Exploration of the inhibiting effect of Notch-1 blocker on tumor growth in osteosarcoma mouse models as well as related molecular mechanisms

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ABSTRACT

Objective: To explore the inhibiting effect of Notch-1 blocker on tumor growth in osteosarcoma mouse models as well as related molecular mechanisms. Methods: SCID mice were selected as experimental subjects, and osteosarcoma mouse models were built through subcutaneous injection of osteosarcoma cells and divided into GSI group and PBS group who received GSI and PBS intervention respectively. 0 d, 5 d, 10 d, 15 d and 20 d after intervention, tumor tissue volume was detected; 20 d after intervention, the contents of pro-apoptosis, pro-proliferation and invasion-related molecules in tumor tissue were detected. Results: On the 0 d of intervention, tumor volume of GSI group was not significantly different from that of PBS group; 5 d, 10 d, 15 d and 20 d after intervention, tumor volume of GSI group was significantly lower than that of PBS group; 20 d after intervention, the contents of pro-proliferation molecules AEG-1, Orai1, STIM1, PAK5, RIPK4 and β-catenin as well as invasion-promoting molecules Gab2, DLK1, Scr and B7-H3 in tumor tissue of GSI group were significantly lower than those of PBS group, and the contents of pro-apoptosis molecules RanBP9, PD-1, PD-L1 and PD-L2 as well as invasion-inhibiting molecules TIMP1 and TIMP2 were significantly higher than those of PBS group. Conclusion: Notch-1 blocker can inhibit tumor growth in osteosarcoma mouse models, and the molecular mechanisms involved in the process include reducing the generation of pro-proliferation molecules and invasion-promoting molecules and increasing the generation of pro-apoptosis and invasion-inhibiting molecules.

1. Introduction

Osteosarcoma is the most common tumor of orthopedics department that is derived from mesenchymal tissue and mostly occurs in metaphysis of the long bone. Teenagers are the high-risk population of osteosarcoma, and osteosarcoma ranks the third of common tumors in teenagers. The tumor has high malignant degree, rapid growth and strong invasion ability, and is prone to hematogenous metastasis and lung metastatic lesions[1]. The main means of clinical treatment of osteosarcoma is surgical resection, but the postoperative incidence rate and metastasis rate are higher, and patients’ survival is not ideal[2]. At present, the molecular mechanism of osteosarcoma is unclear, and it has been reported that abnormal activation of Notch-1-mediated signaling pathway is associated with the occurrence of osteosarcoma[3]. In the following research, osteosarcoma mouse models were built, Notch-1 blocker was provided for intervention, and tumor growth was observed to further verify the relationship between the activation of Notch-1 signaling pathway and osteosarcoma.

2. Materials and methods

2.1 Experimental animals

SCID mice were selected as experimental subjects, all were male
and the body mass was 18-22 g; mice were purchased by animal experiment center of Wuhan University. After adaptive feeding for 2 weeks, 24 mice were randomly divided into GSI group and PBS group, 12 in each group. The research was approved by the hospital ethics committee.

### 2.2 Experimental materials

Osteosarcoma cell lines MG-63 were purchased from Chinese Academy of Sciences, reagents for cell culture were purchased from Thermo Company, Notch-1 blocker GSI was purchased from Calbiochem Company, PBS solution was purchased from Shanghai Beyotime Company and ELISA kits were purchased from Elabscience Company.

### 2.3 Building of osteosarcoma xenograft mouse models

MG-63 cells were cultured, prancreatin-digested, sub-cultured and amplified, amplified cells were collected, the density was adjusted to $2 \times 10^7$/mL, 0.5 mL cell suspension was subcutaneously injected into left posterior limbs of mice, tumor growth was consecutively observed, and after 20 d, the mice with tumor diameter more than 0.5 cm and volume more than 100 mm$^3$ were determined as successfully built osteosarcoma xenograft models. All cell culture and animal experiments were completed in animal experiment center of Wuhan University.

### 2.4 Drug intervention

Successfully established osteosarcoma xenograft mouse models were collected, GSI group received injection of 50 μg GSI in local tumor tissue, and PBS group received injection of equal volume of PBS in local tumor tissue, 1/d for consecutive 1 week.

### 2.5 Tumor growth evaluation

On the 0 d, 5 d, 10 d, 15 d and 20 d of drug intervention, tumor tissue volume was detected; on the 20 d of drug intervention, tumor tissue volume was detected and then mice were executed and anatomized to collect tumor tissue, appropriate amount of tissue was weighed, added to PBS and homogenized, and then Elisa kits were used to detect AEG-1, Orai1, STIM1, PAK5, RIPK4, β-catenin, RanBP9, PD-1, PD-L1, PD-L2, TIMP1, TIMP2, Gab2, DLK1, Scr and B7-H3.

### 2.6 Statistical methods

SPSS 20.0 statistical software was used to input and analyze data, data between two groups was by t test and differences were considered to be statistically significant at a level of $P<0.05$.

### 3. Results

#### 3.1 Changes of tumor volume of two groups of osteosarcoma mouse models

5 d, 10 d, 15 d and 20 d after intervention, tumor volume of both groups gradually increased, and specific analysis of the tumor volume at various points in time showed that on the 0 d of intervention, tumor volume of GSI group was not significantly different from that of PBS group; 5 d, 10 d, 15 d and 20 d after intervention, tumor volume of GSI group was significantly lower than that of PBS group, shown in Table 1.

#### 3.2 Contents of pro-proliferation and pro-apoptosis molecules in tumor tissue

20 d after intervention, analysis of the contents of pro-proliferation molecules in tumor tissue of two groups was as follows: AEG-1, Orai1, STIM1, PAK5, RIPK4 and β-catenin contents in tumor tissue of GSI group were significantly lower than those of PBS group, shown in Table 2; analysis of the contents of pro-apoptosis

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>0 d after intervention</th>
<th>5 d after intervention</th>
<th>10 d after intervention</th>
<th>15 d after intervention</th>
<th>20 d after intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSI</td>
<td>107.6±11.4</td>
<td>120.3±14.7</td>
<td>125.5±13.2</td>
<td>140.2±15.9</td>
<td>148.9±14.3</td>
</tr>
<tr>
<td>PBS</td>
<td>109.3±12.5</td>
<td>218.4±25.6</td>
<td>332.7±39.9</td>
<td>418.9±47.4</td>
<td>624.8±71.5</td>
</tr>
<tr>
<td>$T$</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>AEG-1 (ng/mL)</th>
<th>Orai1 (ng/mL)</th>
<th>STIM1 (ng/mL)</th>
<th>PAK5 (pg/mL)</th>
<th>PIPK4 (ng/mL)</th>
<th>β-catenin (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSI</td>
<td>22.1±2.6</td>
<td>19.3±2.1</td>
<td>56.6±6.1</td>
<td>201.7±23.8</td>
<td>39.1±4.9</td>
<td>3.9±0.5</td>
</tr>
<tr>
<td>PBS</td>
<td>45.4±5.4</td>
<td>34.3±4.3</td>
<td>89.4±10.3</td>
<td>347.4±40.3</td>
<td>66.2±7.8</td>
<td>10.3±1.4</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
molecules in tumor tissue of two groups was as follows: RanBP9, PD-1, PD-L1 and PD-L2 contents in tumor tissue of GSI group were significantly higher than those of PBS group, shown in Table 3.

Table 3. Contents of pro-apoptosis molecules in tumor tissue.

<table>
<thead>
<tr>
<th>Group</th>
<th>RanBP9 (ng/mL)</th>
<th>PD-1 (pg/mL)</th>
<th>PD-L1 (pg/mL)</th>
<th>PD-L2 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSI</td>
<td>93.9±10.5</td>
<td>683.5±94.5</td>
<td>535.3±55.8</td>
<td>881.3±95.9</td>
</tr>
<tr>
<td>PBS</td>
<td>38.5±4.2</td>
<td>314.8±36.9</td>
<td>384.5±0.7</td>
<td>407.9±50.3</td>
</tr>
<tr>
<td>T</td>
<td>14.855</td>
<td>12.183</td>
<td>7.966</td>
<td>11.103</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

3.3 Contents of invasion–related molecules in tumor tissue

20 d after intervention, analysis of the contents of invasion-inhibiting molecules in tumor tissue of two groups was as follows: TIMP1 and TIMP2 contents in tumor tissue of GSI group were significantly higher than those of PBS group, shown in Table 4; analysis of the contents of invasion-promoting molecules in tumor tissue of two groups was as follows: Gab2, DLK1, Scr and B7-H3 contents in tumor tissue of GSI group were significantly lower than those of PBS group, shown in Table 4.

Table 4. Contents of invasion-related molecules in tumor tissue.

<table>
<thead>
<tr>
<th>Group</th>
<th>Invasion-promoting molecules</th>
<th>Invasion-inhibiting molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gab2 (ng/mL)</td>
<td>DLK1 (ng/mL)</td>
</tr>
<tr>
<td>GSI</td>
<td>54.9±6.4</td>
<td>103.4±11.8</td>
</tr>
<tr>
<td>PBS</td>
<td>91.3±10.5</td>
<td>184.5±20.7</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

4. Discussion

Osteosarcoma is the most common malignant bone tumors in 10-20-year old adolescent population, and tumor cell proliferation and invasion ability is extremely strong, thus influencing the effect of surgical resection combined with postoperative chemotherapy. At present, the molecules that play a key role in the process of osteosarcoma are unclear, and specific clinical targeted drugs are also needed.\(^{[4,5]}\) Notch signaling pathway is the intersection of a variety of signal transduction pathways in the body, and is important for the regulation of cell behavior. Notch genes include four types, Notch1-4, all encode type I transmembrane glycoprotein, are a type of evolutionally highly conserved cell membrane surface receptors, and will be activated after combination with the corresponding Notch ligand on adjacent cell surface. Notch-mediated signals are triggered after the combination of transmembrane glycoprotein extracellular fragment with the corresponding ligand, and activated Notch intracellular fragment ICN will fall off and enter into the nucleus, thus regulating the transcription of a variety of target genes and affecting cell growth, proliferation and differentiation. Notch-1 is the most important one of the fourth Notch molecules, and its function determines the overall function of the Notch signaling pathway.

In recent years, more and more scholars begin to pay close attention to the relationship between Notch-1-mediated signaling pathway and malignant tumors. Studies have reported that the expression of Notch-1 significantly increases in lung cancer, liver cancer, osteosarcoma, breast cancer and other malignant tumor tissues, and activated Notch-1 can promote in vitro cultured osteosarcoma cell invasion and metastasis process.\(^{[6-8]}\) According to the results of the above research, it was speculated that enhanced Notch-1 signaling pathway function is related to the occurrence of osteosarcoma, and can promote tumor cell invasion and metastasis. In order to further verify the role Notch-1 signaling pathway in the occurrence and development of osteosarcoma, SCID mice were selected as the research objects to establish osteosarcoma xenograft mouse models, Notch-1 blocker was provided and then tumor growth was determined. Tumor volume could directly reflect tumor growth, and detection and analysis of tumor volume showed that at various points in time after drug intervention, tumor volume of GSI group was lower than that of PBS group. It preliminarily confirmed that Notch-1 blocker could inhibit osteosarcoma growth.

After Notch-1 signaling pathway is activated, Notch-1 molecular intracellular fragment is fractured and translocated into the nucleus, which regulates the production of a variety of transcription factors to influence the expression of multiple genes. The growth of osteosarcoma cells is regulated by a variety of pro-proliferation and pro-apoptosis molecules. Pro-proliferation molecules associated with osteosarcoma include AEG-1, STIM1, PAK5 and RIPK4, and pro-apoptosis molecules include RanBP9, PD-1, etc. The gene encoding AEG-1 belongs to oncogene and can increase the ability of anchorage-independent growth of tumor cells and promote cell proliferation and cell cycle progression.\(^{[9]}\) Orai1 and STIM1 are the key molecules regulating calcium store-dependent calcium influx, and can increase calcium inflow and promote cell proliferation.\(^{[10]}\) PAK5, also called p21 activated kinase 5, is a kind of serine/threonine protein kinase that can regulate cell proliferation by phosphorylation of downstream molecules;\(^{[11]}\) RIPK4 can be combined with receptor LRP6 and increase the content of intracellular \(\beta\)-catenin, thus maintaining cell survival. RanBP9 can interact with the death domain of p75 in tumor necrosis factor receptor family to promote cell apoptosis; PD-1 can be combined...
with PD-L1 and PD-L2 to regulate programmed cell apoptosis. In the research, analysis of the contents of above pro-proliferation and pro-apoptosis molecules showed that AEG-1, Orai1, STIM1, PAK5, RIPK4 and β-catenin contents in tumor tissue of GSI group were significantly lower than those of PBS group, and RanBP9, PD-1, PD-L1 and PD-L2 contents were significantly higher than those of PBS group. It indicated that Notch-1 blocker could increase the generation of pro-apoptosis molecules and inhibit the generation of pro-proliferation molecules in osteosarcoma tissue.

Osteosarcoma patients who receive surgical resection combined with chemotherapy are prone to distant metastasis and tumor cell migration and invasion is the key to cause tumor distant metastasis. Matrix metalloproteinases MMPs family are the key molecules mediating extracellular matrix degradation, cell migration and invasion, and their activity to degrade extracellular matrix is affected by the tissue inhibitor of metalloproteinases (TIMPs)[12]. TIMP-1 and TIMP-2 can form covalent bonding with a variety of MMPS molecules to inhibit MMP degradation of a variety of proteins. In addition to MMP molecules that are associated with tumor cell migration and invasion, Gab2, DLK1, Scr, B7-H3 and other molecules are also involved in this process. Gab2 is one member of the scaffolding protein Gab family, which can cause tumor cell chemotaxis[13]; DLK1 is also known as transmembrane protein 1, its extracellular region contains 6 EGF-like repeating fragments, and it has regulating effect on cell invasion[14]; Scr can promote the invadopodia formation of osteosarcoma cells[15]; B7-H3 is a kind of costimulatory molecule mainly involved in regulation of cell adhesion and migration[16]. In the research, analysis of the contents of above invasion-related molecules showed that TIMP1 and TIMP2 contents in tumor tissue of GSI group were significantly higher than those of PBS group, and Gab2, DLK1, Scr and B7-H3 contents were significantly lower than those of PBS group.

Based on above discussion, it can be concluded that Notch-1 blocker can inhibit tumor growth in osteosarcoma mouse models, and the molecular mechanisms involved in the process include reducing the generation of pro-proliferation molecules and invasion-promoting molecules and increasing the generation of pro-apoptosis and invasion-inhibiting molecules.

References


