IRF2 Destabilizes Oncogenic KPNA2 to Modulate the Tumorigenesis of Osteosarcoma via Regulating NF- κ B/p65

Shuchi Xia

Zhongshan Hospital, Fudan University

Yiqun Ma (✉ ma.yiqun@zs-hospital.sh.cn)

Zhongshan Hospital, Fudan University

Research Article

Keywords: Osteosarcoma, Karyopherin alpha 2, Interferon regulatory factor-2, NF- κ B p65

Posted Date: August 2nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-701134/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Osteosarcomas (OS) are the most frequent primary malignant bone tumor. Emerging evidence revealed that karyopherin alpha 2 (KPNA2) was strongly associated with the tumorigenesis and development of numerous human cancers. The aim of the present study was to investigate the expression pattern, biological functions and underlying mechanism of KPNA2 in OS.

Methods: Bioinformatics TFBIND online was applied to forecast the transcription factor (TF) binding sites in the promoter region of KPNA2. The expression profile of KPNA2 in OS tissues were firstly assessed using TARGET dataset. The expression of KPNA2 in clinical OS samples and normal human adjacent samples were analyzed by RT-qPCR and western blot. CCK8, colony formation, wound-healing, and Transwell assays were used to assess cell viability, proliferation and migration *in vitro*, and *in vivo* experiments were performed to explore the effects of KPNA2 and interferon regulatory factor-2 (IRF2) on tumor growth. In addition, the correlation between IRF2 and KPNA2, and their roles on the NF- κ B/p65 was investigated using chromatin immunoprecipitation (ChIP), RT-qPCR, western blot and dual-luciferase assays.

Results: KPNA2 was obviously upregulated while IRF2 was significantly decreased in OS tissues and cell lines, as well as they were negatively correlated with each other. KPNA2 knockdown remarkably suppressed OS cell growth, migration, invasion *in vitro* and tumor growth *in vivo*, while IRF2 knockdown exerts an opposing effect. IRF2 binds to KPNA2 promoter to modulate the tumorigenic malignant phenotypes of OS via regulating NF- κ B/p65 signaling.

Conclusion: The present study demonstrated that KPNA2 performed the oncogenic function, possibly regulating tumorigenesis through NF- κ B/p65 signaling pathway. Importantly, IRF2 was confirmed to serve a potential upstream TF of KPNA2 involving in the regulation of NF- κ B/p65 pathway in OS.

Introduction

Osteosarcoma (OS) is the frequent primary malignant bone tumor, affecting mainly pediatric and adolescents (1), which is composed of malignant mesenchymal cells producing osteoid and/or immature bone (2). It typically formed in the metaphysis of long bones, specifically the proximal tibia, distal femur, and proximal humerus accompanied by bone swelling and pain (3). It is common that metastasis to the lungs of OS (4). Before the use of neoadjuvant and adjuvant chemotherapy, approximately 90% of OS patients died due to pulmonary metastases (5). OS is characterized by high levels of genomic instability. However, the molecular basis involved in OS remains unclear, and continuing to seek new treatments is urgently needed.

Karyopherin alpha 2 (KPNA2, 58 kDa) is one of seven members of the karyopherin α -family (6, 7); Dysregulation of KPNA2 had been reported serving as a potential biomarker in several malignancies, including breast cancer (8), gastric cancer (9), lung cancer (10) and glioma (11). KPNA2 served as the adaptor to transfer p65 to the nucleus to identify the classic nuclear localization signal (7, 12). A Previous

study had shown that KPNA2 interacts with p65, and facilitated the NF- κ B p65/p50 nuclear transportation in TNF- α -stimulated lung cancer cells (13). As is well-known, NF- κ B/p65 signaling is rest and concentrated in the cytoplasm until being activated and migrating to the nucleus in response to stress. NF- κ B is involved in the regulation of cell growth and survival, and its abnormal activation is associated with the malignant progression of various cancers (14, 15), includes OS (16). Although, a recent study revealed that KPNA2 promoted NF- κ B activation and thus subsequently accelerate the development of osteoarthritis (17). However, the molecular pathways regulated by KPNA2-mediated NF- κ B/p65 in OS are yet to be elucidated.

To better understand the molecular mechanism of KPNA2 in OS, we first applied an online database of TARGET to seek KPNA2-related factors and ultimately found that the interferon regulatory factor-1 (IRF2) expression was positively correlation with KPNA2 in OS tissues. Besides, transcription factors (TFs) are a kind of proteins that specifically recognized DNA through consensus sequences, thus to control chromatin and transcription, guiding expression of the genome (18). To identify the TF of KPNA2 transcription, the online software of TFBIND (<http://tfbind.hgc.jp/>) was employed to identify putative binding sites in the promoter region of KPNA2. Specifically, several canonical IRF2-binding sites on the promoter region of KPNA2 were observed and IRF2 was then extracted from 147 candidate genes. IRF2, belonging to the IRF family, was widely expressed in various tissues (19). Among several cancer types, belong to IRF family, IRF1 signaling pathways may directly induced p21-dependent G0/G1 cell cycle arrest and p21-independent modulation of surviving (20). IRF2 was proved to serves as an important regulator in acute myeloid leukemia by targeting INPP4B (21). Recent study has indicated that IRF2 was able to suppress the strengthen of cell migration and invasion in OS, which were mediated by miR-18a-5p (22). Similarly, *IRF2* was not expressed or at a low level in OS tissues (23). Intriguingly, IRF was already identified as a functional TF in non-small-cell lung cancer (NSCLC) that suppressed KPNA2 expression (24). Therefore, we speculate that IRF2 may negatively regulated KPNA2 as its upstream TF to modulate OS progression by a p65-dependent signaling.

This study aimed to investigate the role of IRF2 in modulating KPNA2 expression, which may serve an important role in p65 nuclear importation in the progression of OS. Here, we found that KPNA2 deficiency suppressed the malignant behaviors of OS cells, and that the underlying mechanisms involved were regulated by IRF2 and associated with NF- κ B signaling pathway by p65 nucleus translocation. Taken together, our findings demonstrated that KPNA2 may serve as a new potential prognostic indicator and therapeutic target for OS.

Materials And Methods

Cell culture

Four human OS cell lines (Saos-2, HOS, U2OS and MG-63) and human normal osteoblastic cell line (hFOB 1.19) were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco)

harboring with 10% fetal bovine serum (FBS) (Gibco). All OS cell lines were cultured in a humidified incubator under 5% CO₂ at 37 °C, while hFOB1.19 cells were grown at 34 °C.

Patients and tissue specimens

Twenty-five paired tumor samples and their adjacent non-tumor tissues from patients who had undergone surgery were obtained from Zhongshan Hospital, Fudan University. This study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University (Y2014-185) according to the Declaration of Helsinki, and written informed consents were obtained from all the patients. No patients had undergone chemotherapy, radiation therapy or other related targeted therapy before surgery. The diagnosis of OS was confirmed by at least two pathologists. All surgical tissue samples used in our study were immediately placed in liquid nitrogen and then stored at -80 °C until use.

RT-qPCR

Total RNA was isolated from OS cell lines and tissue specimens with the TRIzolTM Reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA (cDNA) synthesis was conducted using the PrimeScript RT reagent kit (Takara) for mRNA expression analysis. The cDNA was applied to perform qRT-PCR assay with SYBR Premix Ex Taq kit (TaKaRa) following the protocols. The 2^{- $\Delta\Delta C_t$} method was used to analyze the difference of mRNA level among different groups. Primers used in this study were listed as follows: KPNA2, 5'-ATTGCAGGTGATGGCTCAGT-3' (forward) and 5'-CTGCTCAACAGCATCTATCG-3' (reverse); IRF2, 5'-CATGCGGCTAGACATGGGTG-3' (forward) and: 5'-GCTTTCCTGTATGGATTGCCC-3' (reverse); The GAPDH was used as an internal control and was detected using the following primers: 5'-AATCCCATCACCATCTTCC-3' (forward) and 5'-AGTCCTTCCACGACCAA-3' (reverse).

Lentiviral vector encoding shRNA plasmids

KPNA2 cDNA was cloned into the Lenti-OE vector (Genepharma, Shanghai, China) to generate KPNA2-overexpressing lentiviral vectors. Short hairpin RNAs (shRNAs) were designed by the RiboBio Co., Ltd. (Guangzhou, China). Lentiviral vectors encoding KPNA2 and IRF2 were synthesized and packaged by Genepharma company.

Cell proliferation assay

The cell proliferation assay was performed using a Cell Counting Kit (CCK-8, Dojindo, Japan) following the manufacturer's instructions. Cells at a density of 5 x10³ were added into the 96-well plate and 10 microliters of CCK-8 solution was added to each well at 1, 2, 3, 4 and 5 days at 37°C. An additional 1 h later, the absorbance at wavelength of 450 nm was then measured under a microplate reader.

Colony formation assay

For the colony formation assay, 500 cells were plated into each well of a 6-well culture plate. The plates containing DMEM were incubated at 37°C for 2 weeks. After washed with PBS for three time, cells were fixed by 4% paraformaldehyde for 10 min at room temperature, followed by staining with 0.5% crystal violet solution for another 20 min. Lastly, the visible colonies more than 50 cells were manually counted and imaged under a microscope.

Transwell invasion assay

Cells at the density of 1×10^4 were seeded into a diameter Transwell palte with 8- μ m pores (Sigma-Aldrich). The upper chamber of the plate was added with 50 μ l of Matrigel collagen, and 600 μ L of complete DMEM was added to the lower chambers, and then the cells were incubated for 24 h. The cells on the upper layer were removed and the invasive cells were fixed with 4% formaldehyde for 20 min, and then stained with crystal violet for 15 min. Cells that had invaded the bottom surface of the filter were counted to assess the invasive ability. Invaded cells were quantified by at least five fields of view under a light microscopy (Leica) to obtain the representative images.

Wound healing assay

Cells were cultured in six-well plates. After reaching 90% confluence, a 200 μ l pipette tip was used to create the scratch wounds in the cell monolayer. Representative images of cell migration were photographed under a light microscopy (Leica) at 0 and 24 h after wounding. The migration ability was assessed by measuring changes in the size of the wound width or area with Image J software.

Western blot assay

The protein was lysed from tissues and cells with RIPA buffer (Thermo Fisher Scientific). Protein's concentration was assessed by the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific). Equal amounts of protein samples were separated by 12% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After incubating with 5% not-fat milk for 2 h at room temperature, to hatch the blots with the primary antibodies including anti-KPNA2, IRF2, NF-kB p65, p-NF-kB p65, GAPDH overnight at 4°C. Hatching the horseradish peroxidase-conjugated secondary antibodies at romm temperature. The endogenous GAPDH is the internal reference protein. The protein band signals were visualized on an ECL detector (Pierce) and quantified by scanning the densitometry using image J software.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a kit (Sigma-Aldrich) following the protocol provided by manufacturer. To hatch the diluted DNA-protein complex, the antibodies of anti-IRF2 and mouse IgG (Sigma-Aldrich) were added in the presence of protein A/G beads and incubated at 4°C overnight. RT-qPCR assay was applied to examine the ChIP DNA samples. IgG was the negative control.

Dual luciferase test

The wild (WT) and -mutant (MUT) of KPNA2 were inserted into the pGL3 promoter vector, which was transfected into U2OS and MG-63 cells using Lipofectamine 2000 (Invitrogen) along with plasmid of IRF2 overexpression or empty plasmid (NC). 48 h later, luciferase activity was measured using a dual-luciferase reporter assay system (Promega) following the manufacture's protocols.

Tumor xenograft assay

All animal experiments were compliant with the Guide for the Institutional Animal Care and Use Committee (IACUC) of Zhongshan Hospital, Fudan University (2018-014). Equal number of indicated U2OS cells (5×10^5) were subcutaneously implanted into the right flank of 6-week-old female athymic nude mice (n=10/per group). Tumors were formed and the mice were anesthetized and sacrificed 18 days post-tumor inoculation. After removed, the tumors were imaged and weighed, and the volume of tumors was monitored every 3 or 5 days and calculated as follows: $\text{Volume (mm}^3\text{)} = (\text{length} \times (\text{width}^2)/2)$.

Histological analysis and immunohistochemistry

Xenotransplant tumor samples were isolated and fixed in 4% paraformaldehyde. Paraffin was used to embed the tumor tissues and then sectioned into a thickness of 5- μm . The paraffin sections were dewaxed, hydrated, and then stained with hematoxylin and counterstained with eosin. Antigen were retrieved by citrate buffer and blocked with 3% H₂O₂, immunohistochemistry (IHC) was performed with diluted primary KI67 antibody overnight at 4°C, followed by incubation with secondary antibody at room temperature. The slides were developed by diaminobenzidine (DAB) and stained with hematoxylin. IHC staining pictures were obtained under a light microscope.

Statistical analysis

All obtained data are expressed as the mean $\pm$ standard deviation (SD). Differences Student's t-test or one-way ANOVA followed by a Tukey's post hoc test were used to compare data between two group or among multiple groups. Statistical difference was analyzed using GraphPad Prism 8.0 software. followed by a Tukey's post hoc test.

Results

Overexpression of KPNA2 in the osteosarcoma tissues

Based on the TARGET database, we previously revealed KPNA2 expression was notably upregulated in the OS tissues when compared with the non-tumor tissues (Figure 1A). To examine whether KPNA2 altered in clinical OS, qRT-PCR assay was performed to examine KPNA2 levels in 25 paired of cancerous OS samples and their adjacent normal samples. Figure 1B displayed that the mRNA levels of KPNA2 was obviously up-regulated in OS samples compared with that normal samples. Representative IHC images showed similar results (Figure 1C). Furthermore, the protein levels of KPNA2 in 8 cases of OS samples were obviously elevated as well (Figure 1D). Besides, compared to hFOB1.19 cells, a higher level of KPNA2 in four OS cell lines, including U2OS, HOS, Saos2 and MG-63, was able to be observed (Figure 1E). KPNA2 expression was the highest in U2OS and MG-63, which were chosen to use for subsequent analyzes. These results suggest that KPNA2 might play a vital role in the progression of OS.

Transcription factor IRF2 specifically regulates KPNA2 expression in osteosarcomas

Considering that the regulation of KPNA2 in OS focused on the transcriptional level, we performed bioinformatic analysis for seeking a transcription factor (TF) in the KPNA2 promoter region. Then we extracted 147 TFs of KPNA2 from online dataset. Data from TARGET datasets showed total of 9792 genes were negatively associated with KPNA2 in OS ($R > 0.2$, $FDR < 0.05$). We further found 5950 genes were overlapped in down-regulated genes from GSE157322 dataset, and 31 genes were overlapped in 147 TFs of KPNA2. Therefore, a total of 26 genes were at the intersection of three (Figure 2A). According to the correlation index of these 26 genes with KPNA2, we chosen the top 5 TFs of KPNA2, includes RFX1, STAT3, IRF2, PPARG and MZF1. To assess whether these five TFs could alter KPNA2 expression, we overexpressed these TFs in two OS cell lines. As shown in Figure 2B, these results demonstrated that only overexpression of IRF2 significantly suppressed KPNA2 expression in two OS cell lines, while other four TFs had no significant effect on the KPNA2 expression. Meanwhile, due to KPNA2 was overexpressed in four OS cell lines, we also determined the change of these five TFs after KPNA2 knockdown using RT-qPCR. Silence of KPNA2 markedly upregulated the mRNA levels of IRF2 while other four TFs had not obviously changed response to KPNA2 knockdown (Figure 2C). Therefore, we decided to use IRF2 for subsequent experiments.

Through the TARGET dataset, IRF2 expression was mildly downregulated in OS tissues without significant difference (Figure 2D), while IRF2 expression was strongly negatively correlated with KPNA2 (Figure 2E). Consistently, the mRNA level of IRF2 was significantly downregulated in 25 cases of clinical OS tissue samples when compared with adjacent normal tissues (Figure 2F). Besides, a ChIP test was conducted in two OS cell lines and hFOB1.19 to evaluate the binding relationship of IRF2 with KPNA2, and we found that the enrichment of IRF2 binding was prominently decreased in two OS cell lines

comparing to the hFOB1.19 cells (Figure 2G). The present findings showed that IRF2 might be one of major regulators to regulate KPNA2 expression in OS. To further determine if IRF2 bound to the KPNA2 promoter, a Dual-luciferase assay revealed that IRF2 was able to dramatically reduce the luciferase activity of KPNA2-WT, but not in KPNA2-MUT (Figure 2H). Collectively, IRF2, as a functional TF, could bind to the KPNA2 in OS cells.

IRF2 deficiency may cooperate with KPNA2 to regulate cell proliferation and tumor growth of OS cells *in vivo* and *in vitro*

Since a negative correlation existed among KPNA2 and IRF2, we investigate their effects on cell proliferation, migration, invasion, and cell cycle. Firstly, in four OS cell lines, IRF2 protein expressions were lower than that in hFOB1.19 cells (Figure 3A). Down-regulation of IRF2 or KPNA2 in U2OS cells was achieved by transfections of IRF2 or KPNA2 knockdown vectors (shKPNA2 and shIRF2), as confirmed by western blot (Figure 3B). Regarding the malignant phenotypes of OS cells, KPNA2 knockdown inhibited the cell viability, proliferation while IRF2 knockdown had the opposite effects and partially rescued above-mentioned malignant phenotypes suppressed by KPNA2 knockdown in vitro (Figure 3C-D). In vivo, KPNA2 knockdown remarkably reduced tumor weight and tumor volumes while IRF2 knockdown promoted tumor growth, and IRF2 knockdown could weaken the KPNA2 knockdown-mediated tumor growth inhibition (Figure 3E and 3F). The Ki67 expression was reduced by KPNA2 knockdown while elevated by IRF2 knockdown (Figure 3G). These findings demonstrated that IRF2 silence partially attenuates the impact of KPNA2 knock-down on OS growth.

IRF2/KPNA2 might regulate migration and invasion of in osteosarcoma cells in a p65-dependent manner

Regarding the malignant phenotypes of migration and invasion, KPNA2 knockdown inhibited the abilities of migration and invasion while IRF2 knockdown had the opposite effects and partially rescued above-mentioned malignant phenotypes suppressed by KPNA2 knockdown OS cells (Figure 4A-B). Due to KPNA2 was able to activate NF- κ B/p65 signaling pathway (25), we further clarify the expressions of NF- κ B/p65 and p-NF- κ B/p65 in OS cells after transfected or co-transfected with shKPNA2 or/and shIRF2. The protein level of p-NF- κ B/p65 was apparently decreased by shKPNA2 with a slight change of NF- κ B/p65 expression (Figure 4C). However, the activation protein of p-NF- κ B/p65 increased in shIRF2-infected cells; more importantly, IRF2 silence could obviously attenuate the inhibitory effect of KPNA2 silence on the p-NF- κ B/p65 expression (Figure 4C). Thus, IRF2 could negatively alter KPNA2 by directly binding to the KPNA2 promoter and participated in OS progression through NF- κ B/p65 signaling pathway.

Discussion

Osteosarcomas is relatively rare but devastating (26). Unfortunately, the introduction of novel adjuvant chemotherapy after aggressive surgical resection has temporarily improved overall 10-year survival, but has not significantly improved patient survival since the 1990s (27). Therefore, there is of great significance to identify novel molecules, which further helps to develop effective methods to diagnose and treat this malignant bone tumor. Here, we draw a conclusion that IRF2 binding to KPNA2 to regulate malignant biological properties of OS cells via regulating NF- κ B/p65 signaling pathway. This evidence may provide new ideas for the diagnosis and treatment of osteosarcoma.

Recently, several studies have linked KPNA2 to various cancers, such as lung, breast, colon cancer. High KPNA2 was positively related to cancer invasiveness and poor prognosis, thus regarded KPNA2 as a potentially relevant therapeutic target for patients with different cancers (28). KPNA2 was involved in several cellular biological processes, including cell differentiation, development, viral infection, immune response and transcriptional regulation (29). Similarly, our study illustrated that KPNA2 was dramatically elevated in OS samples comparing to normal samples. Although KPNA2 was proved to frequently express in OS as a novel marker for the diagnosis, as well as in chondrosarcoma and Ewing sarcomas (30), the functions of KPNA2 in osteosarcoma is unclear. In the present study, data mining and bioinformatics analysis indicated that KPNA2 was overexpressed in OS patients from TARGET dataset, and the experiments verified high KPNA2 level in clinical OS samples and OS cell lines. Additionally, KPNA2 knockdown inhibited the proliferation, migration and invasion in two OS cell lines, and remarkably reduced tumor weight and tumor volumes in vivo. These findings revealed that KPNA2 might play a crucial role in the biological progress of OS.

Interferon regulatory factor-2 (IRF2) exerted anti-tumor effects in several human cancers. For instance, *IRF2* could suppress cell proliferation and migrate ability, and promote cell apoptosis in non-small cell lung cancer cells (31). IRF2 might play as a tumor suppressor by regulating P53 signaling in gastric cancer (32). Besides, *IRF2* was proved to serve as a tumor suppressor in patients with hepatocellular carcinoma, whose inactivation led to impaired TP53 function (33). The current study highlighted that KPNA2 could negatively alter the expression of *IRF2* in OS cells. Meanwhile, data mining in the GSE157322 and TARGET datasets, we discovered that *IRF2* could bind to the promoter of KPNA2 and activate KPNA2 expression through bioinformatics analysis for TF prediction. This underlying mechanism was consistent with a previous report that IRF2 could bind to miR-1227 promoter, thus inhibited tumor growth (23). Besides, *IRF2* was downregulated obviously, which was negatively associated with KPNA2 in OS. More importantly, IRF2 knockdown promoted malignant behaviors, which were subsequently suppressed by KPNA2 knockdown. These rescuing effect of IRF2 on KPNA2 was also reflected in tumor growth in vivo. These findings demonstrated that IRF2 silence partially attenuated the impact of KPNA2 knock-down on osteosarcomas progressions.

Previously, researches had shown that KPNA2 played a pivotal role in melanoma development through activating NF- κ B/p65 signaling pathways (25). In addition, IRF could cooperate with NF- κ B p65 to

promote the efficacy of T cell-related immunotherapy in neuroblastoma, which revealed a synergistic regulatory relationship between IRF and p65 (34). What's more, IRF2 could regulate NF- κ B activity via the modulation of NF- κ B subcellular localization (35). In our study, the deletion of KPNA2 reduced p-NF- κ B/p65 expression, and futile total NF- κ B/p65 expression, whereas the opposite results were found after knockdown of IRF2. KPNA2 and IRF2 had opposite regulatory effects on the activation of NF- κ B/p65 pathway. Coincidentally, our results also confirmed that the inhibitory effect of KPNA2 was partially recovered by IRF2 silence. Thus, elevated KPNA2 contributes to the progression of OS by negatively regulating IRF2 via NF- κ B/p65 pathway.

Conclusions

Taken together, our results illustrated that KPNA2 regulated OS development, as well as IRF2 play a potential upstream TF of KPNA2 in regulating the transcription of NF- κ B/p65. This may provide a novel target for OS therapy. Therefore, treatment of OS with restraint of KPNA2 or NF- κ B/p65 signaling can be extra to other therapeutic interventions for the development of this disease.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University (Y2014-185) according to the Declaration of Helsinki, and written informed consents were obtained from all the patients.

Consent for publication

Not applicable.

Availability of data and materials

All the data in the results of this study can be obtained on reasonable request from the corresponding authors.

Conflict of interest

The Authors declare that they have no conflict of interests.

Funding

Authors' Contribution

Conceived and designed the experiments: Yiqun Ma. Performed the experiments: Shuchi Xia. Analyzed the data: Shuchi Xia and Yiqun Ma. Contributed reagents/materials/analysis tools: Shuchi Xia and Yiqun Ma. Wrote the paper: Shuchi Xia. All authors read and approved the final manuscript.

Acknowledgments

We salute all the teachers and colleagues for their valuable advice.

References

1. Kansara M, Teng MW, Smyth MJ, Thomas DM. Translational biology of osteosarcoma. *Nature Reviews Cancer*. 2014;14(11):722–35.
2. Ritter J, Bielack SS. Osteosarcoma. *Annals of Oncology*. 2010;21:vii320-vii5.
3. Taran SJ, Taran R, Malipatil NB. Pediatric Osteosarcoma: An Updated Review. *Indian J Med Paediatr Oncol*. 2017;38(1):33–43.
4. Ferguson JL, Turner SP. Bone Cancer: Diagnosis and Treatment Principles. *American family physician*. 2018;98(4):205–13.
5. Simpson E, Brown HL. Understanding osteosarcomas. *Journal of the American Academy of PAs*. 2018;31(8).
6. Han Y, Wang X. The emerging roles of KPNA2 in cancer. *Life Sciences*. 2020;241:117140.
7. Kelley JB, Talley AM, Spencer A, Gioeli D, Paschal BM. Karyopherin $\alpha 7$ (KPNA7), a divergent member of the importin α family of nuclear import receptors. *BMC Cell Biology*. 2010;11(1):63.
8. Dahl E, Kristiansen G, Gottlob K, Klamann I, Ebner E, Hinzmann B, et al. Molecular Profiling of Laser-Microdissected Matched Tumor and Normal Breast Tissue Identifies Karyopherin $\alpha 2$ as a Potential Novel Prognostic Marker in Breast Cancer. *Clinical Cancer Research*. 2006;12(13):3950.
9. Altan B, Yokobori T, Mochiki E, Ohno T, Ogata K, Ogawa A, et al. Nuclear karyopherin- $\alpha 2$ expression in primary lesions and metastatic lymph nodes was associated with poor prognosis and progression in gastric cancer. *Carcinogenesis*. 2013;34(10):2314–21.
10. Wang C-I, Chien K-Y, Wang C-L, Liu H-P, Cheng C-C, Chang Y-S, et al. Quantitative Proteomics Reveals Regulation of Karyopherin Subunit Alpha-2 (KPNA2) and Its Potential Novel Cargo Proteins in Nonsmall Cell Lung Cancer. *Molecular & Cellular Proteomics*. 2012;11(11):1105.
11. Li J, Liu Q, Liu Z, Xia Q, Zhang Z, Zhang R, et al. KPNA2 promotes metabolic reprogramming in glioblastomas by regulation of c-myc. *J Exp Clin Cancer Res*. 2018;37(1):194-.

12. Guo L, Kim HJ, Wang H, Monaghan J, Freyermuth F, Sung JC, et al. Nuclear-Import Receptors Reverse Aberrant Phase Transitions of RNA-Binding Proteins with Prion-like Domains. *Cell*. 2018;173(3):677 – 92.e20.
13. Liang P, Zhang H, Wang G, Li S, Cong S, Luo Y, et al. KPNB1, XPO7 and IPO8 Mediate the Translocation of NF- κ B/p65 into the Nucleus. *Traffic*. 2013;14(11):1132–43.
14. Baeuerle PA, Baltimore D. NF- κ B: Ten Years After. *Cell*. 1996;87(1):13-20.
15. Zhang Q, Lenardo MJ, Baltimore D. 30 Years of NF- κ B: A Blossoming of Relevance to Human Pathobiology. *Cell*. 2017;168(1–2):37–57.
16. Liao D, Zhong L, Duan T, Zhang R-H, Wang X, Wang G, et al. Aspirin Suppresses the Growth and Metastasis of Osteosarcoma through the NF- κ B Pathway. *Clinical Cancer Research*. 2015;21(23):5349.
17. Tao R, Xu X, Sun C, Wang Y, Wang S, Liu Z, et al. KPNA2 interacts with P65 to modulate catabolic events in osteoarthritis. *Experimental and Molecular Pathology*. 2015;99(2):245–52.
18. Pan Y, Tsai C-J, Ma B, Nussinov R. Mechanisms of transcription factor selectivity. *Trends Genet*. 2010;26(2):75–83.
19. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. *Nat Rev Immunol*. 2014;14(1):36–49.
20. Armstrong MJ, Stang MT, Liu Y, Gao J, Ren B, Zuckerbraun BS, et al. Interferon Regulatory Factor 1 (IRF-1) induces p21(WAF1/CIP1) dependent cell cycle arrest and p21(WAF1/CIP1) independent modulation of survivin in cancer cells. *Cancer letters*. 2012;319(1):56–65.
21. Zhang F, Zhu J, Li J, Zhu F, Zhang P. IRF2-INPP4B axis participates in the development of acute myeloid leukemia by regulating cell growth and survival. *Gene*. 2017;627:9–14.
22. Lu C, Peng K, Guo H, Ren X, Hu S, Cai Y, et al. miR-18a-5p promotes cell invasion and migration of osteosarcoma by directly targeting IRF2. *Oncology letters*. 2018;16(3):3150–6.
23. Jiang X, Chen D. Circular RNA hsa_circ_0000658 inhibits osteosarcoma cell proliferation and migration via the miR-1227/IRF2 axis. *Journal of cellular and molecular medicine*. 2021;25(1):510–20.
24. Huang J-X, Wu Y-C, Cheng Y-Y, Wang C-L, Yu C-J. IRF1 Negatively Regulates Oncogenic KPNA2 Expression Under Growth Stimulation and Hypoxia in Lung Cancer Cells. *OncoTargets and therapy*. 2019;12:11475–86.
25. Yang F, Li S, Cheng Y, Li J, Han X. Karyopherin α 2 promotes proliferation, migration and invasion through activating NF- κ B/p65 signaling pathways in melanoma cells. *Life Sciences*. 2020;252:117611.
26. Simpson E, Brown HL. Understanding osteosarcomas. *JAAPA: official journal of the American Academy of Physician Assistants*. 2018;31(8):15–9.
27. Berner K, Johannesen TB, Berner A, Haugland HK, Bjerkehagen B, Böhler PJ, et al. Time-trends on incidence and survival in a nationwide and unselected cohort of patients with skeletal osteosarcoma.

- Acta oncologica (Stockholm, Sweden). 2015;54(1):25–33.
28. Christiansen A, Dyrskjøt L. The functional role of the novel biomarker karyopherin α 2 (KPNA2) in cancer. *Cancer letters*. 2013;331(1):18–23.
29. Han Y, Wang X. The emerging roles of KPNA2 in cancer. *Life sciences*. 2020;241:117140.
30. Jiang L, Liu J, Wei Q, Wang Y. KPNA2 expression is a potential marker for differential diagnosis between osteosarcomas and other malignant bone tumor mimics. *Diagnostic pathology*. 2020;15(1):135.
31. Liang C, Zhang X, Wang HM, Liu XM, Zhang XJ, Zheng B, et al. MicroRNA-18a-5p functions as an oncogene by directly targeting IRF2 in lung cancer. *Cell Death Dis*. 2017;8(5):e2764.
32. Chen YJ, Wu H, Zhu JM, Li XD, Luo SW, Dong L, et al. MicroRNA-18a modulates P53 expression by targeting IRF2 in gastric cancer patients. *Journal of gastroenterology and hepatology*. 2016;31(1):155–63.
33. Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat Genet*. 2012;44(6):694–8.
34. Lorenzi S, Forloni M, Cifaldi L, Antonucci C, Citti A, Boldrini R, et al. IRF1 and NF- κ B restore MHC class I-restricted tumor antigen processing and presentation to cytotoxic T cells in aggressive neuroblastoma. *PloS one*. 2012;7(10):e46928-e.
35. Chae M, Kim K, Park SM, Jang IS, Seo T, Kim DM, et al. IRF-2 regulates NF- κ B activity by modulating the subcellular localization of NF- κ B. *Biochem Biophys Res Commun*. 2008;370(3):519–24.

Figures

Figure 1

High KPNA2 expression in osteosarcomas samples. (A) KPNA2 mRNA expression in OS and non-tumor tissues based on data from TARGET dataset. (B) The mRNA levels of KPNA2 in 25 paired of OS samples and their adjacent normal samples were detected by RT-qPCR. (C) Representative immunohistochemically (IHC) images of KPNA2 expression in OS tissues and normal tissues. (D) KPNA2 protein expression in 8 pairs of clinical OS samples were examined by western blotting. (E) KPNA2 expression in four OS cell lines and one normal hFOB1.19 cells was assessed using RT-qPCR assay. The data was presented as the mean $\pm$ SD from three independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 2

Identification of transcription factor (TF) of KPNA2. (A) Venn diagram displayed the overlap among differentially expressed protein-coding genes (DEGs) from GSE157322, KPNA2 negative-related genes from TARGET dataset and TF factors of KPNA2 from online TFBIND dataset. (B) The mRNA expression of KPNA2 in response to overexpression of five candidate genes (RFX1, STAT3, IRF2, PPARG and MZF1) in U2OS and MG-63 cells. (C) The mRNA expression of five candidate genes (RFX1, STAT3, IRF2, PPARG and MZF1) in U2OS-shNC, U2OS-shKPNA2, MG-63-shNC and MG-63-shKPNA2 cells. (D) The mRNA expression of IRF2 in Nontumor and OS tissues. (E) The correlation between KPNA2 and IRF2 expression. (F) The relative mRNA of IRF2 in Normal and OS cells. (G) The relative enrichment fold of IRF2-ChIP and IgG-ChIP in hFOB1.19, MG-63 and U2OS cells. (H) The relative luciferase activity (Firefly/Renilla value) in U2OS and MG-63 cells for KPNA2-WT and KPNA2-MUT.

and MZF1) in response to KPNA2 silence in U2OS and MG-63 cells. (D) IRF2 mRNA expression in OS and non-tumor tissues based on data from TARGET dataset. (E) The negatively correlation between KPNA2 and IRF2 was analyzed under a Pearson correlation analysis according to TARGET database. (F) IRF2 mRNA expression in 25 paired of OS and normal samples. (G) The enrichment of IRF2 bind in the KPNA2 promoter was significantly reduced in two OS cell lines when compared with the hFOB1.19 cells from ChIP and qRT-PCR assays. (H) Luciferase assay was performed to detect the luciferase activity of KPNA2-WT and KPNA2-MUT after IRF2 overexpression in U2OS and MG-63 cells. The data was presented as the mean $\pm$ SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 3

Effects of IRF2 and KPNA2 on cell proliferation, colony formation, tumor growth of OS cells in vitro and in vivo. (A) RT-qPCR analysis was performed to measure the relative mRNA levels of IRF2 in four OS cell lines including U2OS, HOS, Saos2, and MG-63 control to the hFOB1.19 cells. (B) U2OS cells were co-transfected with shKPNA2 or/and shIRF2 plasmids and the protein expression of KPNA2 and IRF2 were assessed using western blotting assay. (C) Cell viability was determined using an CCK-8 kit at different time points. (D) Cells were seeded in plates and grown for 14 days. (Left) Cell colonies were stained with 0.1% crystal violet. (Right) Colony numbers were quantified. (E-F) In vivo tumor growth. U2OS cells were injected into mice, and tumor weight (E) and tumor volumes (F) were measured every 5 day during 18 days post-tumor inoculation. n=10. (G) Tumor tissues were separated from mice for HE and IHC staining for detecting KI67 expression. The data was presented as the mean $\pm$ SD from three independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

Figure 4

KPNA2 promoted the activation of NF-κB/p65 signaling via IRF2. (A) Cell migrate capacity of OS cells was examined using wound healing assays at 0 and 24 h. (B) Cells were stained with 0.1% crystal violet (Left). Bars=50 μm. Cell numbers were quantified to value the invasive capacity of OS cells after different transfection (Right). (C) The protein levels of NF-κB/p65, p-NF-κB/p65 in tumor tissues from xenograft mice. The data was presented as the mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.