Systematic Kinome shRNA Screening Identifies CDK11 (PITSLRE) Kinase Expression Is Critical for Osteosarcoma Cell Growth and Proliferation

Zhenfeng Duan1, Jianming Zhang3, Edwin Choy1, David Harmon1, Xianzhe Liu1, Petur Nielsen2, Henry Mankin1, Nathanael S. Gray3, and Francis J. Hornicek1

Abstract

Purpose: Identification of new targeted therapies is critical to improving the survival rate of patients with osteosarcoma. The goal of this study is to identify kinase based potential therapeutic target in osteosarcomas.

Experimental Design: We used a lentiviral-based shRNA kinase library to screen for kinases which play a role in osteosarcoma cell survival. The cell proliferation assay was used to evaluate cell growth and survival. siRNA assays were applied to confirm the observed phenotypic changes resulting from the loss of kinase gene expression. CDK11 (PITSLRE) was identified as essential for the survival of osteosarcoma cells, and its expression was confirmed by Western blot analysis and immunohistochemistry. Overall patient survival was correlated with the CDK11 expression and its prognosis. The role of CDK11 expression in sustaining osteosarcoma growth was further evaluated in an osteosarcoma xenograft model in vivo.

Results: Osteosarcoma cells display high levels of CDK11 expression. CDK11 expression knocked down by either lentiviral shRNA or siRNA inhibit cell growth and induce apoptosis in osteosarcoma cells. Immunohistochemical analysis showed that patients with osteosarcoma with high CDK11 tumor expression levels were associated with significantly shorter survival than patients with osteosarcoma with low level of tumor CDK11 expression. Systemic in vivo administration of in vivo ready siRNA of CDK11 reduced the tumor growth in an osteosarcoma subcutaneous xenograft model.

Conclusions: We show that CDK11 signaling is essential in osteosarcoma cell growth and survival, further elucidating the regulatory mechanisms controlling the expression of CDK11 and ultimately develop a CDK11 inhibitor that may provide therapeutic benefit against osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant tumor of bone. The standard treatment for osteosarcoma incorporates surgery and chemotherapy involving several chemotherapeutic agents which include doxorubicin, cisplatin, ifosfamide, and methotrexate (1, 2). If these agents are unable to lead to favorable tumor response, further chemotherapeutic options are very limited. Despite aggressive chemotherapy, more than 30% of patients with localized osteosarcoma experience metastatic disease. Most of these patients will eventually develop multidrug resistance in late stages of osteosarcoma. The average survival period after metastases is less than one year (1–5). Therefore, there is urgent need to improve the general condition and the overall survival rate of patients with metastatic osteosarcoma by identifying novel therapeutic strategies.

The discovery of oncogenic kinases and target-specific small-molecule inhibitors has revolutionized the treatment of a selective group of cancers, such as chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST). Protein kinases play important roles in regulating tumor cellular functions—proliferation/cell cycle, cell metabolism, survival/apoptosis, DNA damage repair, cell motility, and drug resistance—so it is not surprising that protein kinases are often oncogenic genes. Kinases such as c-Src, c-ABL, PI3K/AKT, EGFR, MAP, IGF-1R, and JAK are commonly activated and highly expressed in cancer cells and are known to contribute to cancer progression (6, 7). Kinases are now firmly established as a major class of anticancer drug targets. Significant progress has been made in understanding kinases and their functions. There has been

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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CDK11 in Osteosarcoma

Translational Relevance
Kinases play an essential role in cancer cell growth and survival; however, the roles of most kinases in osteosarcoma cell growth are largely uncharacterized. In the search for kinases required for osteosarcoma cell growth, we identified CDK11 (PTSLRE) as a potential target by a comprehensive human kinome-wide shRNA screening in osteosarcoma cell lines. Furthermore, knockdown of CDK11 either by lentiviral shRNA, or by synthetic siRNA-independent confirmation, can inhibit cell growth or induces apoptosis in osteosarcoma cells. Immunohistochemical analysis indicated that osteosarcoma patients with high CDK11 tumor expression levels were associated with significantly shorter survival than patients with osteosarcoma low level of CDK11 expression. Systemic in vivo administration of in vivo ready siRNA of CDK11 reduced tumor growth in an osteosarcoma s.c. xenograft model. These observations show that CDK11 signaling is essential in osteosarcoma cell growth and survival, CDK11 may become a promising therapeutic target in the management of osteosarcoma.

Materials and Methods

Cell lines and cell culture
Human osteosarcoma cell line KHOS was kindly provided by Dr. Efstatios Ganos (Institute of Biological Research & Biotechnology, Athens, Greece). Ewing sarcoma cell line TC-71 was provided by Dr Katia Scotlandi (Institute Orthopedics Rizzoli, Italy). The human osteosarcoma cell lines, U-2OS and Saos, human ovarian cancer cell line SKOV-3, and uterine sarcoma cell line MES-SA were purchased from the American Type Culture Collection (ATCC). Osteosarcoma cell line OSA344 was established from primary osteosarcoma tissue. Human osteoblast cells HOB-c were purchased from PromoCell GmbH, osteoblast cells NH osteo were purchased from Lonza Wallkersville Inc., and osteoblast cells hFOB were purchased from ATCC. Osteoblast cells were cultured in osteoblast growth medium (PomoCell) with 10% FBS. All other cell lines were cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen).

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Apoptosis assay
Caspase-cleaved keratin 18-based quantification of apoptosis was evaluated using the M30-Apoptosense ELISA assay kit, as per manufacturer's instructions (Peviva AB). The ELISA apoptosis detects a 21-kDa fragment of cytokeratin 18 that is only revealed after caspase cleavage of the protein. KHOS or U-2OS cell lines reverse transfected with synthetic CDK11 siRNA were seeded at 2,000 cells per well in a 96-well plate. After 48 hours incubation, the cells were then lysed by adding 10 μL of 10% NP-40 per well, and the manufacturer's instructions for the apoptosis assay were then followed. Apoptosis was also evaluated by Western blot analysis using whole-cell lysates immunoblotted with specific antibodies to PARP (Cell Signaling Technologies) and its cleavage products.

Western blotting
The concentration of the protein was determined by Protein Assay Reagents (Bio-Rad) with a spectrophotometer (Beckman Du-640, Beckman Instruments, Inc.). The rabbit polyclonal antibody (sc-928) to human CDK11 (PITSLRE) was purchased from Santa Cruz Biotechnology. The mouse monoclonal antibody to human actin was purchased from Sigma-Aldrich. Goat anti-rabbit horseradish peroxidase (HRP) and goat anti-mouse antibodies were purchased from Bio-Rad. All other antibodies used in this study were purchased from Cell Signaling Technologies. Western blot analysis was conducted as previously reported (13).

Immunofluorescence assay
For immunostainings of cultured osteosarcoma cells, KHOS or U-2OS cells were grown in 8-well chambers for 24 hours and fixed in 3.7% buffered paraformaldehyde. Immunostainings were carried out using antibodies against CDK11 and β-actin. After washing the cells, they were incubated with Alexa Fluor secondary antibodies. Specifically, the slides were stained with Alexa Fluor 488 (Green) conjugated goat anti-rabbit antibody (Invitrogen) for CDK11, and Alexa Fluor 594 (Red) conjugated for goat anti-mouse antibody for β-actin.

Human sarcoma tumor tissues
Six of the osteosarcoma tissue samples (OST1–OST6) were obtained from Massachusetts General Hospital sarcoma tissue bank (Boston, MA) and were in accordance with the policies of the institutional review board of the hospital. All diagnoses were confirmed histologically.

Osteosarcoma tissue microarray and immunohistochemistry
Osteosarcoma tissue microarray was purchased from Imgenex Corp, which contains 57 tumor tissues. Immunohistochemistry was conducted by following the manufacturer’s instructions with HRP-DAB System Cell and Tissue Staining Kit (R&D Systems). In brief, primary antibody of CDK 11 (1:50 dilution) in 1% bovine serum albumin (BSA) was applied to the deparaffinized slide overnight at 4°C. After incubation with the HRP-conjugated goat anti-rabbit antibody, and rinses in PBS thrice, bound antibody was detected with the substrate reagents from HRP-DAB System Cell and Tissue Staining Kit. Finally, slides were counterstained with Hematoxylin QS (Vector Laboratories) and mounted with VectaMount AQ (Vector Laboratories).

Evaluation of immunohistochemical staining
CDK11-positive samples were defined as those showing nuclear staining pattern of tumor tissue. CDK11 staining patterns were categorized into 6 groups: 0, no nuclear staining; 1+, <10% of cells nuclear stained–positive; 2+, 10%–25% positive cells; 3+, 26%–50% positive cells; 4+, 51%–75% positive cells; and 5+, >75% positive cells. The percentage of cells showing positive nuclear staining for CDK11 was calculated by reviewing the entire spot. Categorizing of CDK11 staining was completed by 2 independent investigators. Discrepant scores between the 2 investigators were rescored to get a single final score. CDK11 images were obtained by using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Corp) with a SPORT RT digital camera (Diagnostic Instruments Inc.).

Statistical analysis
Kaplan–Meier survival analysis (GraphPad PRISM Software; GraPhad Software) was used to analyze the correlation between the level of CDK11 expression and prognosis. The Student t test was used to compare the differences between groups. Results are given as mean ± SD and values with P < 0.05 were considered as statistically significant.

CDK11 siRNA osteosarcoma tumor therapy
Ambion In Vivo Ready CDK11 siRNA and nonspecific siRNA were purchased from Applied Biosystems. These In Vivo Ready, validated siRNA were designed using the Silencer Select algorithm and incorporated with additional chemical modifications for superior serum stability with in vivo applications (14–17). The Ctrl:SHO-PhbdScD-Hp* nude female mice at approximately 3 to 4 weeks of age were purchased from The Charles River Laboratories. To determine the effect of CDK11 siRNA on osteosarcoma cell growth in xenograft model, KHOS cells (1 × 10^6) were inoculated subcutaneously with Matrigel from BD Biosciences into the right flank of the nude mouse. Two weeks after injection, the mice were randomized into 3 groups (6 mice/group). Group 1 received injection with sterile saline (0.9% NaCl), group 2 with In Vivo Ready nonspecific siRNA, and group 3 with In Vivo Ready CDK11 siRNA. For intratumoral injections, each animal was injected with 20 μL of PBS containing 10 nmol/L of siRNA. All 3 groups were treated twice a week for 2 weeks. The health of the mice and evidence of tumor growth were examined daily. Tumor volumes were measured at a regular interval of for up to 4 weeks with a digital calipers. Tumor volume (mm^3) was calculated as (width)^2 × length/2 where W is width and L is length. Data are presented as mean ± SD. Tumor tissues from the above treated animals were collected and placed in 10% formalin and embedded in paraffin for histology analysis. The silence efficiency CDK11 siRNA on CDK
proteins were determined by immunohistochemical staining, as described above. Animal experiments in the present study were carried out in compliance with the protocol, which was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (SRAC) under the protocol number 2009N000229.

Results

CDK11 expression is critical for osteosarcoma cell growth and survival

To identify the potential therapeutic kinase targets in osteosarcoma cells, we conducted a comprehensive kinome-wide screening of lentiviral shRNA kinase library in osteosarcoma cell line KHOS. Among the targeted kinase genes, we found that knocking down the expression of CDK11, PLK1, DYRK1B, and ROCK1 led to inhibitory growth effects. Of those kinases that we found critical to osteosarcoma cell growth, the elevated expression of PLK1, DYRK1B, and ROCK1 have been previously reported in various human tumors (6, 12, 18–24). However, direct evidence regarding the relationship between expression of CDK11 and cancer cell growth and survival is lacking. When lentiviral shRNA targeting CDK11 was transduced into osteosarcoma cell lines KHOS and U-2OS, it led to significantly reduced tumor cell growth and eventual cell death as shown by cellular proliferation assay (Fig. 1A). To further characterize the functional role of CDK11 in osteosarcoma, we rigorously validated the results using multiple independent experiments, including multiple shRNA per CDK11 gene and extensively tested with control nonspecific shRNA (The sequence of 5 shRNA target different sites of CDK11 in Supplementary Table S1). The results revealed that 4 out of 5 CDK11 shRNA inhibit osteosarcoma cell growth. We further validated these results with synthetic human CDK11 siRNA. Consistent observations of the dose-dependent CDK siRNA inhibition of osteosarcoma cell growth and survival was established by MTT assay (Fig. 1B). We subsequently measured the expression of CDK11 in siRNA transfected cells. Western blot analysis suggested that down-regulated expression of CDK11 protein by CDK11 siRNA associated with the inhibition of cell growth (Fig. 1B and C).

CDK11 knockdown induces apoptosis in osteosarcoma cell lines

To investigate how CDK11 sustains tumor cell growth and survival, we investigated cellular events during the cell death caused by CDK11 knockdown in osteosarcoma cell lines KHOS and U-2OS. We investigated the potential for the induction of apoptosis using the M-30-Apoptosense...
ELISA and by Western blotting for the cleavage of PARP. Depletion of CDK11 by siRNA resulted in a dose-dependent cell death in KHOS and U-2OS osteosarcoma cell lines, which was not observed with the nonspecific siRNA transfection (Fig. 2A). Consistent with these results, there was also a dose-dependent cleavage of PARP for siRNA-mediated depletion of CDK11 (Fig. 2B). CDK11 has been reported to play an important role in the regulation of gene transcription and pre-mRNA splicing. We hypothesized that inhibition of apoptosis induced by CDK11 may be associated with the deregulation of RNA processing associated with a subsequent decrease of antiapoptotic-dependent proteins. Therefore, we examined whether the inhibition of CDK11 by siRNA could result in decreased expression of antiapoptotic proteins MCL-1, Bcl-XL, survivin, cytochrome C, and cyclin D1. Most of these antiapoptotic proteins such as MCL-1, Bcl-XL, and survivin are highly expressed in osteosarcoma, and downregulation by siRNA can inhibit cell growth and induce apoptosis in osteosarcoma cells. Western blot confirmed that transfection of CDK11 siRNA significantly downregulated the expression of several of these antiapoptotic proteins in both in KHOS and U-2OS cells (Fig. 2C), whereas CDK11 knockdown did not alter actin expression. Thus, these results indicate that CDK11 can control several aspects of apoptosis signaling.

**CDK11 is highly expressed in osteosarcoma cell lines and in tumor tissues**

To further confirm the expression of CDK11 and determine CDK11 protein subcellular localization in osteosarcoma cell lines, immunofluorescence assay was used in KHOS and U-2OS cell lines. Previous studies have reported that the CDK11 protein localizes both to the nucleus and cytoplasm. Our results showed that the CDK11 protein is mainly localized in the nucleus of osteosarcoma cells (Fig. 3A). We also extended our evaluation of the expression of CDK11 in other types of human cancer cell lines including uterine sarcoma (MES-SA), chondrosarcoma (CS-1), synovial sarcoma (SS-1), Ewing sarcoma (TC-71), and ovarian cancer (SKOV-3, 3A, 2008). These tumor cell lines exhibited a variety of expression levels of CDK11 protein as evaluated by Western blot analysis (Fig. 3B). To confirm these data in primary cancer, 6 freshly isolated primary osteosarcoma specimens were also examined by Western blot analysis to exclude the possibility of CDK11 expression being an artifact induced by *in vitro* propagation. Different levels of CDK11 expression were once again observed in these osteosarcoma patient samples, indicating the endogenous expression of CDK11 in tumor cells. In the normal human cells, the expression of CDK11 is tightly regulated, for example, in normal human osteoblast cell lines, HOB-c, NH0st, and hFOB there are...
extremely low and almost undetectable levels of CDK11 (Fig. 3C).

CDK11 expression levels correlates with clinical prognosis in patients with osteosarcoma

To further validate the clinical relevant of CDK11 expression in patients with osteosarcoma, we analyzed CDK11 protein levels by using osteosarcoma tissue microarray. The results showed the majority of tumors present on the tissue microarray had positive staining for CDK11. CDK11 nuclear staining percentage was graded into 6 groups. By comparing the clinical characteristics of low-staining (\(< 3\)) and high-staining (\(> 4\)) osteosarcomas, no correlation existed between CDK11 expression and age or tumor location (\(P > 0.05\), Supplementary Table S2). Kaplan–Meier survival analysis showed that the outcome for patients in the CDK11 high-staining group was significantly worse than for those in the CDK11 low-staining group (Fig. 4A). On the basis of 60 months survival rates, patients were grouped into survivors (survived up to 60 months post follow up) and non-survivors (deceased within 60 months of follow-up). A total of 30 (67%) samples from survivors and 15 (33%) samples from nonsurvivors were collected. Comparison of CDK11 staining intensity between 2 group patients revealed that CDK11 staining for samples from nonsurvivors were significantly higher than that of survivors. The average CDK11 expression levels for survivors and nonsurvivors were 2.8 to 4.3, respectively (Fig. 4B). The immunohistochemical staining of CDK11 protein indicated its location in the nucleus (Fig. 4C) which is consistent with that of osteosarcoma cells, as measured by immunofluorescence assay (Fig. 3A).

CDK11 siRNA inhibit tumor growth

The significant association of CDK11 expression with clinical outcome led us to further verify the essential role of CDK11 in sustaining osteosarcoma growth in vivo. KHOS osteosarcoma cells (1 \times 10^6) were injected subcutaneously into the flank of nude mice. By 2 weeks, visible tumors had developed at injection sites (mean tumor volume = 52 mm^3). CDK11 siRNA was then intratumorally injected, twice a week for 2 weeks. As shown in Fig. 5A, CDK11 In Vivo Ready siRNA significantly suppressed tumor growth as compared with vehicle (saline) and nonspecific siRNA treatment. The immunohistochemical staining indicated a significant decrease of CDK11 expression in tumor treated with CDK11 In Vivo Ready siRNA (Fig. 5B). No gross adverse effects, i.e. the loss of body weight, were observed during the experimental period.

Discussion

To identify essential kinases which are responsible for osteosarcoma growth, we first conducted a kinome-wide shRNA screen. CDK11, PLK1, DYRK1B, and ROCK1 were the primary hits with loss of expression of these kinases.
significantly reduced cell growth and survival. We and others had previously found that targeting PLK1, DYRK1B, and ROCK1 kinases inhibits osteosarcoma cell growth and survival by using similar shRNA or siRNA kinase library screenings (11, 12, 18, 25–27). However, the relationship between the expression of CDK11 and osteosarcoma...
growth and survival was lacking. The important functional role of CDK11 in osteosarcoma cells was further validated by using CDK11 gene-specific synthetic siRNA knockdown endogenous CDK11. Both initial shRNA screenings and follow-up siRNA validation assay results highlighted the importance of CDK11 in osteosarcoma.

CDK11 is a serine/threonine protein kinase and encoded by the CDK11 gene on chromosome 1p36.3 (9, 10). The function of CDK11 has not been described in osteosarcoma. There is only one CDK11 gene in mouse, whereas in humans, there are 2 CDK11 genes that encode 2 almost identical protein kinases. There are 3 CDK11 protein isoforms, p110, p58, and p46 (28). CDK11 p58 protein is specifically translated from an internal ribosome entry site and expressed only in the G2–M phase of the cell cycle. These different CDK11 isoforms seem to play multiple roles in transcription, RNA processing, regulating cell-cycle progression, cytokinesis, and apoptosis (10, 28). CDK11 knockout mice display an earlier phenotype and death during the blastocyst stage of embryonic development (29). The CDK11 null cells exhibit proliferative defects, mitotic arrest, and apoptosis, thus suggesting that CDK11 kinase is critical for embryonic development and cellular viability (29). By kinase-specific siRNA screen for Hedgehog (Hh) regulators, CDK11 has been shown to directly participate in the Hh pathway. CDK11 is necessary and sufficient for the activation of the Hh pathway, functioning downstream of Smo and upstream of the glioma-associated (Gli) transcription factors (19). CDK11 is also a modulator of autophagy in human cells (30). Although the function of CDK11 has been examined in different model systems, its potential role in tumors has not been fully investigated due to a lack of data regarding CDK11 expression and tumor cell growth. CDK11 was initially proposed as a tumor suppressor candidate gene, as the CDK11 chromosomal location region 1p36.3 is frequently deleted or translocated in a number of different human tumors, including neuroblastoma, breast cancer, and melanoma (31–33). However, a more recent study of neuroblastoma by FISH analysis excludes the gene-specific synthetic siRNA knockdown of CDK11 (34). On the other hand, a study of neuroblastoma by FISH analysis excludes the CDK11 genes to be highly expressed in human cancer, especially in high-grade tumors (10, 38). For CDK11, we show that osteosarcoma cell lines and tumors express high level of CDK11 protein in comparison with normal osteoblasts. Most importantly, the levels of CDK11 expression are significantly associated with clinical outcome in osteosarcoma. Overexpression of CDK11 was correlated with poor prognosis. Furthermore, silencing of CDK11 reduced tumor volume in an osteosarcoma xenograft mouse model. These in vivo studies have confirmed the anticancer effects of CDK11 inhibition in vitro and provide a rationale for pharmacologic investigation of CDK11 as a novel therapy target.

Taken together, this study identified that CDK11 as essential for osteosarcoma cell growth and survival. Experiments are underway to understand the mechanisms behind CDK11 signaling in human cancer. In turn, these findings may lead to targeting CDK11 through gene therapy or kinase-specific inhibitors in the treatment of osteosarcoma.

Disclosure of Potential Conflicts of Interest
E. Choy is a consultant/advisory board for Amgen, Sanofi Aventis, and BioMed Valley Discoveries, Inc. No potential conflicts of interest were disclosed by other authors.

Authors' Contributions
Conception and design: Z. Duan, J. Zhang, X. Liu, N.S. Gray, F.J. Hornick
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Duan, J. Zhang, D. Harmon, F.J. Hornick
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Duan, J. Zhang, X. Liu, H. Mankin, F.J. Hornick
Writing, review, and/or revision of the manuscript: Z. Duan, J. Zhang, E. Choy, D. Harmon, P. Nielsen, H. Mankin, N.S. Gray, F.J. Hornick
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