Notch Pathway is Activated in Osteosarcoma and Inhibitors of γ-secretase Prevents Osteosarcoma Growth by Cell Cycle Regulation

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Introduction: The Notch signaling pathway functions as an organizer in development. Notch belong to a family of conserved transmembrane receptors that play a fundamental role in cell fate decisions including cell proliferation, differentiation, and apoptosis. Notch signaling is initiated by receptor–ligand interactions resulting in a successive proteolytic cleavages by γ-secretase (GSI). This processing results in the release of the intracellular domain (NIC), which translocates to the nucleus and binds RBP-jk. RBP-jk/NIC interactions result in the expression of various target genes. Recent studies have shown constitutive activation of the Notch pathway in various types of malignancies. However, it remains unclear whether Notch pathway is activated in human osteosarcoma. In an attempt to better understand osteosarcoma pathogenesis, we investigated the expression and activation of Notch proteins in osteosarcoma and examined the effect of GSI to osteosarcoma growth. And also, we examined the molecular mechanism of osteosarcoma growth inhibition by GSI. The results demonstrated elevated levels of notch signaling molecules compared to normal osteoblast. NIC was accumulated in osteosarcoma cell nucleus. And also, GSI treatment prevented osteosarcoma cells proliferation in vitro and in vivo by cell cycle regulation.

Materials and Methods: RT-PCR was performed with Notch 1-2, Jagged 1-2, Dll1, and Hes1,7 specific primers. Immunohistochemistry for NIC, Jagged1, and Hes1 was performed using osteosarcoma cell lines and human osteosarcoma samples. Cells were treated with GSI to inhibit Notch activation. GSI are considered pan-Notch inhibitors. RBP-jk siRNA was used to confirm the effect of Notch signal inhibition. Cell survival was quantitated using a MTT assay. Cell proliferation was quantified by cell proliferation assay using BrdU incorporation. Nude mice were injected with osteosarcoma cells intradermally, and palpable tumors formed in 7 days. On the seventh day, nude mice were intraperitoneally injected with GSI. Injections continued every day and tumor dimensions were measured. Cell cycle was analyzed by PI and flowcytometry. The expression of the components of cell cycle machinery was analyzed by quantitative RT-PCR and western blot.

Results: RT-PCR revealed high expression of Notch-1, 2, Jagged1, Dll1, and Hes1, 7 in all of osteosarcomas cell lines. And also, 7 osteosarcoma human samples also showed high expression of Notch-1, 2, Jagged1, Dll1, and Hes1, 7 mRNAs. In addition, immunohistochemistry showed high expression of NIC in nucleus of osteosarcoma cell lines and human osteosarcoma samples. And also, expression of Jagged1 and Hes1 was observed in human osteosarcoma samples. These data suggest that notch signaling pathways are activated in osteosarcoma. Next, we investigated the effects of Notch pathway inhibition on osteosarcoma growth. MTT assay showed that GSI and RBP-jk siRNA suppressed the growth of the osteosarcoma cell lines in vitro (Fig.1). Cell proliferation assay showed that GSI suppressed the osteosarcoma cell proliferation. To determine the effects of Notch blockade in vivo, we examined the formation of tumor xenografts. Intraperitoneally GSI administration dramatically inhibited the osteosarcoma xenografts growth in vivo (Fig.2). Kaplan–Meier analysis showed that GSI administration conferred statistically significant survival benefit (Fig.2). Next we examined cell cycle by flowcytometry. When osteosarcoma cell were cultured without GSI, 39.8 % cells were in G1 phase. On the other hand, when cultured with GSI, 53.3 % cells were in G1 phase. These data suggest that GSI promoted G1 arrest. Next we examined the transcription of various target genes which transcription was regulated in cell cycle. Real time RT-PCR revealed that GSI suppressed transcription of cyclin D1, cyclin D3, cyclin E1 cyclin E2, and Skp2. In mammalian cells, many cell cycle regulator proteins are short-lived proteins that are controlled by ubiquitin-dependent proteolysis. Next we performed western blot to examine the expression of component proteins of cell cycle machinery. GSI promoted mild suppression of cyclin D1 and cyclin D3. And also, GSI reduced the expression of cyclin E1, cyclin E2, c-Myc, and Skp2 proteins (Fig.3). Cyclins and c-Myc were reported to promote G1-S phase progression. And also, Skp2 was reported as a component of ubiquitin E3 ligase to regulate the G1/S transition by degradation of p21cip1. p21cip1 can bind to various CDKs, including cyclin D/CDK4, cyclin E, and cyclin A/CDK2, and inhibits their kinase activity. Next we examined the expression of p21cip1. P21cip1 protein was upregulated by GSI treatment (Fig.3). These data suggest that GSI suppressed osteosarcoma growth and promoted G1 arrest by regulation of cell cycle regulators expression.

Discussion: The molecular basis for osteosarcoma tumor cell emergence, survival, and proliferation remains unclear despite active investigation. We demonstrated that osteosarcoma cells both in vitro and in vivo express elevated levels of Notch signal related genes. Notch is constitutively activated as demonstrated by NIC nuclear accumulation and expression of Notch target proteins. Moreover, treatment of osteosarcoma tumor cells with GSI, results in tumor regression in vitro and in vivo model systems. In addition, GSI promoted G1 arrest by regulation of cell cycle machinery proteins expression. These data suggest that inactivation of Notch may be a therapeutic approach for treating patients suffering from osteosarcoma.

![Fig.1](image1.png)

Inhibition of Notch signaling decreased osteosarcoma cell viability

![Fig.2](image2.png)

GSI dramatically reduce the xenograft growth

![Fig.3](image3.png)

Regulation of cell cycle regulators by Notch pathway inhibition

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<thead>
<tr>
<th>Cyclin D1</th>
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<td>Skp2</td>
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