RPL34 regulates osteosarcoma cells proliferation through c-Myc/RPL34 signaling axis

Piwei Huang

Guangxi Medical University First Affiliated Hospital

Minghui Wei

Guangxi Medical University First Affiliated Hospital

Shufan Ji

Guangxi Medical University First Affiliated Hospital

Mitra Fowdur

Guangxi Medical University First Affiliated Hospital

maolin he (✉ hemaolin@stu.gxmu.edu.cn)

Guangxi Medical University First Affiliated Hospital

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Abstract

Background

Ribosomal protein L34 (RPL34) is a member of the L34E ribosomal protein family containing zinc finger domains. This protein plays a key role in regulating the apoptosis, cell cycle progression and proliferation of various cancer including osteosarcoma (OS). The purpose of this study is to clarify the expression of RPL34 in osteosarcoma cells and its molecular mechanism of regulating osteosarcoma cells.

Methods

The expression levels of c-Myc and RPL34 were detected by qRT-PCR and Western blot. Luciferase reporter assays and chromatin immunoprecipitation (ChIP) were used to analyse the binding site of c-Myc and RPL34.

Results

The results showed that c-Myc binds to the E-box region in the RPL34 promoter to regulate RPL34 expression. The results indicated that RPL34 regulates osteosarcoma cells proliferation through c-Myc/RPL34 signaling axis. This research may provide new ideas for targeted therapy of OS.

Conclusion

RPL34 regulates osteosarcoma cells proliferation through c-Myc/RPL34 signaling axis.

Introduction

OS is the most common form of bone cancer, which most commonly affects children and young adults [1]. There is no therapeutic agent currently approved for the targeted treatment of metastatic OS, and outcomes for patients with advanced disease have not improved significantly in the last several decades [2]. The treatment of OS is complicated by the rapid proliferation and metastatic progression, making it a challenge to treat effectively. Therefore, the lack of recent advances in the chemotherapeutic or surgical treatment of OS led us to conduct a study aimed at better understanding the molecular mechanism of OS.

c-Myc is a well-studied transcription factor that functions as a proto-oncogene, being capable of promoting the expression of 10-15% of human genes [3–5]. Dysregulated c-Myc expression or activity is a common facet of oncogenesis [6], with overexpression of this protein resulting in significant changes in the regulation of processes including proliferation [7], cellular differentiation [8], apoptosis [9] and self-renewal [10]. In many cases, several cancers including lung [11], breast [9, 12, 13] and liver cancers have been shown to be driven by c-Myc activity [14]. Similarly, c-Myc has been shown to play key oncogenic roles in OS, functioning to promote cell proliferation [15]. Furthermore, c-Myc is known to directly regulate ribosome biogenesis [16–19], at least in part via a mechanism whereby it controls the auxiliary factor

expression essential for rRNA processing, ribosome assembly, and the nuclear-to-cytoplasmic transport of mature ribosomal subunits [20, 21]. By directly driving enhanced rRNA and ribosomal protein (RPS, RPL, and associated cofactors) expression, c-Myc is also capable of inducing protein synthesis [17, 22–24]. Consistent with these mechanistic functions, c-Myc dysregulation is closely associated with the enhanced expression of ribosomal proteins including RPS3, RPL3, RPL6, RPL23, RPL35 and RPL44 [17, 25].

RPL34 is a ribosomal protein from the L34E family that is dysregulated in oral squamous cell carcinoma [26], non-small cell lung cancer cells [27], and esophageal cancer [28]. Several different proteins have been shown to play essential roles in the regulation of ribosome biosynthesis, such as PI3K [29], MYC [30], mTOR [31], as these factors can directly interact with the micro processing complex. However, how c-Myc regulated the expression of RPL34 has not been definitively established. We previously detected elevated RPL34 levels in OS, and in a review of the UCSC database c-Myc and MAX (Myc associated factor X) were predicted to be capable of regulating RPL34 transcription [32]. Both c-Myc and MAX can dimerize and regulate the proliferation, differentiation, and apoptosis of cells. This led us to hypothesize that RPL34 may regulate the proliferation and apoptosis of OS cells via c-Myc/RPL34 signaling axis. Therefore, in the present study, we sought to better clarify the mechanisms whereby RPL34 regulates the proliferation of osteosarcoma in order to explore novel prognostic or therapeutic biomarker of OS.

Materials And Methods

Cell culture

The Saos-2 and U2OS cells (The Culture Collection of the Chinese Academy of Sciences, China) were cultured in DMEM (Gibco, US) with 10% fetal bovine serum (Gibco, US) containing 1% penicillin-streptomycin Solution (Gibco, US) and placed in a 5% CO₂ humidified incubator at room temperature.

Plasmid transfection and gene knockdown with siRNA

The RPL34 (GenBank accession NM. 000995.4) promoter sequence was cloned into pGL3-Basic Vector having the Mammalian/XbaI sites, while the c-myc (GenBank accession NM. 002467.4) CDS sequence was cloned into pcDNA3.1+ having the KpnI/BglII sites. Successful cloning was then confirmed via sequencing alignment and restriction enzyme digestion, after which plasmids and siRNAs were separately transfected into cells with Lipofectamine 2000 (Invitrogen, USA). Cells were then grown for 6 h, after which media was exchanged for fresh complete media and cells were incubated for a further 24 h. Primers (Shanghai Tuo Ran Biological Technology, China) were as follows: c-Myc-si-1, 5'-CGACGAGACCUUCAUCAAAdTdT-3' and 5'-UUUGAUGAAGGUCUCG UCGdTdT-3'; c-Myc-si-2, 5'-CGAUGUUGUUUCU GUGGAAdTdT-3' and 5'-UUCCACAGAAACAACAUCGdTdT-3'; c-Myc-si-3, 5'-GGAACUAUGACCUCGACUAdTdT-3' and 5'-UAGUCGAGGUCAUAGUUC CdTdT-3'.

qRT-PCR assay

Cellular RNA was isolated using RNAiso Plus reagents (Takara, Japan), after which a 5x Prime Script RT Master Mix (Takara, Japan) was used to prepare cDNA using 500ng of RNA from each sample. qRT-PCR was then performed using 50ng of each cDNA sample with a real-time PCR machine (ABI, USA) using a SYBR GREEN kit (ABI, USA). The PCR cycle parameters were set as follows: 40 cycles of 50°C for 3min, 95°C for 3min, 95°C for 10 s and 60°C for 30s. GAPDH was used for normalization purposes, and relative gene expression was assessed via the $\Delta\Delta C_t$ method [33]. Primers (Gene Wiz, China) were as follows: c-Myc, 5'-CCTG GTGCTCCATGAGGAGAC-3' and 5'-CAGACTCTGACCTTTTGCCAGG-3'; RPL34, 5'-GTTTGACATACCGACGTAGGC-3', 5'-GCACACATGGAACCACC ATAG-3'; GAPDH, 5'-TGACAA CTTTGGTATCGTGGAAGG-3' and 5'-AGGCAG GGATGATGTTCTGGA GAG-3'.

Western blot

Western blot assays were performed as described previously [34] using the following antibody: anti-RPL34 (BBI; 1:1000); anti-c-Myc (CST; 1:1000); Goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch; 1:5000); Goat anti-mouse IgG secondary antibodies (Jackson ImmunoResearch; 1:5000); GAPDH (Proteintech; 1:5000).

Chromatin immunoprecipitation

A Chromatin immunoprecipitation Kit (Millipore, USA) was used based on provided directions. Briefly, Saos-2 cells were added to 10 cm culture plates, then 1% formaldehyde was used to cross-link DNA and proteins within these cells for 10 minutes, after which glycine was added (0.125 M final concentration) to terminate this reaction. Cells were then collected, centrifuged, resuspended in a lysis buffer, and a 100 μ L volume of each lysate underwent sonication to shear the DNA into fragments of approximately 500 bp. A total of 25 μ L of each sample was then reserved as input, while the remaining fractions were mixed for 1 h with protein G agarose at 4°C. Samples were centrifuged and then incubated with 5 μ g primary antibody (CST, USA) or normal IgG overnight with rotation at 4 °C. Then, supernatants were reicubated for 1 h using protein G agarose at 4 °C. Elution was then performed twice to collect purified protein-DNA complexes. Next, 5 M NaCl was added overnight to interfere with cross-linking between chromatin and proteins. RNase A and proteinase K treatments were then used to isolate DNA from input and immunoprecipitated samples, after which the following primers were used to analyze 1 μ L of each sample via qRT-PCR: LEF1-chip: 5'-ATGATGAAGTCAAGCCACTGC-3' and 5'-GCTTAT AACCCACCGAAAGGC-3'; RPL34-chip: 5'-AGGAA AGTTGAGTGGGTA-3' and 5'-GACTTGAGTGTTGGCTTAT-3'.

Dual-Luciferase assay

Using kits (Promega, USA), dual-luciferase reporter assays were performed thrice based on provided directions. Briefly, co-transfection into osteosarcoma cells was done with 500 ng of a pcDNA3.1 carrying c-Myc CDS sequence, 500 ng of a pGL3 reporter containing RPL34 promoter sequence and 500 ng of a pRL-TK (Promega, USA) having Renilla luciferase. Then the luciferase activity of the cells was measured after 24 h.

Identification of RPL34-interacting genes using bioinformatics

In order to identify Transcription factors (TFs) predicted to regulate RPL34 expression, human TF-gene pairs were identified using the UCSC ENCODE Genome Browser (<http://genome.ucsc.edu>). The STRING database was then used to construct a protein-protein interaction network for RPL34 [35], with an interaction pair threshold score of > 0.9 , and with Cytoscape used for visualization purposes [36]. This analysis revealed 11 TFs predicted to regulate RPL34 expression, including MYC and MAX[32], both of which can dimerize to regulate cellular proliferation, differentiation and apoptosis.

Statistical analysis

Data are presented as mean $\pm$ standard deviation, and experiments in this study were conducted in triplicate. Results were compared via one-way ANOVA, with GraphPad Prism 5.0 (CA, USA) or SPSS 20.0 (Chicago, US) used for all statistical testing. $P < 0.05$ was the significance threshold.

Results

The expression of RPL34 in OS cells is positively correlated with the expression of c-MYC

To evaluate the role of c-Myc in the regulation of RPL34 expression, we looked/probed for a possible connection between c-Myc and RPL34. Firstly, to examine whether the expression of RPL34 correlated with c-Myc in response to transfect Saos-2 and U2OS cells with pcDNA3.1+c-Myc plasmid (Figure 1A-C; $p < 0.01$). As observed in the figure, the results showed that expression of RPL34 in both cell lines were significantly elevated (Figure 1D-F; $p < 0.01$). As a result of the positive correlation between c-Myc and RPL34, we further addressed whether RPL34 could be downregulated by the transcription factor c-Myc. To this end, to silence the expression of c-Myc, Saos-2 and U2OS cells were transfected with siRNAs. It was found that suppression of the expression level of RPL34 correlated with c-Myc suppression (Figure 2A-F; $p < 0.01$). Overall, the results showed that c-Myc enhances the RPL34 expression at both the mRNA and protein levels. For all experiments, GAPDH served as a normalization control.

c-Myc binds the E-box region in the RPL34 promoter to regulate RPL34 expression in OS cells

Next, in order to verify whether c-Myc binds to the E-box sequence (5'-CACGTG-3') in the RPL34 promoter, a ChIP-qPCR experiment was performed in Saos-2 cells. It was found that the enrichment of RPL34 was similar to LEF1 by detecting c-Myc CHIP products, indicating that RPL34 can be used as the CHIP binding product of c-Myc (Table I). LEF1 was selected as the positive control. Furthermore, CHIP western blot showed that while c-Myc did not bind to GAPDH, a negative control, c-Myc was found to be bounded to the E-box sequence (5'-CACGTG-3') in the RPL34 promoter in Saos-2 cells (Figure 3C).

Finally, to evaluate whether c-Myc regulates RPL34 transcription through the E-box sequence (5'-CACGTG-3') in RPL34 promoter region, we performed luciferase reporter assays. The results showed that luciferase

activity for a reporter carrying the RPL34 E-box region was significantly upregulated in the presence of c-Myc in Saos-2 (Figure 3A; $P < 0.01$) and U2OS (Figure 1B; $P < 0.01$) cells. These results thus confirmed the ability of c-Myc to directly drive enhanced expression of RPL34 via binding to the E-box region within the RPL34 promoter in OS cells.

Table I ChIP-qPCR

	Input	ERα IP	%(ChIP/Input)	Negative control (IgG)(Ct)	%(Negative control/Input)	Fold Enrichment
LEF1	21.580	27.321	0.373924329	N/A	0.001825077	204.8813446
RPL34	21.580	27.321	0.434180382	N/A	0.001819113	238.6769786

Discussion

In this study, a novel mechanism in which c-Myc can bind to the E-box region in the RPL34 promoter was identified.

As a proto-oncogene with key roles in a wide range of cancers, c-Myc is noted to be central regulator of cellular proliferation, differentiation, growth and apoptosis [19, 36–38]. Previous researches have clearly demonstrated that c-Myc can regulate a number of different proteins that are involved in ribosome biosynthesis and translation, including ribosomal proteins themselves as well as associated translation initiation and elongation factors, and other proteins important for ribosome formation [37, 38]. Furthermore, immunotherapy has made great progress in cancer treatment in recent years. Using antibodies to block the programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway is a good treatment option [39]. Recent studies have found that c-Myc promotes high expression of PD-L1 by directly binding to its promoter, which can help tumor cells evade immune surveillance [40–43], including in osteosarcoma [44]. These findings not only indicated that c-Myc plays an important role in the pathogenesis of malignant tumors, but also suggested that the immune response against cancer can be restored by inhibiting the expression and activity of c-Myc. While we had hypothesized that c-Myc may function as a regulator of RPL34 transcription [32], confirmation of this hypothesis and the underlying molecular basis for such regulatory activity remained to be determined. In this study, the levels of RPL34 protein and mRNA in OS cells, are increased with overexpression of c-Myc and are reduced with knockdown of c-Myc. ChIP and luciferase reporter assays were used to confirm that c-Myc can directly bind to an E-box motif within the RPL34 promoter to regulate RPL34 expression. Together these findings thus revealed that c-Myc can function as a positive regulator of RPL34 expression in OS cells owing to its ability to bind to this E-box motif within the promoter region governing RPL34 transcription. In addition, whether c-Myc promotes tumor cells to evade immune surveillance by regulating the expression of RPL34 requires further experiments.

In the past, RPL34 was considered to be a highly conservative protein, but recent studies have shown that RPL34 dysregulation is an aspect of many different cancers and is closely related to the strong

proliferation ability of these cancer cells [26–28, 32]. Indeed, previous work has shown RPL34 to be upregulated in OS tissue samples relative to normal bone tissues, functioning in an oncogenic manner in these tumor cells [32]. In this study we provided evidence that c-Myc can directly bind to the RPL34 promoter, thereby regulating the transcription of RPL34. Therefore, it is possible that c-Myc may in part enhance OS cell proliferation via this regulatory mechanism, potentially highlighting this pathway as a novel means of target therapy for OS.

Conclusion

RPL34 regulates osteosarcoma cells proliferation through c-Myc/RPL34 signaling axis.

Abbreviations

RPL34: Ribosomal protein L34; OS: osteosarcoma; ChIP: chromatin immunoprecipitation; MAX: Myc associated factor X; TFs: Transcription factors.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

PH and MW designed and carried out the study. PH and JS participated in the experiments and statistical analysis. MW and MF wrote the manuscript. MH revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

No potential conflicts of interest were disclosed.

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Figures

Figure 1

Overexpression c-Myc upregulates the expression of RPL34 in OS cells. A-F: The mRNA and protein levels of RPL34 was significantly increased in Sao-2 and U2OS cells transfected with pcDNA3.1+c-Myc plasmid to upregulate c-Myc. **p < 0.01 compared with the mock group, ##p < 0.01 compared with the pcDNA3.1+ group.

Figure 2

Knockdown c-Myc suppresses the expression of RPL34 in OS cells. A-F: Transfected with siRNAs, Sao-2 and U2OS cells were used to knockdown c-Myc. And following c-Myc knockdown, the expression level of RPL34 was clearly downregulated at both the protein and mRNA levels. ** $p < 0.01$ and ## $p < 0.01$ compared with the mock group and si-NC group respectively.

Figure 3

c-Myc binds to the RPL34 promoter in OS cells in order to regulate its expression. A-B: Saos-2 and U2OS cells were transfected with pGL3 reporter carrying the E-box region in the RPL34 promoter and the control were transfected with pcDNA3.1+or pcDNA3.1+ c-Myc. The luciferase activity induced by c-Myc up-regulation were significantly higher than that of the control group. pRL-TK served as the internal control.

****p** < 0.01 compared with the RPL34+pRL-TK group, **##p** < 0.01 compared with the RPL34+pRL-TK+pcDNA3.1+ group. C: Chromatin immunoprecipitation was carried out with Saos-2 cells. Chromatin from Saos-2 cells was incubated with c-Myc or RPL34 antibody. An unrelated IgG antibody was used as negative control. D: A schematic representation of a luciferase reporter construct comprising of an E-box region in the RPL34 promoter [13].