Original Article
MLLT3 promotes proliferation of osteosarcoma cells by regulating JNK signaling

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Abstract: Osteosarcoma was the most common malignant tumor derived from bone/soft tissues and afflicted mainly the adolescents. Surgical removing assisted with chemotherapy/radiotherapy was the major therapeutics regardless of high mortality. MLLT3 was one of the most common partners fused with mixed lineage leukemia gene and caused leukemia. But its role in osteosarcoma was not previously reported. In this study, we found that MLLT3 was expressed abundantly in osteosarcoma tissues and cell lines including U-2OS, MG-63, Saos-2 and HOS. And by Kaplan-Meier analysis, MLLT3 expression was shown to be clinically correlated with survival of osteosarcoma patients. The 5-year survival rate was 64.3% for patients with low MLLT3 expression (n=14) while it was 36.4% for patients with high MLLT3 expression (n=11). The difference was significant (p=0.026). When MLLT3 was knocked-down, the proliferation ability decreased about 38% in HOS cells and 46% in MG-63 cells. But the migration as well as the invasive ability was not affected. In addition, the expression level of β-catenin was comparable in HOS cells with/without knockdown of MLLT3. The specific inhibitor LGK-974 against Wnt/β-catenin signaling showed little additive effect with MLLT3 knockdown on growth ability of HOS cells. But the level of c-Jun, BCL-2, Akt and c-Myc was significantly decreased in HOS cells with MLLT3 knockdown, which indicated that MLLT3 may at least partially regulate JNK signaling in HOS cells. In conclusion, we proved MLLT3 as an oncogene in osteosarcoma through partially regulating the JNK signaling pathway. And MLLT3 may be a promising target for treatment of patients with osteosarcoma.

Keywords: Osteosarcoma, MLLT3, JNK signaling, HOS cell, BCL-2, c-Myc

Introduction

Osteosarcoma is the most common malignant bone tumor afflicting mainly the children and young adults between 15-25 years old [1, 2]. The estimated new cases of bone and joints tumor in 2016 were about 3,300 in the USA. And the estimated death was 1,490 [3]. But the death rate for sarcoma in China was above 70% in 2015. The estimated total incidence of bone tumor was 28,000 while the total mortality was about 20,700 cases in China [4]. The advanced therapeutic treatments have greatly improved the 5-year-relapse-free survival rate to 65% when the disease is localized [5]. However, the survival rate is only about 20% when the disease is metastatic or relapsed [6]. What’s worse, there is about 20% newly diagnosed patients with lung-related metastasis [1]. Unfortunately, there is no effective marker for diagnosis and prognosis of osteosarcoma although the pathologic characteristics of osteosarcoma have been well established. And little information was reported for the precise molecular mechanism underlying the carcinogenesis, progression and metastasis of osteosarcoma.

To date, apart from the major therapy including surgical remove, chemotherapy and radiotherapy, there is no other effective treatment for osteosarcoma. So it is urgent to develop new drugs or new treatments. Human MLLT3 gene, also called AF9 or LTG9, was located at chromosome 9p22 region. It was one of the most common fifty partners fused with the mixed lineage leukemia (MLL) gene at chromosome 11q23, which resulted in translocation t(9;11)(p22;q23) [7]. The formed fusion gene MLL-AF9 was the most common chromosomal aberrations in pediatric acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) [7]. Also it was reported to be suffi-
The MLLT3 gene was >100 kb and contained a serine/proline-rich domain as well as a nuclear localization signal [7, 8]. It was shown to function as a transcription activator and might be involved in the activity of RNA polymerase II, elongated transcription of target genes and chromatin remodeling [9]. MLLT3 was reported to play essential roles in embryogenesis. The functional loss of MLLT3 in mice caused perinatal lethality or abnormal axial skeleton [8]. Chromosomal translocation was a crucial event in sarcomas and usually resulted in novel fusion genes or elevated oncogene expression. MYC gene was active in many kinds of cancers and was a direct target of super elongation complex containing MLLT3 [9]. However, there is no information about the role of MLLT3 gene in osteosarcoma and the molecular mechanism mediated by MLLT3.

In order to investigate the role of MLLT3 in osteosarcoma, we detected the expression of MLLT3 gene in osteosarcoma tissues and analyzed its clinical correlation. Then the function of MLLT3 gene in osteosarcoma was explored in osteosarcoma with the help of RNAi technology. And the molecular mechanism mediated by MLLT3 was further investigated by western blot analysis.

Materials and methods

Cell culture and cancer tissues

Human osteosarcoma cell lines including U-2OS, MG-63, Saos-2 and HOS cells were purchased from Cell Bank Type Culture Collection of Chinese Academy of Sciences (CBTCCCAS, Shanghai, China) and cultured in DMEM medium (GIBCO, USA) with 10% fetal bovine serum (GIBCO, USA), 100 unit/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO₂.

A total of 25 frozen osteosarcoma tissues were obtained from Cancer Center of the Second Hospital of Shandong University. These osteosarcoma samples were examined carefully with hematoxylin/eosin staining assay and the development and pathogenic progression was diagnosed and evaluated according to the World Health Organization (WHO) criteria. Written informed consent was obtained from each patient. The experimental protocols were approved by the Ethics Committee of the Second Hospital of Shandong University.

Quantitative reverse transcription-PCR (RT-qPCR)

Total RNA was extracted from osteosarcoma tissues and osteosarcoma cell lines with Trizol agent (Invitrogen, USA). Then cDNA was synthesized from 1 μg of total RNA by reverse transcription kit (Promega, USA) according to the manufacturer’s instructions. After that 1 μl of cDNA was used to evaluate the mRNA levels of MLLT3 with the SYBR Green RT-PCR Kit (Takara, Otsu, Japan) on Bio-Rad iQ5 real-time PCR system. GAPDH was used as internal control. The primers were listed in Table 1. Data were analyzed in terms of relative quantification (RQ) to GAPDH based on calculations of $2^{-\Delta\Delta C_{t}}$ where $\Delta C_{t}=C_{t}(\text{MLLT3})-C_{t}(\text{GAPDH})$ [10]. Fold change was obtained with the $2^{\Delta\Delta C_{t}}$ method. All samples were tested in triplicates.

The PCR protocol was as following: Pre-denaturation at 95°C for 1 min, (Denaturation at 95°C for 30 s, annealing and extension at 58°C for 30 s)×40 cycles.

Transfection of osteosarcoma cells with small interfering RNA (siRNA)

The siRNA targeting MLLT3 coding sequence (MLLT3-siRNA) and negative control (NC-siRNA) were designed and synthesized as in Table 2. Then synthesized siRNA were transfected into HOS or MG-63 cells with lipofectamin 2000 (Invitrogen, USA) according to the manufacturer’s protocol. In brief, about $2\times10^{5}$/well HOS or

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>MLLT3</td>
<td>Forward: PF1</td>
<td>TACGCCACCCACACTGATACTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse: PR1</td>
<td>GCAGGACTGGTGTTTAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: PF2</td>
<td>AAGGGTCATACATCTCGCCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: PR2</td>
<td>GGGGCATCCACAGTCTTCTG</td>
</tr>
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Table 2. Designed siRNA fragments targeting MLLT3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
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</thead>
<tbody>
<tr>
<td>MLLT3-siRNA-1</td>
<td>CCACCTGATACCTCCTT</td>
</tr>
<tr>
<td>MLLT3-siRNA-2</td>
<td>GCAAAGATCCACCTTAA</td>
</tr>
<tr>
<td>MLLT3-siRNA-3</td>
<td>GCAGATCGTGAACCTTAT</td>
</tr>
</tbody>
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MG-63 cells were seeded into 12-well plates. After 24 hours, cells were treated with 20 µmol synthesized siRNA by lipofectamin 2000. Culture medium was replaced with 500 µl of fresh DMEM (10% FBS) after 8 hours of incubation. Then transfected cells were cultured at 37°C in 5% CO₂.

Cell proliferation assay

MTT assay was used to evaluate proliferation rate of osteosarcoma cells. About 2×10³ cells/well in 100 µl DMEM were added into a 96-well plate and cultured. 20 µl of MTT solution (Sigma, 5 mg/ml) was added daily from the 2nd to 5th day followed by incubation for another 4 h at 37°C. Then the supernatant was removed and 100 µl dimethyl sulfoxide (Sigma, USA) was added to each well. Absorbance values (A) at 490 nm wavelength were measured on a microplate reader. All experiments were repeated independently for three times.

Wound healing assay

HOS cells transfected with MLLT3-siRNA were grown in a 6-well plate at 1×10⁴ cells/well in 1 ml DMEM. After the cell confluence reached about 90%, a scratch was created with a 10 µl plastic pipette tip, and the cells were washed gently with PBS. Then DMEM without serum was added and cells were cultured for another 24 h. The distance in randomly selected five fields along the scratch was recorded at 0 h, 12 h and 24 h. The migration rate was calculated with the below formula:

Migration rate = (S₀h - S₁2/₂₄h)/S₀h ×100%

Where S₀h stands for the width of the scratch at 0 h while S₁₂/₂₄h stands for the width at 12 h or 24 h.

Transwell assay

This assay was performed with a Transwell (Corning Incorporated, Corning, NY, USA; pore size, 8 µm) in 24-well plate. About 2×10⁴ cells in 200 µl DMEM with no serum were placed in the upper chamber when DMEM supplemented with 10% FBS was placed in the lower chamber. The plates were incubated for 40 h at 37°C. Then the medium was removed and the membranes were washed with PBS twice. The cells on the upper side of membranes were removed using cotton-tipped swabs when the cells on the lower side of the membranes were fixed in 95% alcohol for 10 min and stained with 0.1% crystal violet for 15 min at room temperature. The cells transmitted from the upper to the lower side of the membranes were counted under a light microscope by counting 10 random fields at magnification ×100.

Western blot analysis

HOS cells transfected with MLLT3-siRNA were grown in a 6-well plate for 48 h and total protein was extracted with iced lysis buffer (1% TritonX-100, 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% SDS; 1 mM phenylmethanesulfonyl fluoride; 1 mM EDTA) and quantified by the bicinchoninic acid assay (Generay, China). About 10 µg total proteins were detected on SDS-polyacrylamide gel, electro-transferred to nitrocellulose membranes, and incubated for 12 h with primary mouse monoclonal antibody against β-catenin, c-Jun, TCF and c-Myc (Santa Cruz Biotechnology, USA) or BCL-2, GAPDH (Abcam, USA). Then the membranes were washed and incubated in horseradish peroxidase conjugated secondary antibody for 2 h at room temperature. The target protein was determined with enhanced chemiluminescence (ECL) kit (Amersham Biosciences, USA).

Statistical analysis

All data were expressed as mean ± standard deviation of three independent experiments and the difference between every two groups in the in vitro cell experiments was analyzed by a two-tailed paired Student’s t-test method with SPSS11.0. The difference of MLLT3 expression level between tumor tissues and adjacent normal tissues was analyzed with paired Student’s t-test method on GraphPad Prism. v.5.0. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. P<0.05 was considered as statistically significant.

Results

MLLT3 was expressed abundantly in osteosarcoma tissues and cell lines

To explore the role of MLLT3 in osteosarcoma, RT-qPCR technology was employed to detect the expression level of MLLT3 in osteosarcoma
tissues and cell lines. As shown in Figure 1A, MLLT3 was significantly up-regulated in tumor tissues compared with the adjacent normal tissues. In more than half tissues determined, the mRNA level of MLLT3 was three times higher than that in the adjacent tissues. What’s more, the expression of MLLT3 in four cell lines including U-2OS, MG-63, Saos-2 and HOS was also stronger than that in normal bone cells (Figure 1B). These results demonstrated that MLLT3 was over-expressed in both osteosarcoma tissues and cell lines.

**MLLT3 expression was clinical correlated with osteosarcoma**

Based on the mRNA level of MLLT3 in 25 patients with osteosarcoma, we evaluated the clinical significance of MLLT3. As shown in Figure 1C, the expression level of MLLT3 was significantly correlated with the survival rate. The estimated 5-year survival rate was 64.3% for patients with low MLLT3 expression (n=14) but it was only 36.4% for those with high MLLT3 expression (n=11). And there was significant difference between these two groups (p = 0.026). These data indicated that MLLT3 may be a good prognostic marker for osteosarcoma but data from more patients were needed to support this conclusion.

**Knockdown of MLLT3 suppressed proliferation of osteosarcoma cell lines**

Unlimited growth was one of the ten hallmarks of cancer cells [11]. To investigate the role of MLLT3 in growth of osteosarcoma cell lines, RNAi technology was used to reduce the expression of MLLT3 in HOS and MG-63 cell lines, respectively. As shown in Figure 2A and 2B, the three siRNA fragments targeting coding sequence of MLLT3 reduced the expression of MLLT3 efficiently. Particularly, the knockdown efficiency of MLLT3-siRNA-2 fragment was more than 75% in either HOS or MG-63 cells. After treated with MLLT3-siRNA for 48 h, the
MLLT3 and JNK in osteosarcoma

**Figure 2.** MLLT3 was essential for proliferation of osteosarcoma cells. The mRNA level of MLLT3 was efficiently knocked down by synthesized siRNA in HOS cells (A) and MG-63 cells (B). MTT assay indicated that knock-down of MLLT3 significantly inhibited growth of both HOS cells (C) and MG-63 cells (D). *P<0.05 was considered as significant difference.

proliferation of HOS or MG-63 cells was determined by MTT assay. It was shown that decreased MLLT3 significantly inhibited growth of HOS or MG-63 cells (Figure 2C and 2D). The growth rate decreased about 38% in HOS cells and 46% in MG-63 cells compared with the negative control.

Knockdown of MLLT3 showed no effect on migration and invasion of osteosarcoma cells

Cell migration and invasion to adjacent normal tissue was another hallmark of malignant tumor [11]. And about 20% osteosarcoma patients were diagnosed with lung metastasis [1]. Therefore, the effect of MLLT3 on migration of HOS cells and MG-63 cells was investigated by wound-healing assay while transwell assay was carried out to determine the invasion ability. Unexpectedly, there was no difference between the migration distance of HOS cells treated with MLLT3-siRNA and negative control after 12 h and 24 h (Figure 3A). We got the same results in MG-63 cells (Figure 3B). Furthermore, the number of invaded cells in MLLT3-siRNA treated cells was comparable to that in negative control after 40 h (Figure 3C). These data revealed that MLLT3 had little effect on the migration or invasion ability of HOS cells and MG-63 cells.

MLLT3 activated JNK-dependent signaling

MLLT3 was extensively reported as a fusion partner of MLL gene. But Haribaskar demonstrated that MLLT3 interacted with the planar cell polarity protein Diversin and activated JNK-dependent signaling molecules independent on β-catenin [12]. So we deduced that down-regulation of MLLT3 in osteosarcoma cells may exert a significant effect on JNK signaling. To elucidate the mechanism of MLLT3 in carcinogenesis of osteosarcoma, we detected the expression of downstream molecules of JNK
MLLT3 and JNK in osteosarcoma

was also shown to be expressed abundantly in human osteosarcoma cell lines. Furthermore, the expression level of MLLT3 was associated with the survival rate of patients. In another word, the higher expression of MLLT3 was associated with the poorer prognosis in patients with osteosarcoma. These data indicated that MLLT3 was clinically correlated with osteosarcoma and may contribute to the carcinogenesis of osteosarcoma.

Inter-chromosomal translocations are typical features for many hematological malignancies [14, 15]. And translocation of MLL gene to MLLT3 leaded to a gain of function of MLL gene, in which process a new oncogene MLL-MLLT3 formed and prompted the transformation of normal hematological cells followed by lymphoblastic leukemia [16]. In fact, MLLT3 have a transcriptional activation activity and fusion of MLL with MLLT3 enhanced the transcription of downstream genes [15]. It is known that unlimited growth and potential metastasis are two of ten hallmarks in cancer [11]. Accordingly, MLLT3 was also vital to prolifera-

Discussion

The high mortality rate of osteosarcoma and the limited therapeutics necessitated the development of new drugs [3, 4, 13]. In this study, the role of MLLT3 gene in growth and metastasis of osteosarcoma cells was investigated in vitro. MLLT3 was commonly fused with MLL gene and lead to acute leukemia [7]. But in this study, we found that MLLT3 was overexpressed in human osteosarcoma tissues compared to the adjacent tissues. And MLLT3 signaling as well as the critical molecules of Wnt canonical pathway in HOS cells. Unsurprisingly, expression of c-Jun, BCL-2, Akt and c-Myc were down-regulated in HOS cells treated with MLLT3-siRNA (Figure 4A). But the level of β-catenin molecule was not affected by MLLT3. Moreover, the specific inhibitor against PORCN in canonical Wnt signaling pathway showed little effect on growth of HOS cells (Figure 4B). These data further support that MLLT3 functions as an oncogene, partially if not all, by regulating JNK signaling but not Wnt/β-catenin signaling pathway.

Figure 3. Knock down of MLLT3 showed little effects on migration and invasion of osteosarcoma cells. Decreased MLLT3 did not suppress migration of HOS cells (A) or MG-63 cells (B) in a wound-healing assay. The migrated distance of HOS cells was determined at five-select ed fields at time point 12 h and 24 h after a scratch was produced. MLLT3 knockdown did not affect invasion ability of HOS cells or MG-63 cells in transwell assay (C). The number of invading cells were quantified and averaged at randomly selected 5 fields after 40 h. *P<0.05 was considered as significant difference.
MLLT3 and JNK in osteosarcoma

Figure 4. MLLT3 regulated JNK signaling pathway in HOS cells. A. Western blot analysis indicated that expression of c-Jun, c-Myc, Akt, BCL-2 was downregulated in HOS cells transfected with MLLT3-siRNA. But β-catenin was the same in HOS cells with/without MLLT3-siRNA. GAPDH was used as internal control. B. MLLT3-siRNA but not LGK-974 inhibited HOS cells proliferation and there was no synergic effect between MLLT3-siRNA and LGK-974. Data were displayed as mean ± SD of three independent experiments. *P<0.05 was considered as significant difference.

of osteosarcoma cells. As shown in the text, decreased expression of MLLT3 dramatically inhibited proliferation of HOS and MG-63 cells. However, the migratory and invasive capability was not affected by MLLT3 knockdown in both cells. This was not surprisingly and these data support the deduction that MLLT3 conferred its oncogenic potential to osteosarcoma largely through promoting the growth of osteosarcoma cells but not through metastasis.

The physiological activity of MLLT3 gene was to regulate embryonic development [17]. For example, MLLT3 was shown to affect the development of cerebral cortex by epigenetic modification of histone H3K79 [18]. But MLLT3 was commonly reported to be fused with MLL gene by inter-chromosomal recombination and caused leukemogenesis. One study indicated MLLT3 regulated downstream gene transcription by binding BCL-6 corepressor [14]. In another study, MLLT3 was involved in the formation of SEC-L2 and SEC-L3 complex which directly targeting oncogene MYC [9]. MYC gene was a central player in regulation of cell proliferation and was over-expressed in many kinds of cancer [19]. In consistent, MYC gene was shown to be downregulated in HOS cells when MLLT3 was knocked down. MLL-MLLT3 fusion was shown to induce activation of RacGTPase through Frat2 in leukemia [16]. However, very little information about MLLT3 was reported in solid tumors.

In this study, we demonstrated that the expression of c-Jun, BCL-2, Akt was significantly decreased in MLLT3-siRNA-treated HOS cells. C-Jun was one critical mediator in JNK signaling pathway [20]. And MLLT3 was reported to augment the activation of JNK-dependent gene expression through PCP protein Diversin [12]. Akt was another critical mediator in cell proliferation and was shown to be increased in many kinds of cancer cells [21]. BCL-2 played an anti-apoptosis role and often contributed to carcinogenesis of tumors [22]. So MLLT3 may promote cell proliferation and inhibit apoptosis through up-regulation of Akt and BCL-2 respectively. Wnt/β-catenin was reported to be activated in osteosarcoma by SDF-1/CXCR4 [23]. But MLLT3 was reported to block canonical Wnt signaling [12]. In fact, the expression level of β-catenin was not changed by MLLT3 in HOS cells in our study. Additionally, the proliferation of HOS cells was not affected by the specific inhibitor of Wnt/β-catenin signaling. Therefore, MLLT3 may function as an oncogene by regulating activity of JNK signaling pathway but not canonical Wnt signaling.

Conclusively, in this study, we proved the oncogenic role of MLLT3 gene in carcinogenesis of osteosarcoma. And JNK signaling may be important for MLLT3 in osteosarcoma. Our research expands the role of MLLT3 to solid tumor and provides a promising hope for patients with osteosarcoma.

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MLLT3 and JNK in osteosarcoma

Medicine in the Second Hospital of Shandong University.

Disclosure of conflict of interest

None.

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