Expression of PIM1 Kinase in Osteosarcoma and the Clinical Significance

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Introduction: Osteosarcoma is the most common type of primary malignant bone tumor affecting children and young adults. While surgery alone can only result in long-term survival rates around 10-20%, chemotherapy has increased the disease-free survival rate to more than 60%. However, despite aggressive chemotherapy, one-third of patients with localized osteosarcoma experience recurrent or progressive metastatic disease and the average survival period after developing metastasis is less than 1 year. Therefore, to improve the survival rate of osteosarcoma patients, novel therapeutic strategies are needed.

The serine/threonine-specific PIM1 kinase has been found to be up-regulated in many hematological malignancies and in solid tumors. PIM1 is a 34 kDa protein kinase that is involved in tumor cell proliferation, survival, differentiation, apoptosis, and drug resistance. PIM1 kinase is heavily targeted for anticancer drug discovery, however, very little is known about the relationship between level of PIM1 expression and clinical outcome in osteosarcoma patients. We thus aim to elucidate PIM1 expression in osteosarcoma, using both osteosarcoma cell lines and osteosarcoma tissue microarrays (TMA). We also determined the effects of PIM1 on osteosarcoma cell growth and proliferation.

Methods: Cell lines, cell culture, and drugs

The human osteosarcoma cell lines U-2OS, Saos, KHOS, and MG63 were cultured in RPMI-1640 supplemented with FBS. Human osteoblast cell lines HOB-c and HOst were cultured in osteoblast growth medium. PIM1 inhibitor, benzylidene-thiazolidine-2,4-dione (SMI-4a) was purchased from Sigma-Aldrich.

Synthetic PIM1 siRNA, shRNA, and transfection

Further validation of PIM1 knockdown phenotype in osteosarcoma cell lines was determined by synthetic human PIM1 siRNA and PIM1 lentiviral shRNA (Sigma-Aldrich). Transfection was carried out following the manufacturer’s protocol as previously described.

Cytotoxicity assay, cell proliferation assay

Drug concentrations used in the MTT assay were performed in the absence of cells to verify no change in absorbance. The concentration of drug causing 50% cell death (IC50 of the drug) was determined by plotting percent survival versus cytotoxic drug concentration. Effects of PIM1 knockdown by siRNA or shRNA and PIM1 inhibitor SMI-4a on osteosarcoma cell proliferation were determined by MTT assay.

Western blotting

Western blots were conducted to detect PIM1 protein expression, as well as apoptosis and anti-apoptotic protein expressions. Protein lysates were harvested from osteosarcoma cells using 1×RIPA Lysis Buffer and concentrations were determined using Protein Assay Reagent. Primary antibodies for PIM1 (1:1000 dilution) and actin (1:2000 dilution) were purchased from Abcam and Sigma-Aldrich, respectively. Secondary antibodies were bound to IRDye1 800CW or IRDye1 680LT (LI-COR Biosciences, NE). Western blots were performed as previously described. Actin was used as an endogenous control.
for normalization. Membrane signals were scanned using an Odyssey infrared imaging system and analyzed using Odyssey 3.0 software (LI-COR Biosciences).

**Osteosarcoma tissue microarray (TMA) and IHC**

Osteosarcoma tissue microarray was constructed from the Massachusetts General Hospital Sarcoma Tumor Bank, which contains 114 tumor tissues. IHC was conducted with HRP-DAB System Cell and Tissue Staining Kit. Based on the percentage of cells with positive nuclear staining, the staining patterns were categorized into 6 groups: 0, no nuclear staining; A, 1+, <10% of cells stained positive; B, 2+, 10% to 25% positive cells; C, 3+, 26% to 50% positive cells; D, 4+, 51% to 75% positive cells; E, 5+, >75% positive cells.

**Results:** Osteosarcoma cells showed strong expression of PIM1 while normal osteoblast cells were either negative or showed only weak expression of the protein. TMA showed high PIM1 expression associated with poor clinical outcome in osteosarcoma. Knockdown of PIM1 inhibited osteosarcoma cell growth and proliferation.

**Discussion:** In this study we show that osteosarcoma cells express high level of PIM1 protein compared with normal osteoblast cells. Most importantly, the levels of PIM1 expression are significantly associated with clinical outcome in osteosarcoma. Over-expression of PIM1 was correlated with poor prognosis. Furthermore, PIM1 siRNA or PIM1 inhibitor SMI-4a inhibit the growth and proliferation of the osteosarcoma cells.

**Significance:** These results provide a rationale for investigation of PIM1 as a novel therapeutic target in the treatment of osteosarcoma.

![Image](image_url)

**Figure 1.** PIM1 kinase is over-expressed in osteosarcoma cell lines, while PIM1 is almost undetectable in osteoblast cell lines.
Table1 Over-expression of PIM1 is an independent and significant factor in predicting poor prognosis

<table>
<thead>
<tr>
<th>variable</th>
<th>5-Year survival</th>
<th>5-Year disease-free survival</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>95 % CI</td>
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<tr>
<td>Metastasis</td>
<td>3.56</td>
<td>1.746 - 7.256</td>
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<tr>
<td>PIM1 expression</td>
<td>3.51</td>
<td>2.985 - 4.035</td>
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</tbody>
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