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High Expression of *NETO2* in Osteosarcoma Promotes Cell Proliferation and Migration

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Abstract

Increasing researchers have identified that upregulation of neuropilin (NRP)/tolloid (TLL)-like 2 (*NETO2*) is associated with some of human cancers. However, the expression of *NETO2* and its possible function in osteosarcoma (OS) remain unknown. Herein, this study aimed to investigate the expression and potential action of *NETO2* in OS. In this study, the expression of *NETO2* in clinical tissues based on Gene Expression Omnibus (GEO) data and its expression level in OS cells were investigated. The effect of knockdown of *NETO2* using small interference RNA (siRNA) method on OS cells proliferation and migration was assessed by CCK-8 and scratch wound analyses. Key molecules of mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway was measured by western blot assay. Our data determined that the expression of *NETO2* in OS tissues was prominently higher than that in normal tissues. Consistently, overexpression of *NETO2* was detected in OS cells compared with normal cells. Functionally, downregulation of *NETO2* repressed OS cells proliferation and migration, which was modulated by MEK/ERK signaling. Our findings suggest that *NETO2* may be utilized as a marker of OS and become a target for anti-cancer therapy.

Keywords: Osteosarcoma; *NETO2*; Proliferation; Migration

Introduction

Osteosarcoma (OS) is known as the commonest primary malignant bone tumor and causes substantial number of bone cancer-associated deaths in children and adolescents, which is largely ascribed to its recurrence and metastasis [1-5]. There are limited efficacy in current regiments, for example surgical resection and adjuvant chemotherapy in the treating metastatic and recurrent OS [6,7]. Hereby, there is urgent to have a good understanding of its potential molecular mechanism, attempting to develop novel potential therapeutic targets for OS.

Neuropilin (NRP)/tolloid (TLL)-like 2 (*NETO2*), also termed as BTCL2, encodes a brain-specific transmembrane protein. It is identified as an auxiliary subunit with kainate-type glutamate receptors (KARs) to regulate biophysical properties of KARs, including GluK1-K5 [8-11]. It has been documented that expression of KARs is determined in pediatric CNS tumors, which indicates interference with glutamate signaling may suppress tumor growth [12]. There is an evidence demonstrating that a substantial increase in *NETO2* mRNA level is detected in renal cancer and it is found to be upregulated in cervical carcinoma and colon cancer, suggesting *NETO2* is considered as a potential marker for these cancer [13]. Another report has confirmed that upregulation of *NETO2* expression is associated with tumor progression and poor prognosis in colorectal carcinoma [14]. Increased expression of *NETO2* has also been reported to be involved in proliferating hemangiomas [15]. Nonetheless, there is no available information to show *NETO2* influencing OS progression.

To this end, the goal of this study was to explore the possible involvement of *NETO2* in OS proliferation and migration and the potential mechanism involved. In this study, we firstly analyzed the data downloaded from specific Gene Expression Omnibus (GEO) OS tissue datasets. Our data demonstrated that *NETO2* was upregulated in OS tissues compared to normal tissues, thus showing a gene expression pattern of a tumor promoter gene. Elevated expression of *NETO2* was observed in OS cells in contrast with normal cells. Functional analyses showed that knockdown of *NETO2* constructed using small interference RNA (siRNA) approach inhibited OS cells proliferation and migration, which was regulated by inactivation of mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling. Our investigations indicate that *NETO2* exhibits an oncogenic role in OS and thus it might serve as a therapeutic target for OS.

Materials and Methods

Dataset analysis

The mRNA expression patterns in OS tissues and normal tissues available at the GEO datasets (GSE28424, GSE36001 and GSE49003) were analyzed by GEO online analysis tool GEO2R performing differential gene analysis.

Cell lines and cell culture

OS cell line Saos-2, MG-63, and human osteoblast cells hFOB 1.19 were purchased from (Cell Bank of Chinese Academy of Sciences, Shanghai, China). All cells were cultured in Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS, Thermo), penicillin (100 U/ mL), and streptomycin (100 mg/mL; Thermo) in a 37°C incubator with 5% CO_2.

NETO2 knock-down by small interference RNA (siRNA)

siRNAs were chemically synthesized from Genepharma Co. Ltd (Shanghai, China). The sequences (sense/antisense) for the siRNAs were as follows: si-*NETO2* 1: 5'-UAACA GUACU GGUAG UGGA-

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3'/5'-UCCAC UACCA GUACU GUUA-3'; siENTO2 2: 5'-UCAAG CAUAU UCCUG CAAC- 3'/5'-GUUGC AGGAA UAUGC UUGA - 3'. Non-targeting siRNA controls (si-con) : 5'- UCAUA ACGUG GAUCG AUUC -3'/5'- GAAUC GAUCC ACGUU AUGA -3'. The transfection of si-*NETO2* 1/2 and si-con were performed with Lipofectamine 2000 (Thermo) according to the manufacturer's introductions. These cells were then collected 24 h after transfection to identify the efficiency of siRNA by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analyses.

qRT-PCR analysis

Total RNA from cultured cells was extracted using Trizol reagent (Thermo) in accordance with the manufacturer's protocol. qRT-PCR was performed to synthesize and then amplify the cDNA using the PrimeScript™ RT reagent kit and SYBR Premix Ex Taq kit (TaKaRa Biotechnology, Shiga, Japan) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The conditions of qPCR were as follows: 94°C for 10 min, 40 cycles of 94°C for 15 s, 60°C for 30 s. The primers are as follows: *NETO2*: F: 5'-GGCGTGAAAAGCCCTCCATT -3', R: 5'- GCTCCCGAGAGCTCGAA -3'; GAPDH: F: 5'-GGAGCGAGATCCCTCCAA AAT -3', R: 5'-GGCTGTTGTCATACTTCTCATGG-3'. The relative expression levels of the mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Counting Kit-8

Cells treated with si-*NETO2* and si-con was plated at a density of 3×10^3 cells/well in 96-well plates. At the different time points after transfection, 10 µL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well and the cells were incubated for 1 h at 37°C. The optical density (OD) was measured at 450 nm.

Scratch wound assay

Cells were cultured in 6-well plates in complete medium until they reached 90% confluence. The monolayer cell culture was scratched by a 200 µL pipette tip with a uniform wound. The stripped cells were washed away with serum-free culture medium, and the other cells were cultured in medium containing 10% FBS. Images were captured under an Olympus microscope (Olympus, Japan) at 0 and 24 h after scratch. Migration rate was calculated as: (Migrated distance at measured time-initial distance)/Initial distance × 100%.

Western blot analysis

Total proteins from cultured cells were extracted using radio-immunoprecipitation (RIPA) assay lysis buffer (Beyotime Institute of Biotechnology, Shanhai, China). Then, the protein concentration was quantified using bicinchoninic acid (BCA) method (Beyotime). Equal amounts of proteins (20 µg) were subjected to 10-12% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk, followed by incubation with corresponding diluted primary antibodies (anti-MEK, 1:1,000, ; anti-ERK, 1:1,000; anti-phosphorylated (p-)MEK and anti-p-ERK, 1:1000; anti-*NETO2*, 1:1000, Cell Signaling Technology, Danvers, MA, USA; anti-GAPDH, 1:1000, Beyotime) at 4°C overnight. The membranes were incubated with horseradish peroxidase-labeled secondary antibody (Beyotime) at 37°C for 2 h. Proteins were visualized using electrochemiluminescence (ECL) reagents (Pierce Biotechnology, Inc., Rockford, IL, USA), and the scanned images were analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Statistical analysis was done with using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (San Diego, CA, USA). All experiments were performed in triplicate. Data was expressed as mean and standard deviation (SD). Differences between means were studied using a Student *t*-test when two groups were compared. P value<0.05 was considered to be statistically significant.

Results

Upregulated expression of *NETO2* in OS tissues and cells

The analysis of the available clinical including OS tissues/normal and metastatic/ non-metastatic samples showed that a significant increase of *NETO2* in OS tissues relative to normal tissue (Figures 1A-1C, P<0.01). Moreover, to validate our initial expression profiling data, we performed qRT-PCR of OS cells and observed significant enhanced expression of *NETO2* in OS cells compared with normal cells (Figure 1D, P<0.01). These data suggest a potential oncogenic role played by *NETO2* in OS.

NETO2 silencing impairs proliferation ability of OS cells

After identifying and confirming differential expression of *NETO2* in OS tissues and cells, the effect of *NETO2* on OS cells proliferation was determined. Firstly, we successfully knocked down it by observing a decreased level of *NETO2* both in mRNA and protein level after transfection of si-*NETO2* 1/2 (Figures 2A and 2B, P<0.01). We chose one of si-*NETO2* 1/2 which named as si-*NETO2* to perform the subsequent experiments. Furthermore, OD values of OS cells measured by CCK-8 assay showed that reduced OD values was observed in si-*NETO2* group compared with si-con group with a time-dependent manner (Figure 2C, P<0.01). All results indicate that *NETO2* silencing could inhibit OS cell proliferation.

NETO2 silencing decreases migration ability

To further determine *NETO2* influencing OS cell migration capability, scratch wound assay was performed. The results showed that a lower migration distance was detected in si-*NETO2* group on comparing si-con group (Figure 3). The result suggests a suppressive role of *NETO2* silencing in OS cell migration property.

NETO2 silencing inhibited cell proliferation and migration via suppression of the MEK/ERK pathway

Emerging evidence has reported that MEK/ERK signaling pathway is vital of cancers development, including OS. We next examined the underlying molecular mechanism that contributed to the effects of *NETO2* silencing on OS proliferation and migration. After siRNA-mediated silencing of *NETO2* expression, western blot assay showed that detectable decreased changes in p-MEK and p-ERK, but not MEK and ERK, in si-*NETO2* group in contrast with si-con group (Figure 4, P<0.01). Thus, these data suggest that MEK/ERK signaling is of importance in the process of *NETO2*-mediated OS cell proliferation and migration.

Discussion

Here we present the first study of the role of *NETO2* in OS. In the present study, we identified an elevation of *NETO2* in OS tissues and cells. In addition, knockdown of *NETO2* inhibited OS cells proliferation and migration. Molecularly, *NETO2* silencing resulted in a suppressive effect on MEK/ERK signaling pathway in OS cells. Our results indicate

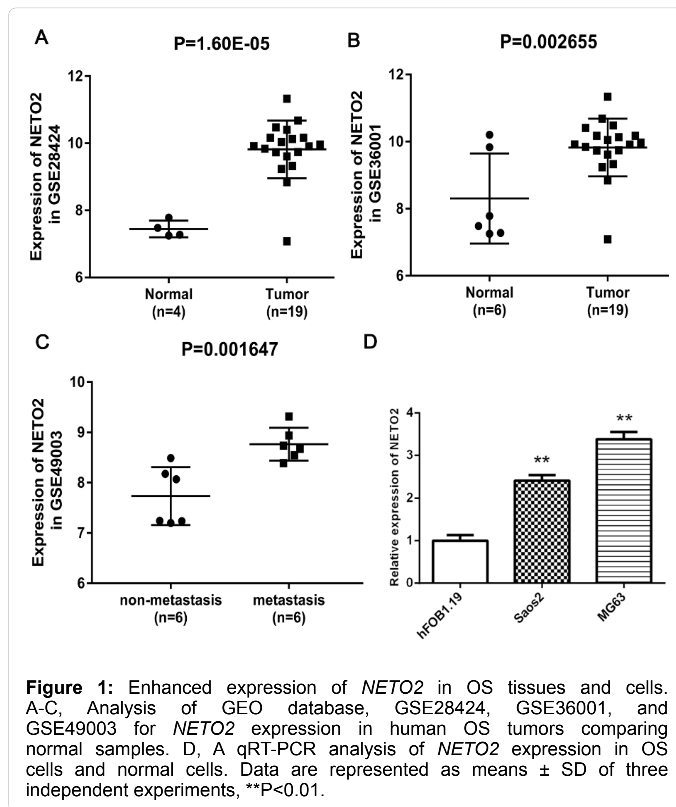

Figure 1: Enhanced expression of *NETO2* in OS tissues and cells. A-C, Analysis of GEO database, GSE28424, GSE36001, and GSE49003 for *NETO2* expression in human OS tumors comparing normal samples. D, A qRT-PCR analysis of *NETO2* expression in OS cells and normal cells. Data are represented as means ± SD of three independent experiments, **P<0.01.

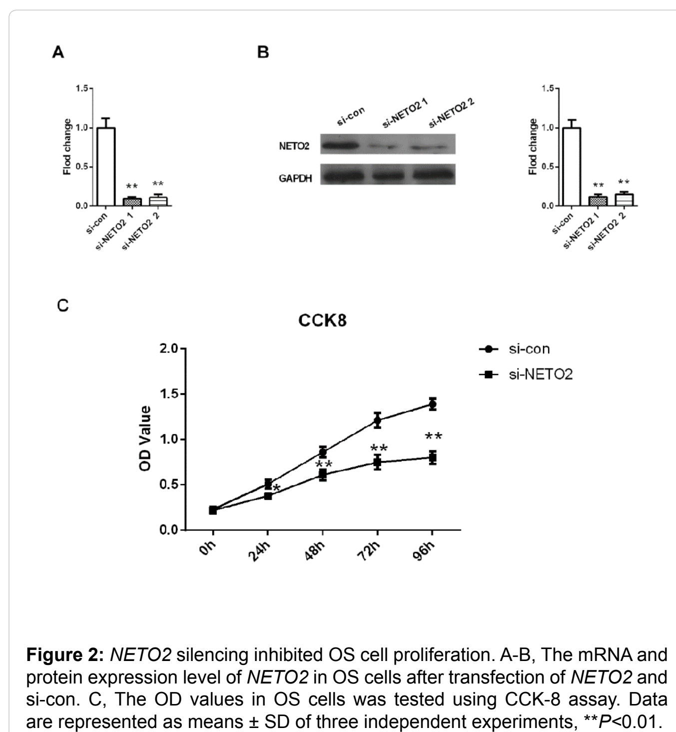

Figure 2: *NETO2* silencing inhibited OS cell proliferation. A-B, The mRNA and protein expression level of *NETO2* in OS cells after transfection of *NETO2* and si-con. C, The OD values in OS cells was tested using CCK-8 assay. Data are represented as means ± SD of three independent experiments, ***P*<0.01.

that *NETO2* may function as an oncogene in OS, implying it will be a potential therapeutic target for the treatment of OS patients.

Cancer initiations and developments is a complex pathogenesis process, implicated various molecules and biological networks, and OS is no exception. It is beyond doubt that bioinformatics provides an expedient tool for studying available molecules. We analyzed herein the mRNA expression patterns in OS tissues and normal samples available from GEO datasets. The data showed that an upregulated level of *NETO2* was identified in OS tissues comparing with normal samples. To confirm it, we performed qRT-PCR to measure *NETO2* mRNA expression level in OS cells. As expected, the result showed that *NETO2* was significantly higher in OS cells than normal cells. Previous studies have suggested that *NETO2* is overexpressed in a variety of cancers, but no reports about effect of it on OS progression. Thereby, this study offers a good insight for comprehending the biological function of *NETO2* on OS.

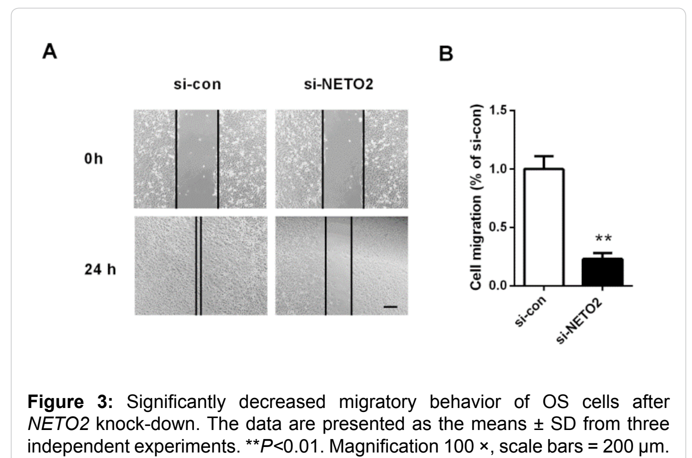

Figure 3: Significantly decreased migratory behavior of OS cells after *NETO2* knock-down. The data are presented as the means ± SD from three independent experiments. ***P*<0.01. Magnification 100 ×, scale bars = 200 μm.

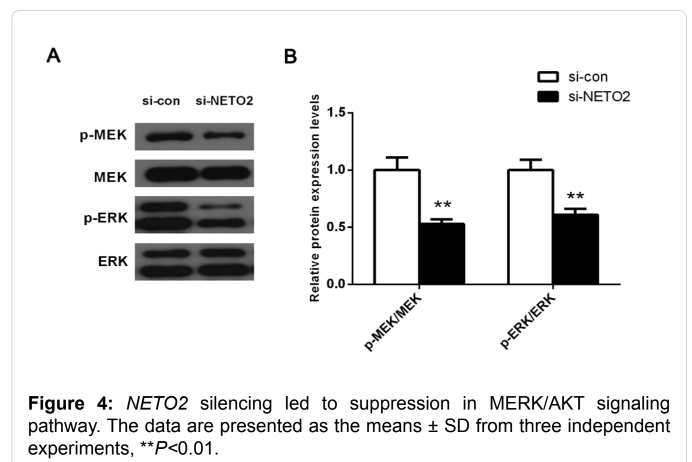

Figure 4: *NETO2* silencing led to suppression in MERK/AKT signaling pathway. The data are presented as the means ± SD from three independent experiments, ***P*<0.01.

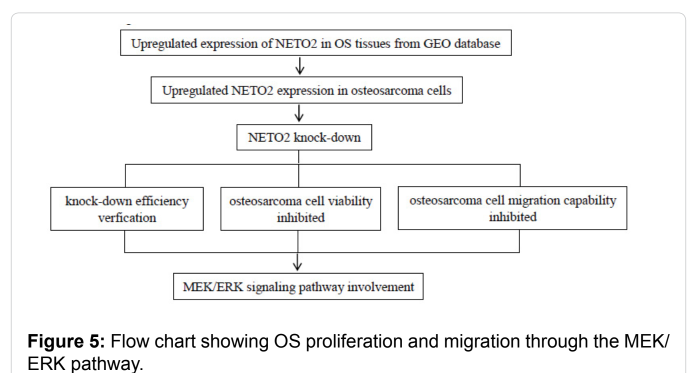

Figure 5: Flow chart showing OS proliferation and migration through the MEK/ERK pathway.

It is well-known that uncontrollable proliferation and aggressive migration are tightly correlated with knotty metastatic and recurrent

OS [16,17]. Hence, to address it, we assessed the effect of *NETO2* on OS proliferation and migration. OD values in OS cells showed that an obvious decreased OD value in si-*NETO2* group was detected on comparing with si-con group. Additionally, the result in scratch wound assay suggested a delayed migration closure in si-*NETO2* group in contrast with si-con group. In short, these data indicate that *NETO2* could exert an inhibitory role in OS proliferation and migration.

Having well-documented that MEK/ERK signaling is crucial for cancer progression, to explore the mechanism underlying *NETO2*-induced OS cell proliferation and migration, we examined its effect on MEK/ERK signaling. In this work, hallmarks of this signaling including MEK/p-MEK and ERK/p-ERK were measured. The result of western blot analysis showed that p-MEK and p-ERK were significantly increased insi-*NETO2* group on comparing si-con group while there were no obvious differences of MEK and ERK in two groups. A previous study has suggested that inhibition in MEK/ERK signaling activity mediates repression in OS metastasis capability [18]. Another report has demonstrated that MEK1/2 and ERK1/2 phosphorylation are involved in Ewing sarcoma metastasis [19]. Altogether, our data in combination with previous studies suggests that MEK/ERK is participated in *NETO2* facilitating OS progression. In spite of this, our current knowledge of the mechanisms responsible for these events and the regulatory components involved is still rudimentary at best and further deeper investigations is necessary [20-28].

Conclusion

Collectively, our present study demonstrated that *NETO2*, which was markedly overexpressed in OS tissues and cells, played a significant role in OS proliferation and migration through the MEK/ERK pathway. A schematic flochart (Figure 5) was used to ravel out our findings. Therefore, these results shed some light on *NETO2* possibly emerging as a promising therapeutic target for treating OS patients in the further clinics. However, our preliminary work still exists some of limitations. Namely, this study is mainly performed in only one cell line, thus more cell lines are required to confirm these determinations. More functional analyses still need in the further studies.

Declaration of Interest

The authors declare that they have no competing interests.

Authors' Contributions

ZMS and TLW designed the study. TLW and TSW performed experiments and wrote the manuscript. XNZ performed the experiments and analyzed the data. All authors have read and agreed the final manuscript.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Consent to Publish

All authors agree to the publication.

References

1. Armakolas, N., Armakolas, A., Antonopoulos, A., Dimakakos, A., Stathaki, M. & Koutsilieris, M.The role of the IGF-1 Ec in myoskeletal system and osteosarcoma pathophysiology.Crit Rev Oncol Hematol, 2016. **108:** 137-147.
2. Berhe, S., Danzer, E., Meyers, P., Behr, G., LaQuaglia, M. P. & Price, A. P. Unusual abdominal metastases in osteosarcoma. J Pediatr Surg Case Rep, 2018. **28:** 13-16.
3. Bhattacharyya, S., Byrum, S., Siegel, E. R. & Suva, L. J. Proteomic analysis of bone cancer: A review of current and future developments. Expert Rev Proteomics, 2007. **4:** 371-378.
4. Botter, S. M., Neri, D. & Fuchs, B. Recent advances in osteosarcoma. Curr Opin Pharmacol, 2014. **16:** 15-23.
5. Brocke, K. S., Staufner, C., Luksch, H., Geiger, K. D., Stepulak, A., Marzahn, J., et al. Glutamate receptors in pediatric tumors of the central nervous system. Cancer Biol Ther 9, 2010. **2:** 455-468.
6. Calicchio, M. L., Collins, T. & Kozakewich, H. P. Identification of signaling systems in proliferating and involuting phase infantile hemangiomas by genome-wide transcriptional profiling. Am J Pathol 2009. **174:** 1638-1649.
7. Cheng, M. L. Iyer, G. Novel biomarkers in bladder cancer. Urol Oncol, 2018. **36:** 115-119.
8. Daw, N. C., Chou, A. J., Jaffe, N., Rao, B. N., Billups, C. A., Rodriguez-Galindo, C., et al. Recurrent osteosarcoma with a single pulmonary metastasis: A multi-institutional review. Br J Cancer, 2015. **112**: 278-282.
9. Durfee, R. A., Mohammed, M. & Luu, H. H. Review of osteosarcoma and current management. Rheumatol Ther, 2016. **3:** 221-243.
10. Gutowski, C. J., Basu-Mallick, A. & Abraham, J. A. Management of bone sarcoma. Surg Clin North Am, 2016. **96:** 1077-1106.
11. He, J. P., Hao, Y., Wang, X. L., Yang, X. J., Shao, J. F., Guo, F. J., et al. Review of the molecular pathogenesis of osteosarcoma. Asian Pac J Cancer Prev, 2014. **15:** 5967-5976.
12. Hu, L., Chen, H. Y., Cai, J., Yang, G. Z., Feng, D., Zhai, Y. X., et al. Upregulation of *NETO2* expression correlates with tumor progression and poor prognosis in colorectal carcinoma. BMC Cancer, 2015. **15:** 1006.
13. Igarashi, K., Yamamoto, N., Shirai, T., Nishida, H., Hayashi, K., Tanzawa, Y., et al. Late recurrence of osteosarcoma: A report of two cases. J Orthop Surg (Hong Kong), 2014. **22:** 415-419.
14. Kafchinski, L. A. Jones, K. B. (2014) MicroRNAs in osteosarcomagenesis. Adv Exp Med Biol **804:** 119-127.
15. Kuijjer, M. L., Hogendoorn, P. C. & Cleton-Jansen, A. M. Genome-wide analyses on high-grade osteosarcoma: Making sense of a genomically most unstable tumor. Int J Cancer, 2013. **133:** 2512-2521.
16. Lagares-Tena, L., Garcia-Monclus, S., Lopez-Alemany, R., Almacellas-Rabaiget, O., Huertas-Martinez, J., Sainz-Jaspeado, M., et al. Caveolin-1 promotes Ewing sarcoma metastasis regulating MMP-9 expression through MAPK/ERK pathway. Oncotarget, 2016. **7:** 56889-56903.
17. Lindsey, B. A., Markel, J. E. & Kleinerman, E. S. Osteosarcoma overview. Rheumatol Ther, 2017. **4:** 25-43.
18. Mittal, N., Kent, P. M. & Ording, J. Metastatic and recurrent bone primary bone cancers. Curr Probl Cancer, 2013. **37:** 215-224.
19. Norimura, S., Kontani, K., Kubo, T., Hashimoto, S. I., Murazawa, C., Kenzaki, K., et al. Candidate biomarkers predictive of anthracycline and taxane efficacy against breast cancer. J Cancer Res Ther, 2018. **14:** 409-415.
20. Oparina, N. Y., Sadritdinova, A. F., Snezhkina, A. V., Dmitriev, A. A., Krasnov, G. S., Senchenko, V. N., et al. Increase in *NETO2* gene expression is a potential molecular genetic marker in renal and lung cancers. Russian Journal of Genetics, 2012. **48:** 506-512.
21. Palacios-Filardo, J., Aller, M. I. & Lerma, J. Synaptic targeting of kainate receptors. Cereb Cortex, 2016. **26:** 1464-1472.
22. Poliakova, M., Aebersold, D. M., Zimmer, Y. & Medova, M. The relevance of tyrosine kinase inhibitors for global metabolic pathways. Cancer, 2018. **17:** 27.
23. Sheng, N., Shi, Y. S., Lomash, R. M., Roche, K. W. & Nicoll, R. A. Neto auxiliary proteins control both the trafficking and biophysical properties of the kainate receptor GluK1. Elife , 2015: **4**: 1
24. Spina, A., Sorvillo, L., Esposito, A., Borgia, A., Sapio, L. & Naviglio, S. Inorganic phosphate as a signaling molecule: a potential strategy in osteosarcoma treatment. Curr Pharm Des, 2013. **19:** 5394-5403.

25. Tang, G., Zhang, Z., Qian, H., Chen, J., Wang, Y., Chen, X., et al. (-)-Epigallocatechin-3-gallate inhibits osteosarcoma cell invasiveness by inhibiting the MEK/ERK signaling pathway in human osteosarcoma cells. J Environ Pathol Toxicol Oncol ,2015. **34:** 85-93.

26. Tang, M., Ivakine, E., Mahadevan, V., Salter, M. W. & McInnes, R. R. *NETO2* interacts with the scaffolding protein GRIP and regulates synaptic abundance of kainate receptors. PLoS One, 2012. **7:** e51433.

27. Varshney, J., Scott, M. C., Largaespada, D. A. & Subramanian, S. Understanding the osteosarcoma pathobiology: A comparative oncology approach. Vet Sci, 2016: **2:** 3.

28. Wyeth, M. S., Pelkey, K. A., Petralia, R. S., Salter, M. W., McInnes, R. R. & McBain, C. J. Neto auxiliary protein interactions regulate kainate and NMDA receptor subunit localization at mossy fiber-CA3 pyramidal cell synapses. J Neurosci, 2014. **34:** 622-628.