Peptide KRP Conjugated with Doxorubicin Exerts Anti-Tumor Activity by Regulating RPS6KA2 in Osteosarcoma

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Abstract

Objectives: Osteosarcoma (OS) is the most common primary solid malignant tumor of the bone in adolescents. Conventional treatment of OS by surgery and chemotherapy is not effective and the prognosis is poor. Our previous study demonstrated that a novel cell-penetrating peptide (KRP) that, coupled to doxorubicin (DOX), allowed specific tumor targeting. However, the underlying molecular mechanisms of the KRP-DOX antitumor effect were not completely elucidated. Therefore, the present work aimed to identify key candidate genes by integrated bioinformatics analysis.

Methods: Differentially expressed genes (DEGs) were screened using the Network Analyst. The functions and pathway involvements of the DEGs were analyzed using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, respectively. The protein-protein interaction (PPI) network was used to identify hub genes. In addition, quantitative RT-PCR (qRT-PCR) and Western blotting were performed to assess the expression level of candidate biomarkers in OS cells after KRP-DOX treatment.

Results: A total of 790 DEGs were identified. GO functional analysis and KEGG pathway analysis demonstrated that the DEGs were mostly enriched in the ribosome. DEGs were visualized by PPI networks. After treatment of OS cells with KRP-DOX, the downregulated ribosomal protein S6 kinase A2 (RPS6KA2) was found to be closely related to inhibition of OS proliferation. In agreement with the bioinformatics analysis, qRT-PCR and western blot results showed low expression of RPS6KA2 in osteosarcoma cells in the KRP-DOX treatment group.

Conclusions: RPS6KA2 is significantly associated with the KRP-DOX anti-tumor effect and may serve as a candidate biomarker and therapeutic target for OS.

1. Introduction

Osteosarcoma (OS), which accounts for 60 percent of all sarcomas, is one of the most prevalent cancers in the world (Zhang et al., 2017). Typically, OS develops in children and adolescents (10–20 years old) and is the second most frequent cause of death in this age group. OS is a rapidly progressing malignancy with a poor prognosis (Kun-Peng et al., 2018). Despite breakthroughs in neo-adjuvant chemotherapy and surgical techniques, only 25–60% of patients with OS have improved survival (Zhang et al., 2017). Chemoresistance represents one of the major obstacles to the treatment of OS. Inactivation of drugs by the tumor or their inability to effectively reach tumor cells are major factors contributing to drug resistance (Wang et al., 2017).

Currently available drugs are limited by their ability to penetrate the cell, which could be the reason for their low biological activity, low therapeutic efficacy, and enhanced toxicity (Wang et al., 2018). Combination therapy is becoming an effective method for the treatment of OS by synergistically acting on tumor cells by a variety of mechanisms, thus reducing drug tolerance (Gurunathan et al., 2019; Silva et al., 2019). In recent years, novel strategies have been developed in the design of molecules with biological
activity and therapeutic properties. However, drug uptake by tumor cells remains a major challenge for their clinical application. A large number of delivery systems, including viral and non-viral carriers, have been developed to overcome low membrane permeability and to promote drug therapeutic activity. However, these delivery methods are associated with high cytotoxicity and, therefore, decrease cellular viability, and, in the case of viral vectors, can potentiate viral recombination. Recent studies have identified protein translocation domains of peptides that are capable of efficient translocation through the cell membrane without affecting its structure and integrity (Kardani et al., 2019). These cell-penetrating peptides (CPPs) are typically up to 30 amino acids in length and, because of their high content of basic residues, are always characterized by a positive net charge. CPPs are superior to other translocation methods because they exhibit high membrane permeability, are capable of migrating to a variety of cells, possess large cargo capacity, have low cell toxicity, and do not trigger immunological responses (Salerno et al., 2016).

Based on these premises, our previous work focused on the design of a novel lysine-rich cell-peptide (KRP). This led to the development of a new tumor-targeted drug delivery system (DDS), linking KRP and doxorubicin (DOX) through stable covalent thioether and amide bonds. Preliminary results demonstrated that KRP-DOX was selectively enriched in tumor tissues in transplanted OS mice, and exhibited better therapeutic efficacy than the negative control (saline) or DOX alone. Furthermore, the KRP-DOX complex escaped lysosomal degradation in OS cells and exerted a cytotoxic effect(Yu et al., 2019).

The aim of this work was to investigate the mechanism of the antitumor activity of KRP-DOX, and to identify potential key genes and pathways using bioinformatic analyses of OS tissues exposed to KRP-DOX, DOX, or saline (Fig. 1).

2. Materials And Methods

2.1 Animal Tissue Samples Preparation.

Animal experiments were conducted under the guidelines approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University (Guangzhou, Guangdong, China). Preparation of DOX and the synthesis of KRP, KRP-DOX, and FITC-KRP-DOX are presented in detail in our previous study(Yu et al., 2019).

Tissue samples (Tissues were obtained after mice were injected with KRP-DOX, DOX, or saline via a tail vein) were washed with 0.9 % sterile saline solution and preserved in RNA later RNA Stabilization Reagent (Qiagen, Germantown, MD, USA) at 37°C for no more than 24 hours. Approximately 10 µL of the RNA later RNA reagent was used for 1 mg of tissue. The samples were cut into 1 g pieces and stored at -80°C After thawing at room temperature, the samples were pulverized with an electric homogenizer. In accordance with the manufacturer’s instructions, total RNA was separated using an RNeasy Mini Kit (Qiagen). The RNA content was determined by a Nanodrop ND-8000 analyzer (Thermo Scientific, Waltham, MA, USA) and preserved at -80°C for later use.
Cell culture

Human OS cell line MG63 was purchased from ATCC (Rockville, MD, USA). These were kept in DMEM medium at 37°C and 5% CO2 (Gibco, USA), with 10% fetal bovine serum (Gibco, USA) and 100 U/mL penicillin streptomycin (Gibco, USA) added.

Preparation of RNA-seq libraries and RNA sequencing:

Mice bearing OS tumors were injected with KRP-DOX, DOX, or saline via the tail vein. After several days, tumor tissues were collected for total RNA extraction, and the library was constructed according to the method of chain specificity. Library preparation and paired-end sequencing were performed. Complementary double-stranded cDNA was synthesized from the total mRNA of cells with reverse transcriptase, and then inserted into the vector and transferred to the host to establish the cDNA library based on the Illumina protocol (San Diego, CA, USA). RNA sequencing was performed on Illumina Genome Analyzer.

2.2 Identification of differentially expressed genes (DEGs)

Raw data were expressed in an expression matrix, which was then normalized according to the robust multi-array average algorithm in the ‘Affy’ package (version 3.4.1) of R software. The ComBat function in the ‘sva’ package was used to correct inter-batch differences. Subsequently, the t-test method in the ‘limma’ package was used to identify the DEGs in tumors treated with KRP-DOX and saline. A |log2 fold change| > 1 and \( P < 0.05 \) were considered to be statistically significant for DEGs identification.

2.3 Functional enrichment analysis of DEGs

GO enrichment analysis.

To further investigate the specific biological functions of differentially expressed genes, the Database for Annotation Visualization and Integrated Discovery (DAVID, version 6.7) was conducted for Gene Ontology (GO) enrichment analyses. The GO enrichment analyses were conducted using the “cluster Profiler” package. The cutoff criteria were set as \( P < 0.05 \) and (log2 fold change) > 1.

2.4 KEGG pathways analyses of DEGs

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was carried out by the Consensus Path DB (http://consensuspathdb.org) to investigate the possible functional approaches implicated in the antitumor activity of KRP-DOX.

2.5 Construction of PPI networks

The relationships between the proteins encoded by DEGs were clarified by mapping the DEGs pair to the search tool for the Retrieval of Interacting Genes (STRING) database (http://stringdb.org/). The construction of the PPI network was achieved using the comprehensive interaction information
downloaded from STRING, and the network was visualized using Cytoscape software (https://cytoscape.org/).

2.6 q RT-PCR

Total RNA was extracted by the RNA simple Total RNA Kit (Tiangen, China) according to the manufacturer’s directions. β-actin was used as an internal control. Genomic DNA was amplified by real-time RT-PCR using the following primers:

RPS6KA2:
forward, 5′-CTCACGGAACACCTTCCATAG-3′,
reverse, 5′-CTGAGCATGAAGAAGTTGCC-3′.

β-actin:
forward, 5′-CCCATCACCATCTTCCAGG-3′,
reverse, 5′-ATGAGTCCTTCCACGATAC-3′.

Samples were amplified with an RT-PCR System (Applied Biosystems). The β-actin gene was used as internal control for qRT-PCR amplification. Pre-denaturation at 95°C for 10 min followed by denaturation at 95°C for 10 s followed by annealing/extension at 55°C for 35 s was performed in an ABI 7500 instrument for 40 cycles. The experiment was repeated three times. The final relative expression of RPS6KA2 mRNA was compared using the 2−ΔΔCt method.

2.7 Western blot

Protein components were extracted from tissues, determined and separated by SDS-PAGE. The protein bands are then transferred to the PVDF membrane. It was incubated overnight with the primary antibody (1:5000) at 4°C and then with the secondary antibody (1:10000) at room temperature for 1 h. Western blot analysis was performed as described in our previous study. Immunoreactivity was detected by chemiluminescence reaction with using an enhanced chemiluminescence (ECL) kit (Pierce/Thermo Fisher Scientific, Rockford, IL, USA). Image J imaging analysis system is used to measure the optical density of the band, and the exposure conditions are adjusted accordingly.

2.8 Statistical analyses

Standardization of gene expression data across tumor tissue chips from mice treated with KRP-DOX and/or DOX alone was performed with Agilent Processed Signal (Agilent Feature Extraction Software) using quantile normalization. Differential gene expression was analyzed by the ‘limma’ package (Bioconductor). Checking and adjusting the p-value many a time was repeated on the basis of Benjamini and Hochberg (Benjamini & Hochberg, 1995; Benjamini and Yekutieli, 2001) using a False Discovery Rate (FDR) of 5%.
3. Results

3.1 Biodistribution and anti-osteosarcoma effect of KRP-DOX

The biological distribution of the injected KRP-DOX, DOX-HCl, and saline in transplanted OS mice were detected utilizing the red fluorescence properties of DOX. KRP-DOX selectively accumulated in tumor tissues 6 h after its administration, while DOX-HCl was diffusely distributed and excreted by liver and kidney metabolism (Fig. 2A). The anti-OS efficacy of KRP-DOX was measured in OS xenograft mice. The tumor volume in saline-treated mice (negative control group) rapidly increased over 4 weeks, whereas tumor growth was significantly suppressed after both DOX-HCl and KRP-DOX treatment (Fig. 2B). There was a statistically significant increase in the inhibition of tumor growth in the KRP-DOX group compared with the DOX-HCl group. We next investigated whether the entire KRP-DOX conjugate or just free DOX, was released from the endolysosome after the hydrolysis of KRP. To achieve this, the KRP moiety was labeled with FITC using 6-aminocaproic acid (Acp) as a linker (Fig. 2C). Confocal microscopy revealed that the FITC (green) and DOX (red) fluorescence co-localized in the MG63 OS cells (Fig. 2C), with molecules reaching the cytoplasm and nuclei 2 h after the drug injection. This result indirectly confirms that the KRP-DOX exerts its anti-tumor activity in MG63 cells as a conjugated molecule rather than as free DOX.

3.2 Identification of DEGs

The mice bearing OS xenografts were treated with KRP-DOX, DOX, or saline, administered via tail vein injection. A total of 790 significantly differentially expressed genes (DEGs) were identified between the KRP-DOX and DOX groups, of which 463 were upregulated and 327 were downregulated. The DEGs are depicted in the Volcano plot (Fig. 3A), in which \( P < 0.05 \) was set as the cut-off criterion of significant difference. The red points represent upregulation and the green points represent downregulation (Table 1). The value of the log2 fold change was normalized (scale number) and clustered using the hierarchical clustering method. Finally, a heatmap was generated in which red represents highly expressed genes and blue indicates low expression levels (Fig. 3B).
### Table 1
DEGs between KRP-DOX and DOX

| Gene symbol | Log2 FC | p-Valu  | p-adj   |
|-------------|---------|---------|---------|
| **up-regulation**                                    |
| CXCL8       | 2.104936975 | 2.60E-28 | 1.53E-24  |
| TNFAIP3     | 1.554407398  | 3.80E-12 | 2.78E-09  |
| RRAD        | 1.546905962  | 1.33E-32 | 1.56E-28  |
| AC004816.2  | 1.408210052  | 1.76E-05 | 0.001210915 |
| AC073115.2  | 1.344504722  | 2.34E-07 | 3.97E-05  |
| KRT17       | 1.275748093  | 0.000130752 | 0.00555602 |
| AL360181.2  | 1.242614463  | 1.38E-10 | 5.60E-08  |
| ATF3        | 1.175709315  | 1.59E-12 | 1.43E-09  |
| RAPH1       | 1.173471546  | 0.000227174 | 0.00809818 |
| IGFBP3      | 1.172571463  | 4.45E-06 | 0.000426356 |
| **down-regulation**                                  |
| AC145098.2  | -0.88360285  | 4.38E-05 | 0.002463659 |
| BMF         | -0.91503599  | 2.34E-06 | 0.000254246 |
| OSGEPL1     | -0.9232445   | 1.23E-05 | 0.000919613 |
| TRNP1       | -0.93828139  | 0.002533284 | 0.04137932 |
| QRICH2      | -0.98442754  | 0.001054995 | 0.023582962 |
| LDLRAP1     | -0.98453657  | 3.91E-06 | 0.000395658 |
| **RPS6KA2** | -1.02513904  | 0.00262754 | 0.042178689 |
| RN7SL5P     | -1.0593151   | 3.00E-05 | 0.001853761 |
| BTN3A2      | -1.06179827  | 0.00259683 | 0.041892198 |
| SLC6A9      | -1.17164151  | 0.000260639 | 0.008891938 |

### 3.3 GO enrichment analysis of DEGs in KRP-DOX- and DOX-treated tissue

DEG functions were analyzed using the Gene Ontology (GO) resource based on three categories related to virulence: “biological processes (BP),” “cell components (CC),” and “molecular functions (MF)” with the
help of the DAVID gene annotation tool. In the BP category, DEGs were mostly enriched for SRP-dependent co-translational protein targeting to cell membranes and co-translational protein targeting to membranes. In the CC category, genes were primarily enriched for the cytosolic ribosome, ribosomal subunit, and cytosol. DEGs in the MF category were mostly involved in structural components of the ribosome and cadherin binding (Fig. 4A). Thus, the analysis indicated that DEGs are primarily involved in the biological functioning of ribosomes, suggesting that the KRP-DOX complex may exert its anti-tumor effects in OS cells via ribosome-related pathways.

### 3.4 Signaling pathway enrichment analysis

To further elucidate the mechanism of KRP-DOX activity against OS, KEGG analysis of DEGs was conducted. The 20 most significantly enriched path entries of upregulated and downregulated DEGs identified by KEGG are shown in Fig. 4B. The selection standards were the \( p \)-value and the \( q \)-value; the closer the values were to zero, the more significant the enrichment. Among these pathways, those related to ribosomes, glycolysis/gluconeogenesis, and biosynthesis of amino acids were most significantly enriched. Additionally, the highest level of enrichment was identified for eukaryotic translation elongation, formation of a pool of free 40S subunits, and peptide chain elongation. These findings suggest that the ribosome pathway was significantly different from other pathways identified by the analysis. This is consistent with the GO results and supports the possibility that KRP-DOX may inhibit OS growth by affecting ribosomal pathways.

### 3.5 Construction of PPI network

The results discussed above, in combination with the analysis of functions of DEGs by GeneCards, indicated that the most likely DEG related to ribosomes was ribosomal protein S6 kinase A2 (\( RPS6KA2 \)). Interactions between the identified DEGs were examined by constructing a PPI network in which DEG-encoded proteins were selected by the STRING database, and pairs with a combined score higher than 700 were extracted for visualization by Cytoscape (Fig. 5A). In this figure, the yellow nodes indicate the downregulated genes while upregulated genes are shown by orange nodes. The PPI results demonstrated that \( RPS6KA2 \) was significantly associated with MAPK8, suggesting that the anti-tumor effect of the KRP-DOX complex could be related to the MAPK8 signaling pathway.

### 3.6 q RT-PCR and Western blot

Quantitative RT-PCR and Western blot assays were performed to measure the expression levels of the candidate biomarker, \( RPS6KA2 \), in OS cells after KRP-DOX treatment. Treatment with KRP-DOX was seen to inhibit \( RPS6KA2 \) expression in these cells (Fig. 5B,C).

### 4. Discussion

We have previously demonstrated that after KRP-DOX injection, OS xenograft-bearing mice showed almost no dissociated DOX in their circulation. This indicated that normal tissues are protected from the toxic side effects of DOX in the KRP-DOX treatment group. Additionally, KRP-DOX could change the
biodistribution pattern of the small molecule DOX from simply spreading into multiple organs to targeted accumulation through enhanced permeability in solid tumors and the retention effect (EPR effect). Binding to tumor cells is achieved through electrostatic interaction between the positively charged KRP and the cell's negative surface charge, allowing KRP-DOX to be internalized by cancer cells and avoiding lysosomal degradation. KRP-DOX can also enter the nuclei of tumor cells, triggering their death (Yu et al., 2019).

In the present work, gene expression was analyzed in three groups of microarray data of OS tissues obtained from mice treated with KRP-DOX, DOX-HCl, or saline. Comparison of the KRP-DOX and DOX-HCl groups identified a total of 790 common DEGs, including 463 upregulated and 327 downregulated genes, with \( P < 0.05 \) and \([\log 2 \text{ FC}] > 1\). Subsequently, both upregulated and downregulated DEGs were subjected to GO term enrichment analysis for functional annotation. The GO analysis classified the DEGs into three groups: BP, CC, and MF. These analyses showed that the functions of the DEGs are related to ribosomes. Ribosome biogenesis is essential for the survival, growth, and proliferation of cells. Numerous studies have confirmed that the hyperactivation of ribosome biogenesis plays a vital role in the development and progression of cancer. Ribosome biogenesis is generally found to be more active in tumor cells due to the need to adapt to the increased metabolic demands of neoplastic cells. In addition, the ribosome acts as a central information hub, regulated by many oncogenic signaling pathways (Catez et al., 2019; Pelletier et al., 2018; Penzo et al., 2019). Several studies on OS have demonstrated an association between increased ribosomal biogenesis and poor prognosis (Montanaro et al., 2007). The DEG analysis showed that \( RPS6KA2 \) (one of the major DEGs) was closely related to ribosome biogenesis and, therefore, may be involved in the anti-tumor effect of the KRP-DOX complex (Gianferante et al., 2017; Kim et al., 2013; Mitchell et al., 2015).

The analysis of the interrelationships between pathways showed that most DEGs were associated with pathways related to ribosomes, glycolysis/gluconeogenesis, or biosynthesis of amino acids. The important role of increased ribosome biogenesis and protein synthesis in maintaining tumor cell growth and proliferation has been discussed previously in the literature (Prakash et al., 2019). Furthermore, recent studies have shown that tumorigenesis is driven not only by an increased number but also by modifications of ribosomes (de Las et al., 2014; Kim et al., 2017; Sulima et al., 2017). Consistent with the current results, the ribosomal pathway was found to be one of the most significant pathways in an OS polygenic interaction study. The KRP-DOX complex may inhibit the growth of OS by affecting ribosome biogenesis.

In addition, the analysis of differentially expressed genes has shown that RPS6KA2 is closely related to ribosome biogenesis and, therefore, may be involved in the anti-tumor effect of the KRP-DOX complex (Montanaro et al., 2007). In combination with a review of the literature, it was concluded that the downregulated \( RPS6KA2 \) was closely related to ribosomal processes. The \( RPS6KA2 \) gene encodes ribosomal protein S6 kinase A2, which is an important member of the ribosomal protein S6 kinase (S6Ks) family. Biochemical and genetic studies have established that S6Ks are major kinases responsible for the phosphorylation of ribosomal protein S6 (RPS6) \textit{in vivo} (Pende et al., 2004). Studies of the
phosphorylation levels of RPS6 in rps6ka2-deficient mouse cells have found that RPS6KA2 is an essential S6K responsible for the phosphorylation of RPS6 (Chauvin et al., 2014). RPS6 is an important component of the 40S subunit of ribosomes, and RPS6 post-translational modification plays an important role in the biogenesis of ribosomes. In addition, studies have reported that over 75% of the transcription factors associated with ribosomal biogenesis are regulated by S6Ks (Orsolic et al., 2016), including Nop14, Nop56, Gar1, Rrp9, Rrp15, Rrp12, and Pwp2 nucleoli proteins. Thus, current studies suggest that RPS6KA2 gene expression plays an important role in ribosomal biogenesis. On the other hand, qRT-PCR and Western blot analysis verified that the expression of RPS6KA2 was significantly downregulated by KRP-DOX treatment in OS. The finding that the ribosomal biogenesis-related oncogene RPS6KA2 was differentially and significantly downregulated in the KRP-DOX group raises the hypothesis that the KRP-DOX complex may inhibit ribosome biogenesis by downregulating RPS6KA2, thus enhancing the anti-tumor effect. In the past decade, studies have indicated that the over-activation of ribosomal biogenesis plays an important role in tumor development, and drugs that inhibit ribosome biogenesis may provide a new means for tumor treatment (Orsolic et al., 2016), including Nop14, Nop56, Gar1, Rrp9, Rrp15, Rrp12, and Pwp2 nucleoli proteins. Thus, current studies suggest that RPS6KA2 gene expression plays an important role in ribosomal biogenesis. On the other hand, qRT-PCR and Western blot analysis verified that the expression of RPS6KA2 was significantly downregulated by KRP-DOX treatment in OS. The finding that the ribosomal biogenesis-related oncogene RPS6KA2 was differentially and significantly downregulated in the KRP-DOX group raises the hypothesis that the KRP-DOX complex may inhibit ribosome biogenesis by downregulating RPS6KA2, thus enhancing the anti-tumor effect. In the past decade, studies have indicated that the over-activation of ribosomal biogenesis plays an important role in tumor development, and drugs that inhibit ribosome biogenesis may provide a new means for tumor treatment (Ma et al., 2011; Park et al., 2016). The activation of RPS6KA2 leads to the phosphorylation of functionally diverse p90RSK substrates in the cytoplasm and nucleus, thereby promoting the occurrence and development of carcinomas. The members of the P90RSK family have been shown to be overexpressed or hyper-activated in several cancers including breast cancer, lung cancer, and OS. Given that RPS6KA2 increases cell apoptosis, enhances cell chemosensitivity, inhibits proliferation and migration, and suppresses tumor formation, this protein might be a potential target of biotherapy for OS (Hirashita et al., 2016; Poomakkoth et al., 2016). Additionally, the downregulation of RPS6KA2 affects the activity of human OS cells through inactivation of the AKT/mTOR signaling pathways (Qiu et al., 2016). Pathway cards revealed that the RPS6KA2 upstream of AKT/ mTOR is ERK1/2, and that downstream is mTORC1.

Thus, it is hypothesized that the KRP-DOX complex downregulates RPS6KA2, inhibiting the MAPK-RPS6KA2-mTOR signaling pathway, and consequently blocks ribosomal biogenesis, ultimately inhibit tumor growth (Fig. 6).

In summary, RPS6KA2 is significantly associated with an anti-OS effect and can serve as a potential biomarker and therapeutic target for OS. Further studies are necessary to identify the specific molecular mechanisms by which RPS6KA2 affects the initiation and progression of OS.
**Declarations**

**Author Contributions:**

Hua Wang, Mei Yu and Jiameng Liu conceived and designed the study; Zhiyuan Lu, Yiyang Chen, Dongjie Zhang, Rui Liang performed data analysis; Mei Yu and Jiameng Liu wrote the paper.

**Conflicts of Interest:**

The authors declare no conflict of interest.

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Figures
Figure 1
Flow chart of study. Data collection, processing and analysis were shown in the flow chart. DEGs: differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PPI: protein–protein interaction; RPS6KA2: ribosomal protein S6 kinase A2; qRT-PCR: Quantitative reverse transcription-PCR.

Figure 2
Biodistribution and Antitumor Efficacy of KRP-DOX A. Fluorescence intensity within the tumor 6 h after administration; B. After treatment with DOX-HCL and KRP-DOX respectively, tumor growth was significantly inhibited, while the tumor volume in the saline group increased rapidly; C. Confocal laser scanning micrograph (63× oil-immersion objective) showing the distribution of FITC (green fluorescence) and DOX (red fluorescence) from FITC-KRP-DOX in MG63 cells. Both signals can be seen in the cytoplasm and nuclei 2 h after administration.
Figure 3

Volcano plot of the differentially expressed genes. The X coordinate is $|\log_2(\text{fold change})|$ and the Y coordinate is $-\log_{10}(\text{p adj})$. Each dot represents a gene. Red dots are significantly upregulated genes. Green dots are significantly downregulated genes. Blue dots are non-significant genes; B Heatmap of all differentially expressed genes (DEGs). Clustering of the samples is shown at the bottom of the heatmap. DEGs: differentially expressed genes; log2 FC: log2 fold change.

Figure 4
GO functional and KEGG pathway enrichment analyses. A. Histogram of GO enrichment analysis of DEGs; B. Histogram of KEGG enrichment analysis of DEGs.

**Figure 5**

A. The protein–protein interaction network for the differentially expressed genes (DEGs); B. q RT-PCR verified that KRP-DOX significantly downregulated RPS6KA2 expression in OS cells; C. Western blot showing levels of RPS6KA2 in OS cells after treatment with KRP-DOX.
Figure 6

Proposed mechanism of KRP-DOX inhibition of osteosarcoma.

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