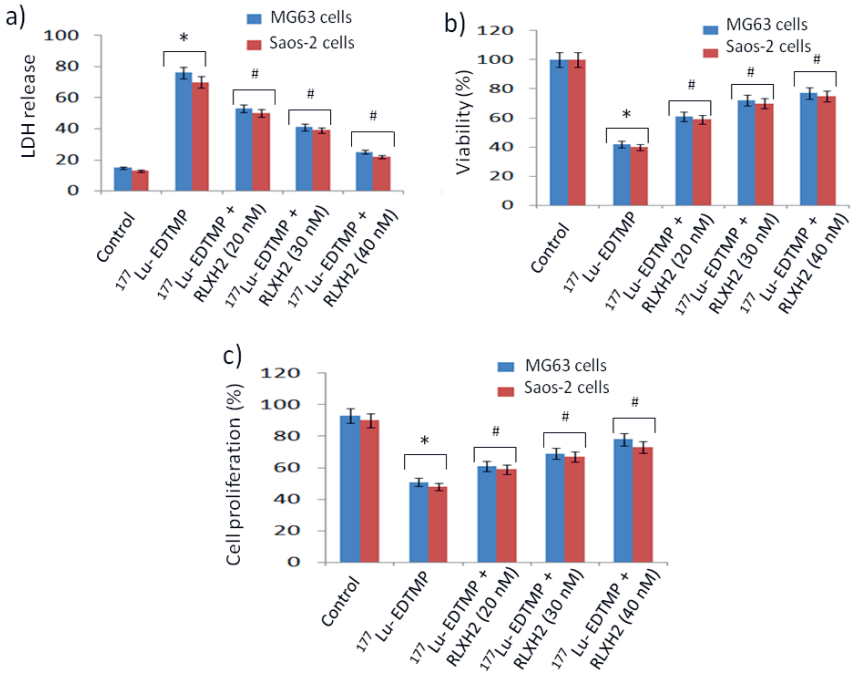

Fig. 2. Cellular toxicity of MG63 and Saos-2 cells treated with either ^{177}Lu-EDTMP alone and together with different concentrations of RLXH2. Data were shown as mean ± SD. (*) shows $p < 0.05$ compared to control, whereas (#) shows $p < 0.05$ compared to ^{177}Lu-EDTMP.

Impact of ^{177}Lu-EDTMP and RLXH2 on apoptotic cell death

To look for the protective role of RLXH2 against ^{177}Lu- EDTMP induced death in MG63 and Saos-2 cells, the apoptotic assay was carried out by using Cell Death Detection ELISA method. During apoptosis, cells exclude fragmented DNA and histone from the nucleus to the cytoplasm, which can be detected by the ELISA method. We found that ^{177}Lu-EDTMP treatment significantly ($p < 0.05$) increases apoptosis compared to control, while as RLXH2 treatment significant ($p < 0.05$) decreases apoptosis in ^{177}Lu-EDTMP treated MG63 and Saos-2 cells (Fig. 3).

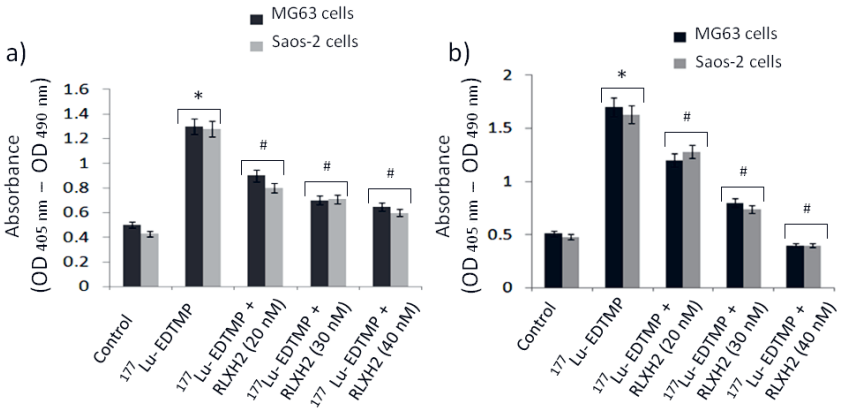

Fig. 3. Cell death of MG63 and Saos-2 cells was determined with apoptosis-based assay (ELISA based). MG63 and Saos-2 cells were treated with either ^{177}Lu-EDTMP alone or in the combination with RLXH2 for: a) 12 h or b) 24 h. Data were shown as mean ± SD. (*) shows $p < 0.05$ compared to control, whereas ($^{\#}$) shows $p < 0.05$ compared to ^{177}Lu-EDTMP.

Impact of ^{177}Lu-EDTMP and RLXH2 on apoptotic protein levels

Cellular survival and death are mainly decided by the anti-apoptotic and pro-apoptotic proteins (24). Expression levels of anti-apoptotic proteins like Bcl2 get up-regulated during cell survival, while their down-regulation allows cell death (25, 26). We observed down-regulation of Bcl2 protein during ^{177}Lu-EDTMP treatment however RLXH2 significantly increases its expression (Fig. 4). Cleavage of PARP proteins (113 kDa) into two smaller fragments 89 and 24 kDa is observed during apoptosis (27, 28). As shown in Fig. 4, expression levels of cleaved PARP and Bcl2 are increased after ^{177}Lu-EDTMP treatment, whereas RLXH2 treatment significantly decreased their expression levels.

Various cellular stress inducers enhance and activate p38 protein (29). Activation of p38 occurs due to its phosphorylation, and after its activation, it gets translocated to the nucleus and activates various transcriptional factors and proteins (30). We observed decreased expression of p38 and phospho-p38 after RLXH2 treatment in ^{177}Lu-EDTMT challenged cells (Fig. 4). The decreased expression levels of p38 and phospho-p38 may be associated with the increased survival of MG63 cells (31, 32). It was observed that ^{177}Lu-EDTMP

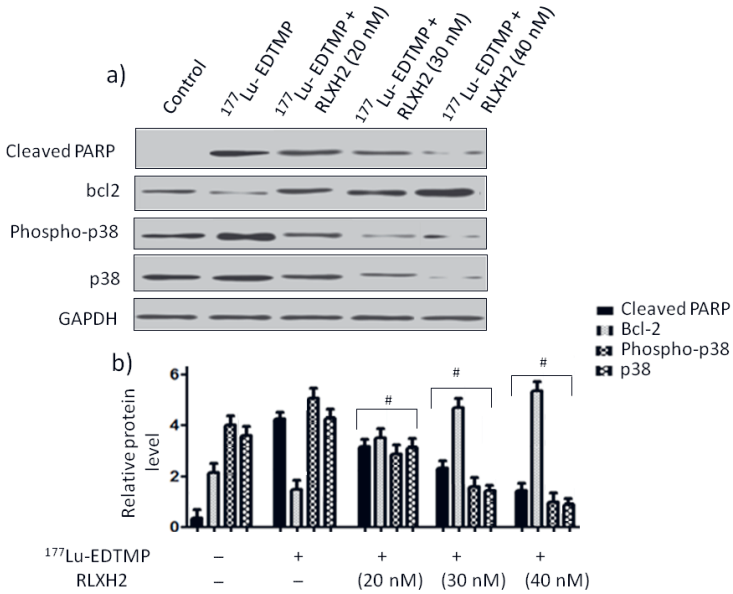

Fig. 4. a) Western blotting analysis of apoptotic proteins after cells treatment with ^{177}Lu-EDTMP alone or in the combination with RLXH2. b) Densitometry analysis of immunoblots. Data were shown as mean ± SD. ($^{\#}$) shows $p < 0.05$ compared to ^{177}Lu-EDTMP.

treatment significantly increases the expression of p38 and phospho-p38, while RLXH2 significantly decreased their expression level. As shown in the densitometry analysis, ^{177}Lu-EDTMP treatment increases expression levels of cleaved PARP by 5 times compared to the control, however, RLXH2 treatment significantly decreases its expression level in a concentration-dependent manner. Furthermore, ^{177}Lu-EDTMP treatment decreases expression of Bcl2, which was upregulated by RLXH2 treatment. Similarly, ^{177}Lu-EDTMP treatment up-regulates expression levels of phospho-p38 and p38 by 3.5 and 3 times compared to control. However, expression levels of phospho-p38 and p38 were significantly down--regulated after treatment with different concentrations of RLXH2.

Impact of RLXH2 on the Notch-1 signalling pathway

Earlier studies have reported that the activation of the Notch-1 signalling is commonly observed in osteosarcoma (33, 34). Activation of the Notch-1 signalling pathway has been reported to induce cell death (35). In osteosarcoma tumor cells, ^{177}Lu-EDTMP has been associated with cellular toxicity and apoptosis. It should be noted here that the impact of Notch signalling on osteosarcoma chemotherapy is yet to be explored. We, therefore, set an experiment to looks for a possible role of the Notch-1 signalling pathway during ^{177}Lu-EDTMP exposure in osteosarcoma cells. We found that ^{177}Lu-EDTMP induces cell death in MG63 and Saos-2 cells *via* the Notch-1 signalling pathway. It is well reported that the protective effects of RLXH2 may depend on the mechanism of Notch1 activation (36, 37). We further set the experiment to search for a possible impact of RLXH2 on the Notch-1

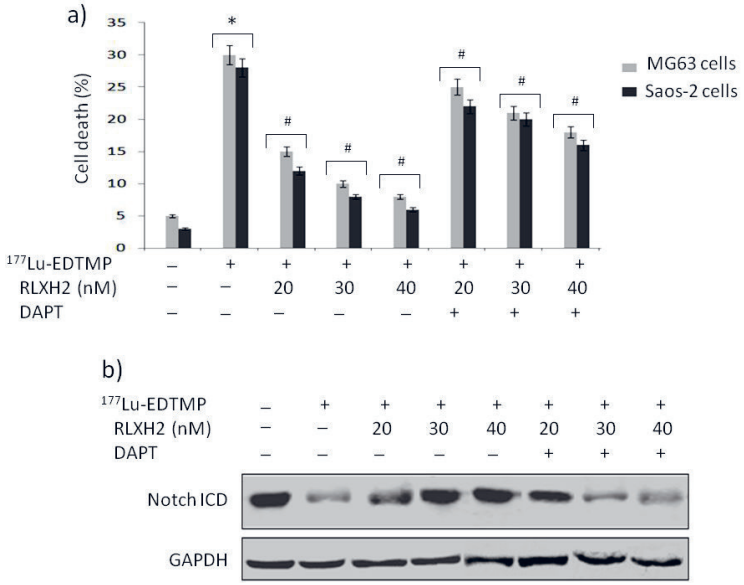

Fig. 5. a) Cell death of MG63 and Saos-2 cells was determined using an apoptosis-based cell death assay (ELISA based). b) Western blotting analysis. Data are shown as mean ± SD. (*) shows $p < 0.05$ compared to control, while as ($^{\#}$) shows $p < 0.05$ compared to ^{177}Lu-EDTMP.

signalling pathway during ^{177}Lu-EDTMP exposure in osteosarcoma cells. We reported that RLXH2 was able to inhibit ^{177}Lu-EDTMP associated cell death by activating the Notch-1 signalling pathway. The protection offered by RLXH2 against ^{177}Lu-EDTMP induced cell death got abolished by inhibiting the Notch-1 signalling using DAPT; a specific Notch-1 inhibitor (Fig. 5a). Furthermore, RLXH2 treatment was able to increase the expression levels of Notch intracellular domain (Notch-ICD), as shown in Fig. 5b. We, for the first time, report that RLXH2 activates the Notch-1 signalling pathway in ^{177}Lu-EDTMP challenged MG63 and Saos-2 cells and thereby allowed their proliferation and avoiding cell death.

CONCLUSIONS

^{177}Lu-EDTMP, being a chemotherapeutic agent, is used for pain palliation in bone cancer patients. However, ^{177}EDTMP induces cellular toxicity and apoptotic cell death in bone cells. RLXH2 was able to counter cellular toxicity and apoptotic cell death associated with ^{177}Lu-EDTMP in MG63 and Saos-2 cells through activating the Notch-1 signalling pathway. The results obtained in the current study are encouraging to carry out future work on the detailed role played by the RLXH2 in osteosarcoma.

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Conflicts of interest. – The authors declare no conflict of interest.

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Authors contributions. – Conceptualization, J.X and Z.J.; methodology J.X and W.S.; analysis S.W.; investigation, W.S and W.C.; writing, original draft preparation, Y.Z.; writing, review and editing, Y.Z and Z.J. All authors have read and agreed to the published version of the manuscript.

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