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SS18-SSX-regulated miR-17 promotes tumor growth of synovial sarcoma by inhibiting p21WAF1/CIP1

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Key words Cyclin-dependent kinase inhibitor p21, drug resistance, hsa-mir-17 microRNA, SS18-SSX fusion protein, synovial sarcoma

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Synovial sarcoma is a high-grade malignancy and accounts for approximately 5–10% of soft tissue sarcomas. Synovial sarcomas mainly develop in the para-articular regions in adolescents and young adults. The combination of surgery and chemotherapy has resulted in an approximate 60% 5-year survival rate, but the 10-year survival rate is still miserably low; therefore, identification of effective therapeutics for this sarcoma is critical. Synovial sarcoma is characterized by a chromosomal translocation between chromosomes 18 and X, generating oncoproteins such as SS18-SSX1 and SS18-SSX2. This translocation is present in >95% of cases and is likely to be the driving oncogenic event in the development of this tumor; however, the precise mechanisms of SS18-SSX transformation remain controversial.

MicroRNA (miRNA) can function as tumor suppressors or oncogenes, and also as potential specific cancer biomarkers; however, there are few published studies on miRNA in synovial sarcomas, and their function remains unclear. We transfected the Oncomir miRNA Precursor Virus Library into synovial sarcoma Fuji cells followed by a colony formation assay to identify miRNAs to confer an aggressive tumorigenicity, and identified miR-17-5p from the large colonies. MiR-17 was found to be induced by a chimeric oncoprotein SS18-SSX specific for synovial sarcoma, and all examined cases of human synovial sarcoma expressed miR-17, even at high levels in several cases. Overexpression of miR-17 in synovial sarcoma cells, Fuji and HS-SYII, increased colony forming ability in addition to cell growth, but not cell motility and invasion. Tumor volume formed in mice in vivo was significantly increased by miR-17 overexpression with a marked increase of MIB-1 index. According to PicTar and Miranda algorithms, which predicted CDKN1A (p21) as a putative target of miR-17, a luciferase assay was performed and revealed that miR-17 directly targets the 3’-UTR of p21 mRNA. Indeed, p21 protein level was remarkably decreased by miR-17 overexpression in a p53-independent manner. It is noteworthy that miR-17 succeeded in suppressing doxorubicin-evoked higher expression of p21 and conferred the drug resistance. Meanwhile, introduction of anti-miR-17 in Fuji and HS-SYII cells significantly decreased cell growth, consistent with rescued expression of p21. Taken together, miR-17 promotes the tumor growth of synovial sarcomas by post-transcriptional suppression of p21, which may be amenable to innovative therapeutic targeting in synovial sarcoma.
doxorubicin treatment-evoked massive expression of p21, resulting in profound tumor growth in mice both in vitro and in vivo. These findings suggest that miR-17 is a potentially powerful therapeutic agent in synovial sarcoma.

Materials and Methods

Cell lines, cell culture and synovial sarcoma cases. The human synovial sarcoma cell lines SYO-1, Fuji and HS-SYII were established and maintained as described previously.\(^{(24)}\) SYO-1 and Fuji cells genetically possess the SS18-SSX2 fusion transcript, whereas HS-SYII cells occur as SS18-SSX1. Human oral cancer cells (HSC-2, HSC-3 and HSC-4), prostate cancer cells (LNCap), fibrosarcoma (HT1080), osteosarcoma (SaoS-2), embryonic kidney 293T cells and BJ/² foreskin fibroblast were maintained in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Cansera, Toronto, Ontario, Canada). Human Ewing’s sarcoma cells (TC71) were maintained in RPMI1640 with 10% FBS. Mir-17-overexpressing cells were established using the BLOCK-IT HiPerform Lentiviral PolII miR RNAi Expression System with EmGFP (Invitrogen, Carlsbad, CA, USA). Seven surgically resected tumors diagnosed as synovial sarcoma were used to assess the expression levels of miR-17. The present study was approved by the Medical Ethics Committee of the Hokkaido University Graduate School of Medicine.

Reagents, antibodies and immunoblotting. The following antibodies were purchased: antibodies to p21, CIP1, phospho-Akt (clone: D9E), Cyclin D1 and actin (I-19) were purchased from Santa Cruz (Cyclin D1, fibrosarcoma (HT1080), osteosarcoma (SaoS-2), embryonic kidney 293T cells and BJ/² foreskin fibroblast were maintained in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Cansera, Toronto, Ontario, Canada). Human Ewing’s sarcoma cells (TC71) were maintained in RPMI1640 with 10% FBS. Mir-17-overexpressing cells were established using the BLOCK-IT HiPerform Lentiviral PolII miR RNAi Expression System with EmGFP (Invitrogen, Carlsbad, CA, USA). Seven surgically resected tumors diagnosed as synovial sarcoma were used to assess the expression levels of miR-17. The present study was approved by the Medical Ethics Committee of the Hokkaido University Graduate School of Medicine.

Identification of microRNA to promote tumorigenicity in synovial sarcoma. The OncomiR miRNA Precursor Virus Library (System Bioscience, Mountain View, CA, USA) was infected into Fuji and HS-SYII cells, and a colony formation assay in soft agar was performed as described below. RNA was isolated from the two isolated colonies formed by Fuji cells, and semi-quantitative RT-PCR using the OncomiR miRNA Precursor Virus Library primer (System Bioscience) and the following sequencing were performed to identify infected oncomiR.

Analysis of cell proliferation and colony formation assay. Analysis of cell proliferation was conducted as described previously.\(^{(25)}\) To assess the effect of miR-17 on cell growth, cells were transfected with anti-miR-17 reagent (miRCURY LNA microRNA Inhibitors, hsa-miR-17, 410087-00, EXIQON, Vedbaek, Denmark) and counted after 4 days. For the colony formation assay, the numbers of colonies after 4 weeks of <100 μm, 100–250 μm and more than 250 μm in diameter were counted.\(^{(25)}\)

Analysis of cell motility and invasion assay. A wound healing assay assessing cell motility and a Matrigel invasion assay (Corning, Bedford, MA, USA) were performed as described previously.\(^{(26)}\)

RNA isolation and RT-PCR. RNA isolation, cDNA synthesis and RT-PCR for human miR-17, p21, SS18-SSX1, SS18-SSX2, RN6b and GAPDH (conventional and quantitative real-time PCR) were performed as described previously.\(^{(26)}\) Primers used were as follows: miR-17 forward, 5’-GCCGGCGGTCCAGAATACGTCAAGGTCGCCAGTCG-3’; and reverse, 5’-ACACCCATATGGCTACAAGTCTTCTCAGGAA-3’. For the colony formation assay in soft agar was performed as described below. RNA was isolated from the two isolated colonies formed by Fuji cells, and semi-quantitative RT-PCR using the OncomiR miRNA Precursor Virus Library primer (System Bioscience) and the following sequencing were performed to identify infected oncomiR.

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Analysis of cell motility and invasion assay. A wound healing assay assessing cell motility and a Matrigel invasion assay (Corning, Bedford, MA, USA) were performed as described previously.\(^{(26)}\)

Results

SS18-SSX-regulated miR-17 functions as oncogene in synovial sarcoma cells. To identify whether microRNA causes marked tumorigenicity on synovial sarcoma, cancer-related miRNA library screening was performed, the OncomiR miRNA Precursor Virus Library was infected into independent human synovial sarcoma cell lines Fuji and HS-SYII, and a colony formation assay in soft agar was performed (Fig. 1a). Fuji cells infected with the OncomiR Library, but not HS-SYII, formed numerous large colonies compared to cells without the infection (Fig. 1b). Total RNA was isolated from the largest two colonies formed by infected Fuji cells, and semi-quantitative RT-PCR using the OncomiR miRNA Precursor Virus Library primer and the
MiR-17 increases growth of synovial sarcoma cells. To clarify the functions of miR-17 in synovial sarcoma cells, we stably established miR-17-overexpressing Fuji and HS-SYII cells by infecting the cells with miR-17-producing lentivirus (Fig. 2a). MiR-17 overexpression significantly promoted cell proliferation in both Fuji and HS-SYII cells, reaching 1.6-fold and 4.0-fold increases compared to the control cells, respectively (Fig. 2b). In addition, forced expression of miR-17 in Fuji and HS-SYII cells increased the colony formation ability compared to the corresponding control cells (Fig. 2c). Based on these findings, miR-17 might have a similar biological effect on cell proliferation and colony formation both in SS18-SSX1 and SS18-SSX2-harboring synovial sarcoma cells. In contrast, cell motility and invasion capabilities were not altered by miR-17 overexpression (Fig. 2d,e). MiR-17 overexpression did not lead to significant alterations on the cell morphologies (data not shown).

MiR-17 promotes tumor development of synovial sarcoma in in vivo mice. To evaluate the effect of miR-17 on in vivo tumorigenicity of synovial sarcoma, miR-17-overexpressing Fuji cells were injected s.c. into nude mice, and the tumor volume was measured twice a week. Twenty-nine days post-implantation, miR-17 overexpression produced a significant increase in tumor volume compared with the control cells. Ultimately, massive tumors developed, with a 6.5-fold increase at 50 days (Fig. 3a,b). Average weights of the formed tumors with or without miR-17 overexpression were 1.4 and 0.2 g, respectively (Fig. 3c).
mainly involved in cell-cycle progression. We employed this gene as a potential target of miR-17 (Table 1). To examine the effectiveness of miR-17 in molecular targeted therapy of synovial sarcomas, we established Fuji and HS-SYII cells transduced by anti-miR-17 vector fused to the 3′-UTR of p21 (Fig. 4a). In stably miR-17-overexpressing Fuji cells, p21-3′UTR luciferase activity was significantly suppressed compared with the control cells (Fig. 4b, left). A similar result was obtained even upon temporary overexpression of miR-17 in Fuji cells (Fig. 4b, right), together verifying p21-3′UTR as a direct target of miR-17. Overexpression of miR-17 notably attenuated p21 protein levels in Fuji cells in a p53-independent manner, albeit with an increased p21 mRNA level (Fig. 4c,d), indicating miR-17-regulated post-transcriptional degradation of p21. Phosphorylation levels of ERK and Akt remained almost unchanged, irrespective of miR-17 overexpression (Fig. 4d). It is noteworthy that marked p21 expression evoked by doxorubicin treatment, a conventional chemotherapy for synovial sarcoma, was also strikingly reduced by miR-17 overexpression (Fig. 4e). Consistent with this reduction, forced expression of miR-17 rescued doxorubicin-induced growth suppression in Fuji cells (Fig. 4f).

Anti-miR-17-reagent suppresses cell proliferation through restored p21 expression. To assess the effectiveness of miR-17 in molecular targeted therapy of synovial sarcomas, we established Fuji and HS-SYII cells transduced by anti-miR-17 reagent suppression in Fuji cells (Fig. 4f).
reagent. The depletion of miR-17 by anti-miR-17 treatment reliably rescued p21 protein levels in Fuji cells (Fig. 5a), and this was followed by a substantial decline in the cell growth (Fig. 5b). Similar suppression of the cell growth was also observed in HS-SYII cells treated with anti-miR-17 reagent (Fig. 5b).

Discussion

Synovial sarcoma is a relatively common soft tissue sarcoma with poor prognosis. Synovial sarcomas occur in adolescents and young adults mainly in para-articular regions, and an effective therapy bringing an improved prognosis is urgently desired. Functional analyses of miRNA have demonstrated the potential for a novel molecular targeted therapy; however, little is known concerning the engagement of miRNA in synovial sarcoma. In the present study, we found that forced expression of miR-17 in synovial sarcoma cells strikingly promoted tumorigenicity in in vivo mice (Fig. 3) by directly targeting p21CIP1/WAF1 (Fig. 4b,d). It is noteworthy that SS18-SSX fusion oncoprotein possesses an ability to induce miR-17 expression (Fig. 1f). MiR-17 overexpression reduced p21 protein level (Fig. 4e). These lines of evidence indicate that miR-17 elevates the growth of synovial sarcoma cells by promoting cell cycle progression due to elimination of the inhibitory machinery of G1-S transition, targeting p21.

We previously demonstrated that SS18-SSX1 per se has an ability to induce p21 protein in an Sp1/Sp3-dependent manner but not hBRM and p53, leading to premature senescence in synovial sarcoma cells. Therefore, for full transforming activity of SS18-SSX to overcome such senescence, it has been assumed that other factor(s) should contribute to escape...
miR-17 that disrupts 3′UTR of p21 mRNA, leading to increased cell proliferation (Fig. 5c). Hence, our findings propose miR-17 as a candidate for p53-independent p21 downregulation in synovial sarcoma. We further revealed the SS18-SSX2-dependent, SS18-SSX1-independent induction of miR-17 in synovial sarcoma cells (Fig. 1f, data not shown). SS18-SSX was recently shown to induce the assembly of aberrant SWI/SNF (BAF) chromatin remodeling complexes by excluding BAF47, and reversing H3K27me3-mediated repression, leading to proliferation of this tumor. The ability of SS18-SSX to disrupt BAF complexes depends on two regions of the SSX protein: the C-terminal 8 aa (SDPEEDDE) and a polar region of 2 aa. (29) The fact that the latter 2 aa is different between SSX1 (KR) and SSX2 (ER) might affect the ability of inducing miR-17. Alternatively, SS18-SSX1 and SS18-SSX2 have been shown to distinctly regulate the expression of Snail and Slug, respectively. (30,31) Because these transcription factors were demonstrated to regulate expression of several miRNA such as miR-128 and miR-221 in breast cancer, (32,33) they might provide SS18-SSX2/Slug/miR-17 axis in synovial sarcoma.

Previous papers revealed the overexpression of miR-17 in various types of human cancers (19,20) and its involvement in cell cycle regulation. (20) p21 is an inhibitory regulator in cell cycle progression of G1-S transition. Regarding synovial sarcoma cells, previous works have revealed that p53 protein acting upstream of p21 is effectively induced in response to DNA damage. (34) When DNA damage occurs after radiation therapy

Table 1. Prediction scores of PicTar and Miranda algorithms in miR-17-targeted genes previously reported

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<tr>
<td>E2F1</td>
<td>Positive regulation of cell proliferation, Proapoptotic proteins</td>
<td>6.57</td>
<td>-1.06</td>
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<tr>
<td>E2F2</td>
<td>Positive regulation of cell proliferation, Proapoptotic proteins</td>
<td>ND</td>
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<tr>
<td>E2F3</td>
<td>Positive regulation of cell proliferation, Proapoptotic proteins</td>
<td>1.24</td>
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<tr>
<td>CDKN1A</td>
<td>Negative regulator of (p21) G1-S checkpoint</td>
<td>4.19</td>
<td>-0.8</td>
</tr>
<tr>
<td>BIM</td>
<td>Proapoptotic protein</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tsp1</td>
<td>Anti-angiogenic protein</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>CTGF</td>
<td>Anti-angiogenic protein</td>
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<td>AIB1</td>
<td>Oncogenic protein</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CyclinD1</td>
<td>Oncogenic protein</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>p21</td>
<td>3'UTR of p21 utilized in luciferase assay. Sequences</td>
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Fig. 4. MiR-17 directly targets p21-3′UTR and induces drug resistance in synovial sarcoma cells. (a) Diagram of luciferase reporter vector fused to 3′-UTR of p21 utilized in luciferase assay. Sequences of miR-17-5p and the targeted p21 3′-UTR are shown. (b) Dual luciferase assay. p21-3′UTR luciferase activities were measured in stably (left) and temporarily (right) miR-17-overexpressing Fuji cells. (c) p21 mRNA expression levels were examined in parental and miR-17-overexpressing Fuji and HS-SYII cells by semi-quantitative RT-PCR. (d) In Fuji cells with or without miR-17 overexpression, expression levels of indicated proteins were investigated by immunoblotting. (e) Expression levels of p21 protein in miR-17-overexpressing Fuji and its control cells were examined by immunoblotting in the presence of doxorubicin treatment. (f) Cell proliferation of Fuji cells overexpressing miR-17 was investigated in the presence or absence of doxorubicin treatment.
and conventional chemotherapy in synovial sarcoma, a depletion of miR-17 might therefore reinforce the effect of p21-induced growth suppression. Indeed, we revealed that miR-17 overexpression conversely promotes the growth of Fuji and HS-SYII cells even in the presence of doxorubicin at an effective dose (Fig. 4f).

It has been reported that several molecules, such as cyclin D1 and insulin-like growth factor-1 receptor, are essential for cell proliferation and oncogenesis of synovial sarcoma. Cai et al. suggest that the SS18-SSX protein plays an important role in synovial sarcoma cell growth through the ERK pathway. The MAPK tyrosine kinase inhibitor sorafenib inhibits cell growth in synovial sarcoma, and PI 3-kinase/AKT signaling is also important for cell proliferation of this sarcoma. We have previously shown that the Crk adaptor protein enhances proliferation of synovial sarcoma cells through the phosphorylation of Gab1 by Src and focal adhesion kinase and the consequent activation of a DOCK180-p38 MAPK signaling. Furthermore, our data demonstrated that dual inhibition of Src and Aurora kinases is a powerful approach to suppress the growth of this sarcoma. Although kinase-targeted therapy is, therefore, a promising approach in the treatment of this sarcoma, a kinase-irrelevant strategy using anti-miR-17 reagent might be innovative and effective in avoiding the emergence of serious drug resistance in the clinical application.

Among the synovial sarcoma cell lines we tested, miR-17 overexpression produced a significant increase in proliferation of Fuji and HS-SYII cells, but not SYO-1 distinctly (data not shown). Given that the PicTar algorithm predicted 711 genes as direct targets of miR-17 with prediction scores ranging from 8.63 to 0.83, of which CDKN1A (p21) is ranked as 140/711 genes with the score of 4.19, miR-17 may become exhausted by many of the targets in SYO-1 cells. Supporting this possibility, the effect of miR-17 on cell motility and invasion has been controversial in a cellular context. Thus, special attention should be paid to assess the balance of miR-17-targeted genes such as oncogenes or tumor suppressors, which definitely determines the physiological property of synovial sarcoma cells.

In the present study, our findings provide the first evidence that miR-17 plays an important role in regulating the growth of human synovial sarcoma. Forced expression of miR-17 drives colony formation capacity, cell proliferation and tumorigenicity both in vitro and in vivo by directly targeting the p21-3'UTR. Synovial sarcoma is largely resistant to conventional chemotherapy. Upregulation of p21 protein by miR-17 depletion might exert potent effects in the therapy of human synovial sarcoma.

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**Disclosure Statement**

The authors have no conflict of interest to declare.
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