Abstract. High-dose chemotherapy and surgical treatment have improved the prognosis of osteosarcoma. However, more than 20% of patients with osteosarcoma still have a poor prognosis. We investigated the expression and function of casein kinase 2 (CK2) in osteosarcoma growth. We then examined the effects of CX-4945, a CK2 inhibitor, on osteosarcoma growth in vitro and in vivo to apply our findings to the clinical setting. We examined the expression of CK2α and CK2β by western blot analysis, and performed WST-1 assays using CK2α and CK2β siRNA or CX-4945. Flow cytometry and western blot analyses were performed to evaluate apoptotic cell death. Xenograft models were used to examine the effect of CX-4945 in vivo. Western blot analysis revealed upregulation of CK2α and CK2β in human osteosarcoma cell lines compared with human osteoblast cells or mesenchymal stem cells. WST assay showed that knockdown of CK2α or CK2β by siRNA inhibited the proliferation of human osteosarcoma cells. Treatment with 3 µM of CX-4945 inhibited osteosarcoma cell proliferation; however, the same concentration of CX-4945 did not affect the proliferation of human mesenchymal stem cells. Additionally, treatment with CX-4945 inhibited the proliferation of human osteosarcoma cells in a dose-dependent manner. Western blot and flow cytometry analyses showed that treatment with CX-4945 significantly prevented osteosarcoma growth in vivo compared with control vehicle treatment. Our findings indicate that CK2 may be an attractive therapeutic target for treating osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant bone tumor in children, adolescents, and young adults. Osteosarcoma has been treated using various chemotherapy regimens that have been developed over more than 40 years (1). Approximately 20% of patients with osteosarcoma develop metastases. More than 85% of metastatic disease occurs in the lung, which is the most common site of metastasis (2). Metastatic osteosarcoma exhibits resistance to chemotherapeutic treatment (3). There has been little improvement in the survival rates of osteosarcoma patients since the 1980s (1).

To improve the prognosis of osteosarcoma, many researchers have investigated molecular targets with which to inhibit osteosarcoma growth and metastasis. Casein kinase 2 (CK2) is a highly conserved serine/threonine kinase that comprises two α catalytic (α and α′) and two β regulatory subunits (4). Hundreds of CK2 substrates have been identified, and many substrates continue to be discovered (5). CK2 has important roles in cell growth and cell fate. In addition, deregulated CK2 activation promotes many types of human cancers (6-9).

CX-4945 is a potent and selective orally bioavailable small molecule inhibitor of CK2 that has been investigated in clinical trials (10-14). CX-4945 promotes significant reductions in the proliferation and survival of non-small cell lung carcinoma, squamous cell carcinoma, breast cancer, pancreatic cancer, and B-cell lymphoma cells (10,15,16). In this study, we found that the expression of CK2 is upregulated in human osteosarcoma cells. We evaluated the function of CK2 in human osteosarcoma using siRNA and CX-4945.

Materials and methods

Cell lines and reagents. The human osteosarcoma cell lines 143B, SaOS-2, U2OS, and MG63 were purchased from the...
American Type Culture Collection (Manassas, VA, USA). The human osteosarcoma cell lines NY and Hu09 were purchased from the Health Science Research Resources Bank (Osaka, Japan). The human osteosarcoma cell lines NOS1 and HSOS1 were purchased from Riken Cell Bank (Tsukuba, Japan). Normal human osteoblast cells (NHost) were purchased from Sanko Junyaku (Tokyo, Japan). The human mesenchymal stem cell line UBE6T15 was purchased from Health Science Research Resources Bank. The cell lines were cultured at 37°C in 5% CO2. CX-4945 was purchased from MedChem Express (Princeton, NJ, USA). Control siRNA, CK2α siRNA, and CK2β siRNA were purchased from dharmacon (Pittsburgh, PA, USA).

**Analysis of cell viability.** Cells were treated with CX-4945 and a control vehicle. Cell viability was evaluated by a WST-1 assay (Roche, Basel, Switzerland) for mitochondrial dehydrogenase activity, as described previously (17).

**Western blot analysis.** Cells were lysed using Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA, USA), 3 mM pAPMSF (Wako Chemicals, Kanagawa, Japan), 5 mg/ml aprotinin (Sigma-Aldrich, St. Louis, MO, USA), and 2 mM sodium orthovanadate (Wako Chemicals). SDS-PAGE and immunoblotting were performed, and the following antibodies were used: CK2α (Merck Millipore, Billerica, MA, USA), CK2β (Merck Millipore), cleaved-PARP (Cell Signaling, Danvers, MA, USA), cleaved caspase-3 (Cell Signaling), Bcl-xL (Cell Signaling), Bcl-2 (Cell Signaling), and tubulin (Sigma-Aldrich). ECL Western Blotting Reagent (GE Healthcare, Amersham, UK).

**Flow cytometry.** I43B and Saos-2 were cultured with CX4945 or vehicle at 37°C in 5% CO2 for 24 and 48 h. Cells were treated with the Annexin V-FITC/7-AAD kit (Beckman Coulter, Brea, CA, USA), and fluorescence-activated cell sorting was performed with a CyAn ADP analyzer (Beckman Coulter).

**Animal studies.** Mouse xenograft models were created as previously described (17,18). For CX-4945 and control vehicle treatment, I43B cells (1x10⁶) were suspended in 100 μl of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Five-week-old nude mice were subcutaneously inoculated...
with the mixture of 143B cells and Matrigel. The tumor volume was evaluated using the formula LW^2/2, where L and W represent the length and width of the tumor, respectively. Xenograft models were randomly treated with either CX-4945 (150 mg/kg/day) or an equal volume of vehicle as a control administered via oral tube every day. All animal experiments were performed in compliance with the guidelines of the Institute of Laboratory Animal Sciences, Graduate School of Medical and Dental Sciences, Kagoshima University (permit no. Md15045). Every effort was employed to minimize both the number of animals used and animal pain.

Statistical analysis. The Kolmogorov-Smirnov test was performed to examine the distribution of data. Statistical analyses were performed using the Mann-Whitney U test. The survival rate was evaluated using the Kaplan-Meier method and the log-rank test. All statistical analyses were performed using BellCurve for Excel 2015 (SSRI, Osaka, Japan). P<0.05 was considered statistically significant.

Results

Upregulated expression of CK2 in human osteosarcoma cells. Western blot analysis showed that the expression of both CK2α and CK2β protein was upregulated compared with NH6T15 normal human osteoblasts and UBE6T15 human mesenchymal stem cells (Fig. 1).

Knockdown of CK2α or CK2β inhibited the proliferation of human osteosarcoma cells. We used siRNA to determine whether CK2α promoted the proliferation of osteosarcoma cells. Western blot analysis revealed that CK2α siRNA decreased the expression level of CK2α protein (Fig. 2A and C). WST assay revealed that knockdown of CK2α inhibited the proliferation of the 143B, SaOS-2, NY, and U2OS human osteosarcoma cell lines (Fig. 2B and D). In addition, we used siRNA to determine whether CK2β promoted the proliferation of osteosarcoma cells. Western blot analysis revealed that CK2β siRNA decreased the expression of CK2β protein (Fig. 2E). WST assay revealed that knockdown of CK2β inhibited the proliferation of the 143B and SaOS-2 human osteosarcoma cell lines (Fig. 2F).

CX-4945 inhibited the proliferation of human osteosarcoma cell lines, but not the proliferation of mesenchymal stem cells. We used 4 human osteosarcoma cell lines, 143B, SaOS2, NY, and U2OS which expressed high levels of CK2α for further examinations. Treatment with 3 µM CX-4945 did not inhibit the proliferation of UBE6T15 human mesenchymal stem cells (Fig. 3A); however, treatment with 3 µM CX-4945 caused

Figure 3. CX-4945 inhibited the proliferation of human osteosarcoma cell lines but not the proliferation of mesenchymal stem cells. WST assay revealed that treatment with 3 µM CX-4945 did not inhibit the proliferation of UBE6T15 human mesenchymal stem cells (A). Treatment with 3 µM CX-4945 significantly prevented the proliferation of osteosarcoma cells (B). WST assay revealed that treatment with CX-4945 inhibited the proliferation of the 143B, SaOS-2, NY, and U2OS human osteosarcoma cell lines in a dose-dependent manner (C).
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a significant reduction in osteosarcoma cell proliferation. (Fig. 3B). WST assay revealed that treatment with CX-4945 inhibited the proliferation of the 143B, Saos-2, NY, and U2OS human osteosarcoma cell lines in a dose-dependent manner (Fig. 3C).

**CX-4945 promotes apoptotic cell death of human osteosarcoma cell lines.** Western blot analysis revealed that treatment with CX-4945 increased the expression of cleaved PARP and cleaved caspase-3 (Fig. 4). Treatment with CX-4945 also decreased the expression of Bcl-2 and Bcl-xL. Analysis by flow cytometry showed that treatment with CX-4945 increased the population of early and late apoptotic cells in 143B and SaOS-2 osteosarcoma cells at 24 and 48 h (Fig. 5). These findings indicate that CX-4945 treatment promoted apoptotic death of human osteosarcoma cells.

**CX-4945 prevents osteosarcoma growth in vivo.** Palpable tumors were confirmed in nude mice 7 days after inoculation with 143B osteosarcoma cells. Compared with control vehicle treatment, treatment with CX-4945 significantly inhibited the growth of the osteosarcoma xenografts (Fig. 6A). Kaplan-Meier analysis showed that CX-4945 treatment provided a significant survival benefit (Fig. 6B).

**Discussion**

CK2 is upregulated in many types of malignant tumors, and maintains the phenotype of malignancy (6,19). CK2 is a potential therapeutic target for human cancers, and has been tested in clinical trials (10-14). We found that human osteosarcoma cells upregulated the expression of CK2α and CK2β. To the best of our knowledge, this is the first report to show the upregulation of CK2 in human osteosarcoma. We showed that treatment with 3 µM CX-4945 inhibited osteosarcoma growth in vitro, but did not inhibit mesenchymal stem cell proliferation. These findings suggest that 3 µM of CX-4945 might be a safe and effective dose for osteosarcoma treatment. We also showed that CX-4945 prevented the growth of osteosarcoma in vivo.

CX-4945 reportedly has a long half-life, high oral bioavailability, and non-cardiac toxicity (20). Furthermore, CX-4945 inhibits osteoclast differentiation and enhances osteoblast differentiation (21), indicating that CX-4945 inhibits osteosarcoma growth while promoting the regeneration of affected bone. As CK2 reportedly drives the metastatic development of lung cancer, prostate cancer, squamous cell carcinoma, and breast carcinoma (9,22-25), the CK2 inhibitor CX-4945 is a promising drug for the treatment of metastatic bone tumors.

Human osteosarcoma specimens and cell lines reportedly have an overexpression of Hedgehog pathway-related genes, including SMO and GLI2. Furthermore, inhibition of the Hedgehog pathway prevents osteosarcoma growth and metastasis (17,18,26-30). CK2 activates the Hedgehog pathway in many types of cells, including mesothelioma, ovarian cancer, hepatocellular carcinoma, and lung cancer cells (31-36). There is a possibility that CX-4945 inhibits the Hedgehog pathway via inhibition of CK2. Osteosarcoma growth is inhibited in vitro by a combination of arsenic trioxide, a Hedgehog signal inhibitor, with conventional FDA-approved anticancer agents including cisplatin, ifosfamide, or doxorubicin. In vivo tumors treated with a combination of arsenic trioxide with either cisplatin or ifosfamide grew significantly less than tumors treated with the vehicle alone (17,27,29). These findings suggest that combining CX-4945 with conventional anticancer agents might more effectively prevent osteosarcoma growth, and that this combination therapy might decrease the required concentration of each drug. The lower levels of each agent in these combinations might reduce the toxicities associated with the use of each single drug. However, osteosarcoma is an extremely heterogeneous tumor. Preselection of patients with osteosarcoma exhibiting upregulated CK2 is required for CK2-targeted treatment.

CX-4945 reportedly modulates not only CK2 activity, but also PI3K-Akt-mTOR signaling, the Notch pathway, the PI3K-Akt-mTOR pathway, the focal adhesion kinase-Src-paxillin signaling cascade, ER stress signaling, NF-κB, and Bcl-xL, ERK, AP-1, and IL-8 gene activities (37-42). The pleotropic effect of CX-4945 and off-target effects of CK2 might potentially affect the inhibition of osteosarcoma growth.

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Figure 4. CX-4945 increased the expression of apoptosis markers. Western blot analysis revealed that CX-4945 treatment increased the expression of cleaved PARP or cleaved caspase-3. CX-4945 treatment decreased the expression of Bcl-2 or Bcl-xL. The experiment was performed in triplicate, producing similar results.
Figure 5. CX-4945 promoted the apoptotic cell death of human osteosarcoma cell lines. Osteosarcoma cells were treated with DMSO, 5 µM CX-4945, or 10 µM CX-4945 for (A) 24 or (B) 48 h. Flow cytometry showed that CX-4945 treatment increased the population of early and late apoptosis in the 143B and SaOS-2 osteosarcoma cell lines at 24 and 48 h. The experiment was performed in triplicate, producing similar results.

Figure 6. CX-4945 inhibits the growth of osteosarcoma growth in vivo. Compared with vehicle treatment, treatment with CX-4945 significantly inhibited the growth of the osteosarcoma xenograft in nude mice (A and B). Kaplan-Meier analysis showed that CX-4945 treatment provided a significant survival benefit (C).
Nonetheless, CX-4945 showed promising therapeutic efficacy for osteosarcoma in this study. Taken together, our findings indicate that CK2 might be an attractive therapeutic target, and that CX-4945 might be a promising new reagent for the treatment of osteosarcoma.

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