



## Anticancer effect of crizotinib on osteosarcoma cells by targeting c-Met signaling pathway

Tao Jia<sup>#</sup>, Mengmeng Cai<sup>#</sup>, Zengkun Wang, Tianxin Chen<sup>\*</sup>

The Second Affiliated Hospital of Harbin Medical University, Harbin, China

<sup>#</sup> Contributed equally to this work

### ARTICLE INFO

#### Original paper

#### Article history:

Received: February 08, 2023

Accepted: May 18, 2023

Published: May 31, 2023

#### Keywords:

Osteosarcoma, c-Met, crizotinib, HGF

### ABSTRACT

C-Met receptor and its ligand hepatocyte growth factor (HGF) are overexpressed in a variety of osteosarcoma cell lines and osteosarcoma pathological samples. It is suggested that c-Met/HGF plays an important role in the development of osteosarcoma. This study aimed to explore the anticancer effect of the c-Met-targeted drug crizotinib on osteosarcoma (OS) cells. The effects of crizotinib on the proliferation of osteosarcoma cells (SaOS2, MG-63 and MNNG) at different concentrations were detected by CCK8. Human osteosarcoma cell line MG-63 was used as an in vitro model to evaluate the effects of 2.5  $\mu$ M crizotinib, 5.0  $\mu$ M crizotinib and DMSO on cell apoptosis, cell cycle, migration and invasion. The expression of the c-Met signaling pathway in osteosarcoma cells was detected by western blot. The results showed that crizotinib inhibited the proliferation of cell lines in a concentration-dependent manner. Crizotinib significantly increased the number of apoptotic cells compared with the control group. Compared with the control group, crizotinib increased G0/G1 phase cells and decreased S phase cells. Compared with the control group, crizotinib inhibited the migration and invasion of osteosarcoma cells and decreased the expression of c-Met/Gab1/STAT5. This study will provide a promising therapeutic target and theoretical basis for the clinical application of crizotinib in osteosarcoma.

Doi: <http://dx.doi.org/10.14715/cmb/2023.69.5.27>

Copyright: © 2023 by the C.M.B. Association. All rights reserved.

### Introduction

Osteosarcoma is the most common primary bone malignancy derived from osteoid or immature bone produced by malignant mesenchymal cells (1-3). Osteosarcoma occurs most frequently in adolescents aged 15 to 19 years, and the etiology is unknown. It is associated with rapid bone proliferation, and a few may be caused by radiation or exposure to alkylating agents (4, 5). Pain, swelling, and limited joint movement are typical signs and symptoms of osteosarcoma. Metastases occur in about 15% of patients, most commonly in the lung, followed by bone, and rarely in the lymph nodes (6, 7). Current treatment options for osteosarcoma, which include neoadjuvant induction chemotherapy before surgery, followed by radical or limb salvage surgery, followed by postoperative adjuvant chemotherapy, cure approximately two-thirds of patients with localized disease (3, 8). Although multidisciplinary therapies have made remarkable progress in the treatment of osteosarcoma, with a 5-year survival rate of 80%, the survival rate for patients with metastatic disease remains low (8). Moreover, high-dose methotrexate (MTX) is highly toxic, has many side effects and is easy to produce drug resistance, which is accompanied by varying degrees of limb function loss, bringing great psychological pressure and heavy economic burden to patients and their families. Therefore, it is urgent to explore more effective and safe treatment strategies.

Mesenchymal-epithelial transition tyrosine kinase receptor, c-Met, and its ligand hepatocyte growth factor (HGF) play a key role in embryonic development, tumor and tissue damage repair (9, 10). Abnormal activation of c-Met/HGF plays an important role in the occurrence and development of a variety of human cancers, regulating a variety of biological processes of cancer cells, such as cell proliferation, survival rate, apoptosis inhibition, migration, invasion, metastasis, and drug resistance (11-13). c-Met is overexpressed in most osteosarcoma tissues (14). The proliferation of osteosarcoma cells is reliant on the overexpression of c-Met, which has been established as a significant contributor to osteosarcoma formation and maintenance in vitro (15). Targeting c-Met/HGF with specific drugs, such as small molecules (e.g., Crizotinib, Tivantinib, and Capmatinib) or antibodies (such as Leronlimab and Onartuzumab), has shown promise in various tumor types (12).

In this study, we demonstrated the anticancer effect of Crizotinib on a variety of osteosarcoma cells. Then, we found that crizotinib promoted apoptosis and inhibited the migration and invasion ability of osteosarcoma cells. In addition, crizotinib decreased c-Met/Gab1/Stat5 protein expression. In this study, the effect and potential mechanism of crizotinib in the treatment of osteosarcoma were investigated through in vitro trials, which provided a promising approach for crizotinib in the treatment of OS patients.

\* Corresponding author. Email: [mengmengcai890526@gmail.com](mailto:mengmengcai890526@gmail.com)

## Materials and Methods

### Cell culture and treatment

Human osteosarcoma cell lines SaOS2, MG-63 and MNNG were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in MEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin mixture. All cells were cultured in an incubator at 37°C and 5% CO<sub>2</sub>.

### Cell proliferation assay

Human osteosarcoma cell lines SaOS2, MG-63 and MNNG cells were cultured to the third generation, digested, centrifuged, re-suspended in the culture medium, thoroughly blown and mixed, and cell count was performed. 3000-5000 cells/well were inoculated in 96-well culture plates. After 24 hours of cell attachment, different concentrations of crizotinib (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 μM) or dimethyl sulfoxide (DMSO) were added. After 72 hours, 10 μL cell counting kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added to each well. After incubation in the incubator for 2 hours, the light absorption value was measured at 450 nm.

### Cell apoptosis assay

MG-63 cells were cultured overnight on 6-well plates at a density of 1×10<sup>6</sup> cells/well, and then crizotinib (2.5, 5.0 μM) or DMSO with different concentrations was added into the medium. Apoptosis analysis was performed 72 h later. Suspension cells were stained with Annexin V and PI with the Annexin V-FITC apoptosis assay kit, detected by Attune NxT flow cytometry, and analyzed by FCS Express software (BD Biosciences, Franklin Lakes, NJ, USA).

### Cell cycle assay

MG-63 cells were cultured overnight on 6-well plates at a density of 1×10<sup>6</sup> cells/well, and then crizotinib (2.5, 5.0 μM) or DMSO with different concentrations was added into the medium. The cells were collected 72 hours later, washed twice with pre-cooled PBS, and fixed with pre-cooled 70% ethanol at 4°C overnight. The cells were then stained with 50 μg/mL PI and 20 μg/mL RNase A at 37°C for 30 min, and analyzed by flow cytometry.

### Cell migration assay

MG-63 cells were evenly seeded in 6-well plates and allowed to form a monolayer overnight. A slit of uniform width was created using a 200 μL pipette tip, followed by washing with PBS buffer solution. The medium was then replaced with serum-free medium containing different concentrations of crizotinib (2.5, 5.0 μM) or DMSO. Cell migration through the slit was observed using an inverted phase contrast microscope (Nikon Eclipse TE2000-S, Tokyo, Japan) at 0 hours and 24 hours.

### Cell invasion assay

A 24-well Transwell cell culture chamber (Corning Costar, Corning, NY, USA, membrane with aperture of 8 μm) was used. The upper chamber was coated with Matrigel. 1×10<sup>5</sup> MG-63 cells were inoculated in each well and serum-free medium was added. 10% FBS was added into the serum-free medium in the lower chamber. Different concentrations of crizotinib (2.5, 5.0 μM) or DMSO were

added to the upper and lower compartments, incubated for 24 h, and cells on the upper compartment were removed. Cells infiltrated into the lower compartment were fixed with methanol, stained with crystal violet, and counted under a light microscope.

### Western blot

MG-63 cells were divided into 6 well plates with 3×10<sup>5</sup> cells per well. After cell adhesion, different concentrations of crizotinib (2.5, 5.0 μM) or DMSO were added. After 72 hours of culture, the protein was extracted for detection. The cells were rinsed with pre-cooled PBS and lysed with radioimmunoprecipitation test buffers (RIPA) containing protease and phosphatase inhibitor, centrifuged at 12000 rpm at 4°C for 15 min and taken supernatant. Protein concentration was determined using a bicinchoninic acid (BCA) protein analysis kit. They were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, transferred to polyvinylidene fluoride (PVDF) membrane (0.22 μm) for closure (Roche, Basel, Switzerland), and incubated overnight at 4°C with the primary antibodies. After incubation with secondary antibodies, immunoreactive bands were developed using Bio-Rad-ChemiDocXRS (Hercules, CA, USA). The gray value is measured by software and analyzed statistically.

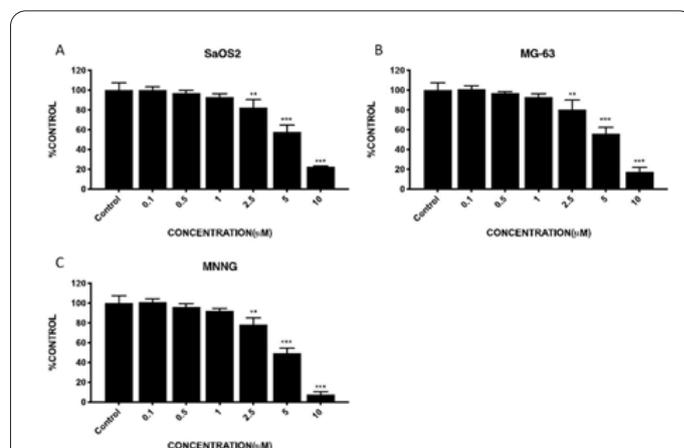
### Statistical analysis

All experiments were repeated three or more times, and relevant data were recorded in statistical software Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA), in which the results after scientific treatment were expressed as mean ± standard deviation. Then *t* test and  $\chi^2$  test were taken, *P*<0.05 indicates statistically significant difference (\*, *P*<0.05; \*\*, *P*<0.01; \*\*\*, *P*<0.001; \*\*\*\*, *P*<0.0001).

## Results

### Crizotinib inhibits the proliferation of osteosarcoma cells

Human osteosarcoma cell lines SaOS2, MG-63 and MNNG were sensitive to crizotinib when different concen-



**Figure 1.** Human osteosarcoma cell lines SaOS2 (A), MG-63 (B) and MNNG (C) were added with different concentrations of crizotinib (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 μM) or DMSO. The cell activity was measured by CCK-8 assay. The data were expressed as mean ± standard deviation from three independent experiments. Compared with the control group, \*\*, *P*<0.01; \*\*\*, *P*<0.001, *t* test.

trations of crizotinib (0.1, 0.5, 1.0, 2.5, 5.0, 10.0  $\mu\text{M}$ ) or DMSO were added, and crizotinib inhibited the proliferation of the three cell lines in a concentration-dependent manner. When the dosage of crizotinib was 2.5  $\mu\text{M}$ , 5.0  $\mu\text{M}$  and 10.0  $\mu\text{M}$ , the difference was statistically significant compared with the Control group (Figure 1).

### Crizotinib promotes apoptosis of osteosarcoma cells

In order to explore the potential mechanism of crizotinib inhibiting the proliferation of osteosarcoma cells, a cell apoptosis experiment was conducted. Human osteosarcoma cell line MG-63 was selected for the experiment. Based on the analysis of CCK-8 results, the dosage of crizotinib was 2.5  $\mu\text{M}$  and 5.0  $\mu\text{M}$ . The experiment was divided into three groups, adding different concentrations of crizotinib (2.5, 5.0  $\mu\text{M}$ ) or DMSO. Flow cytometry showed that crizotinib significantly increased the number of apoptotic MG-63 cells compared with the control group (Figure 2).

### Crizotinib blocks the cell cycle of osteosarcoma

To explore the potential mechanism by which crizotinib inhibits the proliferation of osteosarcoma cells, we also performed cell cycle experiments. After the addition of different concentrations of crizotinib (2.5, 5.0  $\mu\text{M}$ ) or DMSO, the number of G0/G1 phase cells was significantly increased, and the number of S phase cells was significantly decreased by flow cytometry compared with the control group (Figure 3).

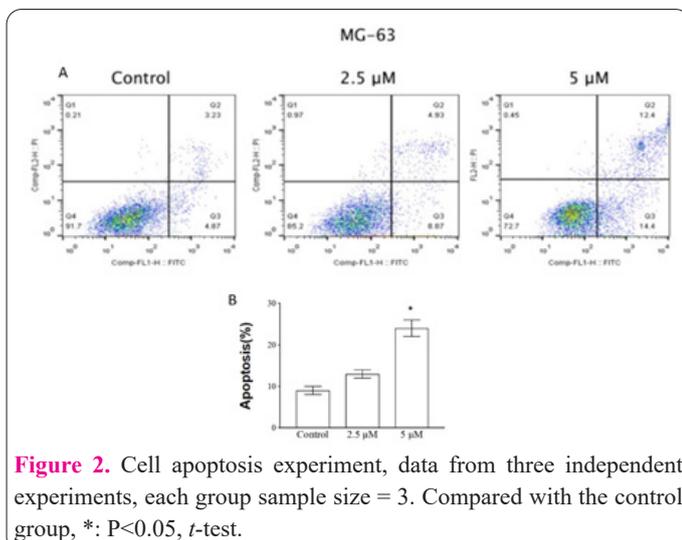
### Crizotinib inhibits the migration and invasion of osteosarcoma cells

To further evaluate the effect of crizotinib on the migration function of osteosarcoma cells, a cell scratch assay was performed. Crizotinib inhibited the migration of osteosarcoma cells compared with controls (Figure 4).

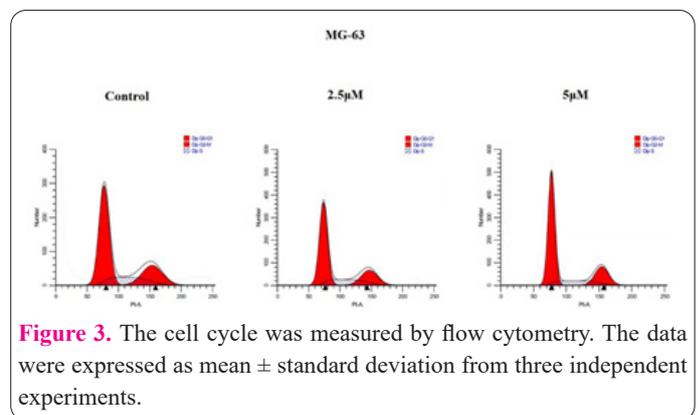
To further evaluate the effect of crizotinib on the function of osteosarcoma cells, the cell invasion assay was also performed. Crizotinib significantly reduced cell invasion compared with the control group (Figure 5).

### Crizotinib inhibits the c-Met signaling pathway

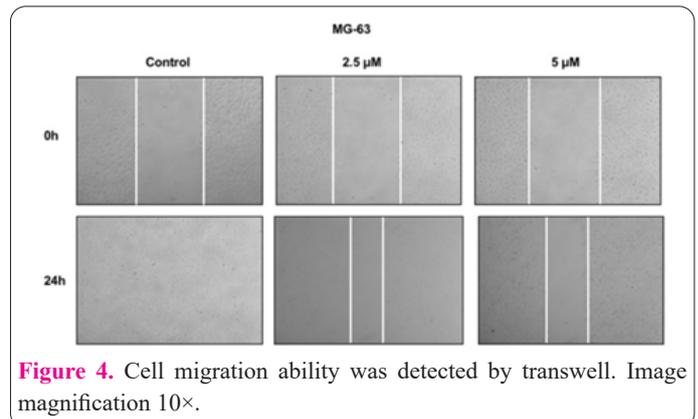
To explore the potential mechanism by which crizotinib inhibits the proliferation of osteosarcoma cells, we evaluated the changes of the c-Met pathway-associated proteins c-Met, Gab1, and Stat5 by Western Blot. Human



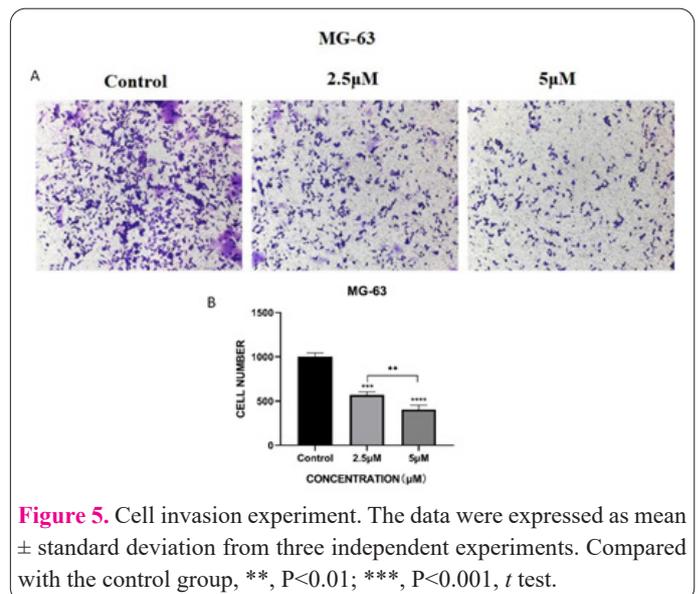
**Figure 2.** Cell apoptosis experiment, data from three independent experiments, each group sample size = 3. Compared with the control group, \*:  $P < 0.05$ ,  $t$ -test.



**Figure 3.** The cell cycle was measured by flow cytometry. The data were expressed as mean  $\pm$  standard deviation from three independent experiments.



**Figure 4.** Cell migration ability was detected by transwell. Image magnification 10 $\times$ .

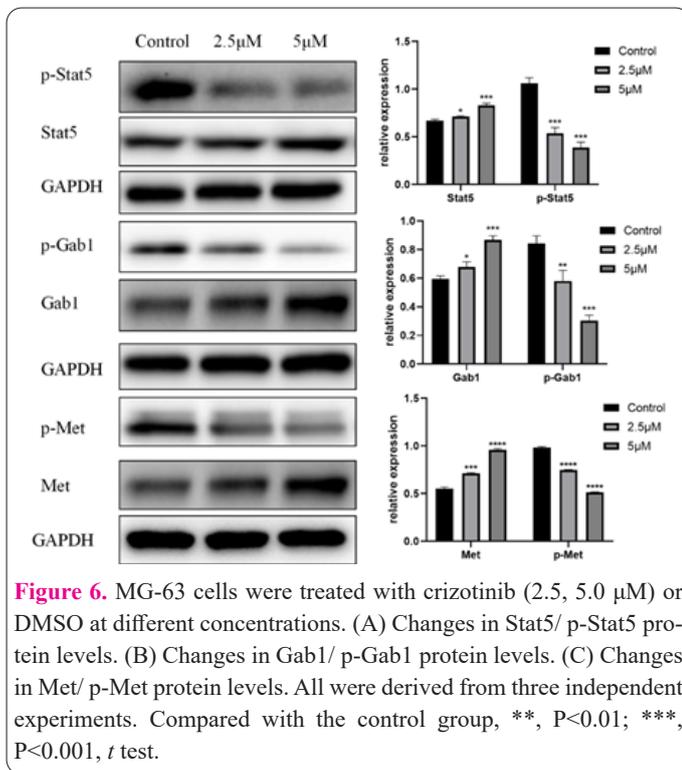


**Figure 5.** Cell invasion experiment. The data were expressed as mean  $\pm$  standard deviation from three independent experiments. Compared with the control group, \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ,  $t$  test.

osteosarcoma cell line MG-63 was treated with crizotinib (2.5, 5.0  $\mu\text{M}$ ) or DMSO at different concentrations. It was found that crizotinib decreased the expression of c-Met, Gab1 and Stat5 proteins (Figure 6).

## Discussion

c-Met is one of the most commonly altered tyrosine kinases in human cancer (9, 16). c-Met is overexpressed in most osteosarcoma tissues (14, 17, 18). The overexpression of c-Met is a common feature of osteosarcoma and soft tissue sarcoma. The overexpression of c-Met in sarcoma cell lines and clinical sarcoma specimens suggests that the imbalance of the HGF/c-Met signal plays an important role in the development of osteosarcoma (18, 19). Abnormal activation of the c-Met pathway is mainly caused by Met14 exon jump mutation, Met amplification or Met pro-



**Figure 6.** MG-63 cells were treated with crizotinib (2.5, 5.0  $\mu\text{M}$ ) or DMSO at different concentrations. (A) Changes in Stat5/ p-Stat5 protein levels. (B) Changes in Gab1/ p-Gab1 protein levels. (C) Changes in Met/ p-Met protein levels. All were derived from three independent experiments. Compared with the control group, \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$ ,  $t$  test.

tein overexpression during tumor development (10). After specific binding of HGF to the c-Met receptor in the extracellular domain, c-Met undergoes polymerization and tyrosine phosphorylation in the cell, leading to receptor internalization and recruitment of adaptor proteins including Gab-1, Grb2, and Shc (20). Activated adaptor proteins become binding sites for downstream proteins and mediate various physiological effects through downstream signaling pathways such as RAS-MAPK, PI3K-AKT, FAK and STAT (9, 10). In a variety of tumors, due to the overexpression of c-Met and HGF, and the positive paracrine and autocrine feedback loops formed by HGF and c-Met. The HGF/c-Met signaling pathway is abnormally activated, which promotes the growth, invasion, migration and angiogenesis of tumor cells (21-23). The development of multiple malignancies in c-Met and HGF transgenic mice also confirms the importance of HGF/c-Met signaling in cancer (24, 25). HGF transgenic mice developed fibrosarcoma (25). Wild-type or transfected activated c-Met drives osteoblast and fibroblast transformation (18). The growth of osteosarcoma and soft tissue sarcoma cells depends on c-Met because silencing c-Met will inhibit the formation of tumors in these cells in nude mice (26, 27). Antitumor therapies targeting overactivated tyrosine kinases, such as Imatinib mesylate (28) and Trastuzumab (29), have demonstrated significant efficacy. Therefore, c-Met has become a molecular target for the treatment of osteosarcoma. This study will investigate the potential mechanism of the c-Met inhibitor crizotinib in the treatment of osteosarcoma.

In this study, osteosarcoma cell lines SaOS2, MG-63 and MNNG were used as models in vitro. The proliferation of osteosarcoma cell lines was significantly inhibited after crizotinib treatment, and there was no significant difference in the sensitivity of the three cell lines to crizotinib. And we found that crizotinib significantly inhibited the proliferation of osteosarcoma cells in a dose-dependent manner. In order to investigate the potential mechanism of crizotinib inhibiting the proliferation of osteosarcoma

cells, we performed cell apoptosis and cell cycle experiments. Flow cytometry results showed that crizotinib could induce the cell cycle block in the G0/G1 phase and trigger cell apoptosis. It indicated that crizotinib regulates the development and growth of osteosarcoma by affecting cell cycle progression and apoptosis.

The ability of osteosarcoma cells to migrate is critical for early metastasis and disease recurrence, and early lung metastasis is a major cause of death in patients (30). Therefore, treatments that inhibit the ability of osteosarcoma cells to migrate may reduce local recurrence or early metastasis, thereby improving survival. Therefore, we evaluated the effect of crizotinib on the migration and invasion ability of osteosarcoma cells. The results of the scratch test and transwell invasion test showed that crizotinib could inhibit the migration and invasion of osteosarcoma cells. The c-Met signaling pathway is transduced by a number of cytoplasmic effector proteins, including Grb2, Gab1, and signal transduction and transcriptional activator protein 5 (Stat5). In order to confirm that the anti-proliferative effects of crizotinib are related to c-Met signaling, the expression of phosphorylated c-Met, Gab1 and Stat5 was evaluated by Western blot. Crizotinib reduces the expression of phosphorylated c-Met and downstream signaling proteins in a dose-dependent manner without inhibiting total protein levels.

In conclusion, crizotinib, a c-Met inhibitor, inhibits the proliferation of osteosarcoma cells by blocking the cell cycle and promoting cell apoptosis. Crizotinib's inhibition of migration and invasion of osteosarcoma cells suggests that crizotinib may reduce the occurrence of early lung metastasis or reduce local recurrence. Crizotinib acts by inhibiting the c-Met/Gab1/Stat5 signaling pathway.

We demonstrate that crizotinib inhibition of the c-MET signaling pathway is a key anticancer mechanism of crizotinib in osteosarcoma cells. We further provide evidence that crizotinib therapy has an enhanced inhibitory effect on the growth of osteosarcoma cells in vitro, which is a promising new therapy for patients with osteosarcoma but remains to be studied in animal models.

#### Data availability

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

#### Funding acknowledgements

This work was supported by the Subject of Heilongjiang Provincial Health and Family Planning Commission (2017-065) "Crizotinib, Xalcori, Pfizer Inhibitory effect and mechanism of combined low-dose MTX on osteosarcoma".

#### Conflict of interests

The authors declared no conflict of interest.

#### References

- Doyle LA. Sarcoma classification: an update based on the 2013 World Health Organization Classification of Tumors of Soft Tissue and Bone. *Cancer-Am Cancer Soc* 2014; 120(12): 1763-1774.
- Guo W, Zhang X, Lin L, et al. The disulfiram/copper complex induces apoptosis and inhibits tumour growth in human osteosarcoma by activating the ROS/JNK signalling pathway. *J Biochem*

- 2021; 170(2): 275-287.
3. Arndt CA, Crist WM. Common musculoskeletal tumors of childhood and adolescence. *New Engl J Med* 1999; 341(5): 342-352.
  4. Stiller CA, Bielack SS, Jundt G, Steliarova-Foucher E. Bone tumours in European children and adolescents, 1978-1997. Report from the Automated Childhood Cancer Information System project. *Eur J Cancer* 2006; 42(13): 2124-2135.
  5. Stiller CA, Craft AW, Corazziari I. Survival of children with bone sarcoma in Europe since 1978: results from the EUROCARE study. *Eur J Cancer* 2001; 37(6): 760-766.
  6. Choi JH, Ro JY. The 2020 WHO Classification of Tumors of Bone: An Updated Review. *Adv Anat Pathol* 2021; 28(3): 119-138.
  7. Bielack SS, Kempf-Bielack B, Delling G, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol* 2002; 20(3): 776-790.
  8. Alexander JH, Binitie OT, Letson GD, Joyce DM. Osteosarcoma: An Evolving Understanding of a Complex Disease. *J Am Acad Orthop Sur* 2021; 29(20): e993-e1004.
  9. Birchmeier C, Birchmeier W, Gherardi E, Vande WG. Met, metastasis, motility and more. *Nat Rev Mol Cell Bio* 2003; 4(12): 915-925.
  10. Christensen JG, Burrows J, Salgia R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. *Cancer Lett* 2005; 225(1): 1-26.
  11. Zhou J, Zheng J, Zhang X, et al. Crizotinib in patients with anaplastic lymphoma kinase-positive advanced non-small cell lung cancer versus chemotherapy as a first-line treatment. *Bmc Cancer* 2018; 18(1): 10.
  12. Moosavi F, Giovannetti E, Peters GJ, Firuzi O. Combination of HGF/MET-targeting agents and other therapeutic strategies in cancer. *Crit Rev Oncol Hemat* 2021; 160: 103234.
  13. Ma PC, Tretiakova MS, Nallasura V, Jagadeeswaran R, Husain AN, Salgia R. Downstream signalling and specific inhibition of c-MET/HGF pathway in small cell lung cancer: implications for tumour invasion. *Brit J Cancer* 2007; 97(3): 368-377.
  14. Pathania H, Chauhan P, Chaudhary V, et al. Engineering core-shell mesoporous silica and Fe(3)O(4)@Au nanosystems for targeted cancer therapeutics: a review. *Biotechnol Genet Eng* 2022: 1-29.
  15. Coltella N, Manara MC, Cerisano V, et al. Role of the MET/HGF receptor in proliferation and invasive behavior of osteosarcoma. *Faseb J* 2003; 17(9): 1162-1164.
  16. Uchikawa E, Chen Z, Xiao GY, Zhang X, Bai XC. Structural basis of the activation of c-MET receptor. *Nat Commun* 2021; 12(1): 4074.
  17. Rong S, Jeffers M, Resau JH, Tsarfaty I, Oskarsson M, Vande WG. Met expression and sarcoma tumorigenicity. *Cancer Res* 1993; 53(22): 5355-5360.
  18. Patane S, Avnet S, Coltella N, et al. MET overexpression turns human primary osteoblasts into osteosarcomas. *Cancer Res* 2006; 66(9): 4750-4757.
  19. Scagliotti GV, Novello S, von Pawel J. The emerging role of MET/HGF inhibitors in oncology. *Cancer Treat Rev* 2013; 39(7): 793-801.
  20. Ponzetto C, Bardelli A, Zhen Z, et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* 1994; 77(2): 261-271.
  21. Vadaye KE, Fazly BB, Kerachian MA. Implantation of stem cells on synthetic or biological scaffolds: an overview of bone regeneration. *Biotechnol Genet Eng* 2021; 37(2): 238-268.
  22. Whang YM, Jung SP, Kim MK, Chang IH, Park SI. Targeting the Hepatocyte Growth Factor and c-Met Signaling Axis in Bone Metastases. *Int J Mol Sci* 2019; 20(2): 384.
  23. Basilico C, Modica C, Maione F, Vigna E, Comoglio PM. Targeting the MET oncogene by concomitant inhibition of receptor and ligand via an antibody-"decoy" strategy. *Int J Cancer* 2018; 143(7): 1774-1785.
  24. Avan A, Maftouh M, Funel N, et al. MET as a potential target for the treatment of upper gastrointestinal cancers: characterization of novel c-Met inhibitors from bench to bedside. *Curr Med Chem* 2014; 21(8): 975-989.
  25. Takayama H, LaRochelle WJ, Sharp R, et al. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. *P Natl Acad Sci Usa* 1997; 94(2): 701-706.
  26. Guo Y, Xie J, Rubin E, et al. Frzb, a secreted Wnt antagonist, decreases growth and invasiveness of fibrosarcoma cells associated with inhibition of Met signaling. *Cancer Res* 2008; 68(9): 3350-3360.
  27. Rees H, Williamson D, Papanastasiou A, et al. The MET receptor tyrosine kinase contributes to invasive tumour growth in rhabdomyosarcomas. *Growth Factors* 2006; 24(3): 197-208.
  28. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *New Engl J Med* 2001; 344(14): 1031-1037.
  29. McNeil C. Herceptin raises its sights beyond advanced breast cancer. *Jnci-J Natl Cancer I* 1998; 90(12): 882-883.
  30. Jaffe N. Osteosarcoma: review of the past, impact on the future. The American experience. *Cancer Treat Res* 2009; 152: 239-262.