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Accumulation of pre-let-7g and downregulation of mature let-7g with the depletion of EWS

Eun Jung Sohn [a,b,*], Junhong Park [a], Soo-im Kang [a], Yun-Ping Wu [a]

[a] Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20871, USA
[b] Korean Research Center for Asbestos-Related Diseases, Pusan National University School of Medicine, Yangsan, Gyeongnam 626-870, Republic of Korea

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ABSTRACT

EWS functions in RNA splicing and transcription by encoding an RNA binding protein, which results in the chromosomal translocation t(11;22)(q24;q12) found in Ewing sarcoma. EWS interacts with the microprocessor complex involving Drosha and DGCR8, which play roles as the cofactors of primary microRNA processing. However, the role of EWS in microRNA biogenesis has not been investigated. Here, we show that endogenous EWS interacts with endogenous Drosha by IP–western blotting. In addition, EWS knockout mouse decreased the expression of Drosha. The depletion of EWS results in the accumulation of precursor let-7g but down-regulates mature let-7g in U2OS cells. Consistently, mature let 7g was suppressed in both Ewing sarcoma cell and primary Ewing sarcoma. Also, expression levels of *Dicer* and *CCND1* (*Cyclin D1*), which are known target genes of the let-7 family were upregulated. Our findings suggest that EWS mediates generation of mature let-7g from pre-let-7g.

1. Introduction

EWS (Ewing sarcoma protein) belongs to the TEF (TLS/EWS/TAF15) family of RNA-binding proteins, along with FUS/TLS and TAF15/TAFII68, and plays a role in transcription and splicing [1]. The NH_2 terminus of the TET family functions in transcription activation by being rich the serine–tyrosine–glycine glutamine (SYGQ) motif in addition to a C-terminal domain containing an RNA-binding domain possessing an RNA-recognition RRM motif and at least 1 arginine–glycine–glycine (RGG)-rich motif [2]. The N-terminal domain of EWS interacts with the RNA Pol II subunit and its C-terminal domain binds to splicing factors such as TASR, U1C, and YB-1 [3–5]. It is suggested that EWS functions in precursor mRNA splicing. Recently, EWS was found to mediate the regulation of alternative splicing patterns for the p53 repressor, MDM2, or in response to DNA damage [6]. EWS-null mice exhibit defects in meiosis and B-cell lymphocyte development, and showed an accelerated aging phenotype [7]. Ablation of EWS promotes senescence in the hematopoietic stem cells [8].

EWS forms part of the microprocessor complex at which primary microRNA (miRNA) is generated. This complex is composed of ribonuclease III, Drosha; the double-stranded binding protein, DiGeorge syndrome critical region 8 (DGCR8); heterogeneous nuclear ribonucleoproteins (hnRNP); and several RNA-binding proteins such as DEAD box RNA helicase p68 (DDX5), p72 (DDX17), EWS, TLS, and NF90-NF45 [9]. The hairpin-shaped precursor miRNA (pre-miRNA) is exported from the nucleus into the cytoplasm by the Exportin5/RanGTP heterocomplex [9,10]. It is then cleaved by the RNase III enzyme, Dicer, to generate an miRNA/miRNA duplex complex 21–25 nucleotides long [11–13]. Each miRNA is selected or degraded by RNA-induced silencing complex, which is the effector of ribonucleoprotein complex.

Previously, EWS is suggested to be a microprocessor, but its role has not been studied in detail. In this study, we demonstrate that the depletion of EWS leads to the accumulation of precursor let-7g (pre-let-7g) and downregulates mature let-7g.

2. Materials and methods

2.1. Cell culture

CHP100 and A4573 cells [14] were incubated with Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Gibco Cell culture, Portland, OR, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin (Invitrogen). U2OS and TC71 cells [14] were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, glucose, and penicillin/streptomycin. The following antibodies were used: α-Drosha (Cell Signaling Technologies, Beverly, MA, USA), α-DGS8 (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for detecting the

* Corresponding author. Address: Korean Research Center for Asbestos-Related Diseases, Pusan National University School of Medicine, Beomeori, Mulgeumeup, Yangsan, Gyeongnam 626-870, Republic of Korea. Fax: +82 515108134.
E-mail address: eunjungs93@gmail.com (E.J. Sohn).

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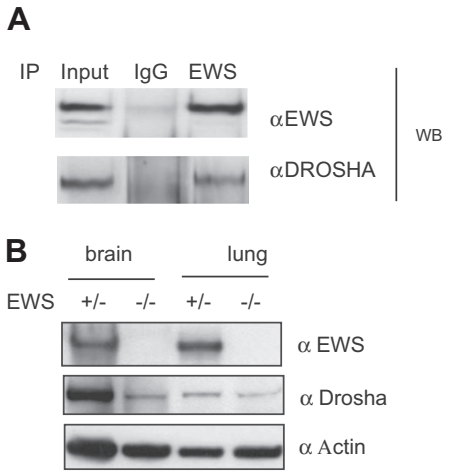

Fig. 1. EWS interacts with Drosha in U2OS cells (A) Interaction between EWS and Drosha in U2OS cells. U2OS cells were immunoprecipitated with α-EWS antibody and immunoblotted with α-Drosha. (B) Decrease in the Drosha level in EWS-null mice. Lysates of the brain and lungs from postnatal day 0 mice ($P = 0$) were immunoblotted with α-Drosha, α-EWS, and α-actin.

DGCR8, α-actin (Sigma Aldrich, St. Louis, MO, USA), α-FLI-1 (Santa Cruz Biotechnology), α-cyclin D1 (Santa Cruz Biotechnology).

2.2. EWS-deficient mouse

Homozygous EWS−/− mice were generated from crossing out heterogeous EWS mouse [7]. All animal procedures were approved and handled according to the guidelines provided by the NIH Animal Research Advisory Committee.

2.3. Immunoprecipitation–western blot analysis (IP–western blot)

U2OS cells were washed with PBS and solubilized in lysis buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM NaF, and 1 mM Na_3VO_4) for 30 min on ice. The extracts were clarified by centrifugation at 13,000 rpm for 20 min at 4 °C. The supernatants were incubated with rabbit IgG antibody and Protein A agarose (Millipore) beads for 30 min at 4 °C. After brief centrifugation, the supernatants were mixed with anti-rabbit IgG or α-EWS (Bethyl Laboratories), pre-immune and rabbit α-EWS [7], α-Ago2 (Cell Signaling Technologies), α-Drosha (Cell Signaling Technologies), α-DGS8 (H-300) (Santa Cruz Biotechnology), α-PABPI (Cell Signaling Technologies), and α-Dicer-1 (Santa Cruz Biotechnology) antibodies overnight at 4 °C. The beads were washed four times in lysis buffer, and protein was analyzed by western blotting.

2.4. Quantitative real-time RT-PCR

Total RNA was extracted using STAT 60 according to the manufacturer's protocol (AMSBIO). cDNA was generated using Superscript III cDNA (Invitrogen). The primary and precursor miRNAs were determined by qRT-PCR using CyberGreen Mixture (Applied Biosystems, Austin, TX, USA) with the following primers: pre-miR let-7g forward, 5′-GGCTGAGGTAGTAGTTGTACAGTT-3′ and reverse, 5′-AGGCAGTGGCCTGTACAGTTA-3′; pre-miR214 forward, 5′-GGCC TGGCTGGACAGAGT-3′ and reverse, 5′-AGGCTGGGTTGTCATGTGA-3′; pri-let-7g; forward 5′-GGCTGAGGTAGTAGTTGTACAGTT-3′, reverse TATCTCCCAGTGGTGGGTGT [15]; GAPDH; forward, CCATCACCATCTTCCAGGAGCGAG; reverse, primer; GCAGTGATGG-CATGGACTGTG. For the mature miRNA, cDNA was generated using the mirVana™ miRNA Kit (Applied Biosystems). Mature miRNA levels were measured by qRT-PCR using the following Taq Man probes: let-7g, mir214, let-7a, and U6 (Applied Biosystems).

2.5. Transfection with siRNAs

U2OS cells were cultured in 6-well plates until they reached 30% confluence. siRNA oligo for EWS were purchased from Ambion as follows: EWS siRNA #1, Ambion ID 3017; EWS siRNA #2, Ambion ID 241464; EWS siRNA #3, Ambion ID 146641 (Ambion/Applied Biosystems, Austin, TX, USA). EWS siRNA #4 was purchased from MWG biotech with the sequence EWS #4, GACUCUGACAACAGUGCAATT. They were subsequently transfected with four different EWS siRNAs (100 nM each) and scrambling siRNA (Qiagen, Hilden, Germany) using 5 μL Lipofectamine (Invitrogen). One day after the first transfection, the cells were transfected again using Lipofectamine with 100 nM EWS and scrambling siRNA. Forty-eight hours after

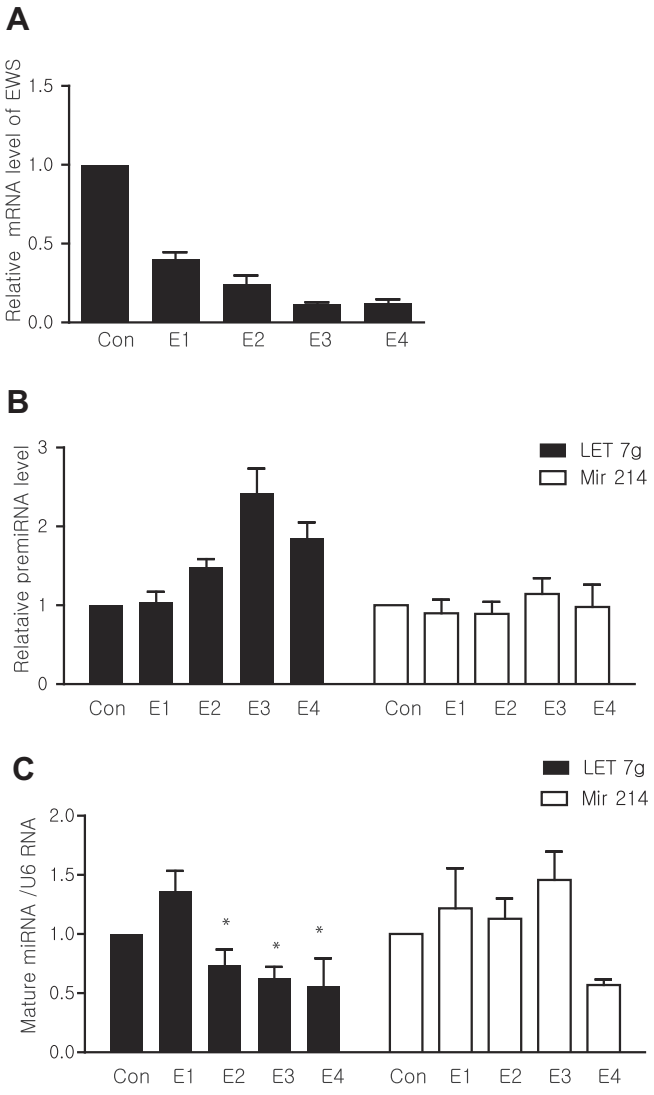

Fig. 2. Expression level of let-7g in EWS siRNA-treated U2OS cells (A) Expression level of EWS after EWS siRNA knockdown in U2OS cells. The expression level of EWS was determined by qRT-PCR ($n = 3$) (left panel) and western blotting (right panel); GAPDH was used for normalization for qRT-PCR. (B) Increase in pre-let-7g in the EWS siRNA-treated cells. The level of pre-let-7g was determined by qRT-PCR ($n = 3$). GAPDH was used for normalization. (C) Mature let-7g levels in EWS siRNA-treated cells. Mature let-7g, let-7a, and miR 214 were determined by real-time RT-PCR using TaqMan probes (Applied Biosystems) ($n = 3$). U6 RNA was used for normalization. (*$P < 0.05$).

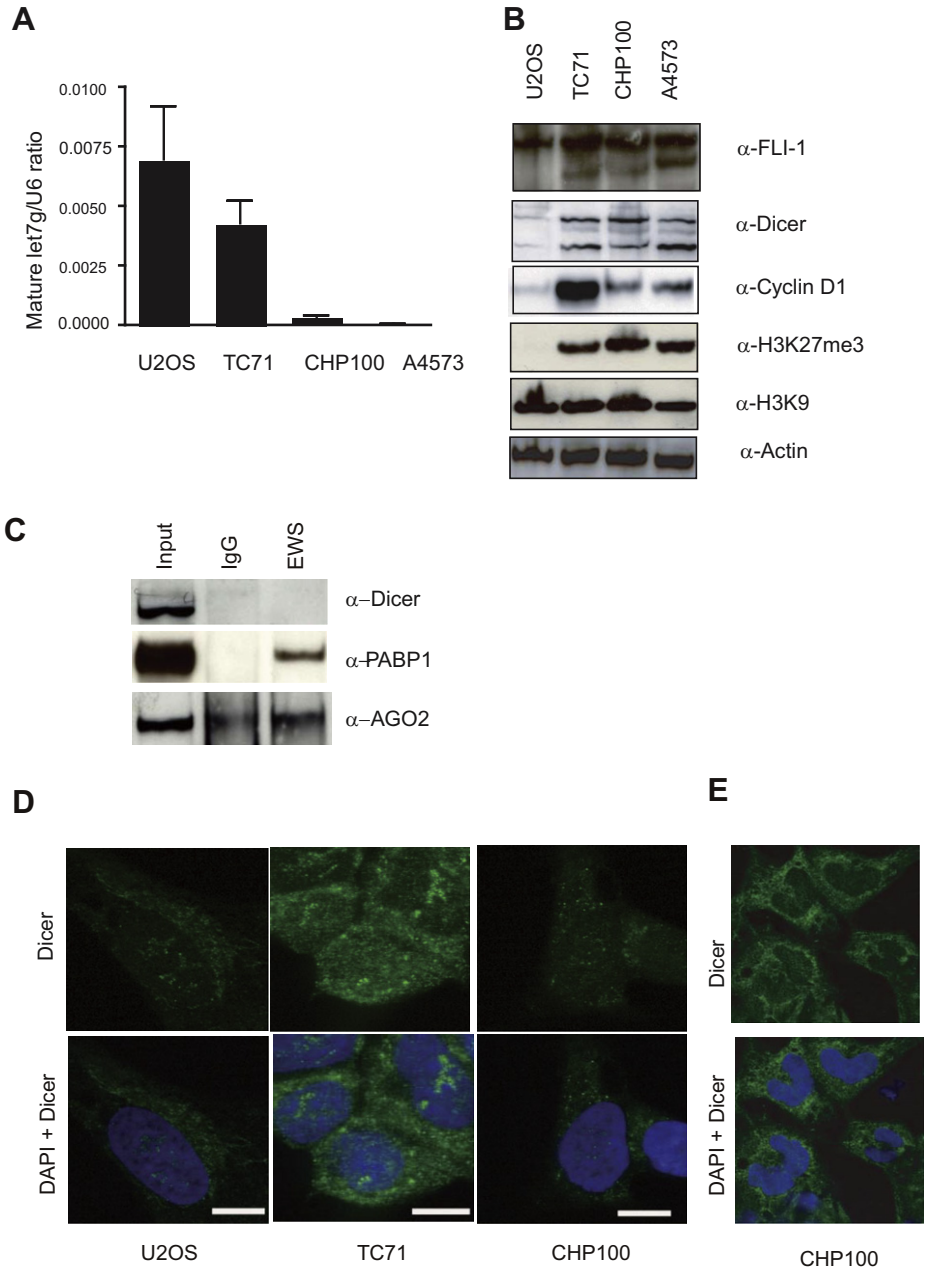

Fig. 3. Downregulation of let-7g and up-regulation of Dicer, H3K27me3, and CCND1 in Ewing sarcoma cells. (A) Reduced expression level of mature let-7g in Ewing sarcoma cells. Total RNA from U2OS and Ewing sarcoma cells (i.e., TC71, CHP100, and A4573) was analyzed for mature let-7g (upper panel) and miR 214 (lower panel) by real-time PCR using miRNA TaqMan probes (Applied Biosystems). U6 RNA was used for normalization. The data represent the mean ± SEM of 3 independent experiments. (B) Increase in the expression levels of Dicer and CCND1. The lysates from Ewing sarcoma tumors (TC71, CHP100, and A4573) and U2OS cells were immunoblotted with α-Dicer, α-actin, α-cyclin D1, and α-H3K27me3 antibodies. (C) Interaction between EWS, and PABP1, and AGO2 in U2OS cells. U2OS cells were immunoprecipitated with C-terminal EWS and immunoblotted with α-Dicer, α-PABP1, α-EWS, and α-AGO2 antibodies. (D and E) Localization of Dicer in Ewing sarcoma cells by immunofluorescence. Ewing sarcoma cells (TC71, CHP100, and A4573), U2OS cells (D) and CHP100 (E) were fixed and immunostained with α-Dicer. The localization of Dicer was analyzed by confocal microscopy. Nuclei were stained with DAPI. Scale bar, 10 μm.

the second transfection, the cells were washed with cold PBS and harvested for analysis.

3. Results

3.1. EWS binds with the Drosha/DGCR8 complex

In order to elucidate the role of EWS in miRNA biogenesis, we first examined the interaction between EWS and the Drosha/DGCR8 complex, which processes primary miRNA. We observed that endogenous EWS interacts with Drosha in U2OS cells (Fig. 1A), but we could not detect DGCR8 (data not shown). To further determine the relationship between Drosha and EWS, we determined the level of Drosha from both homozygous and heterozygous EWS mouse brains and lungs ($P = 0$). EWS-null mice exhibited postnatal lethality [7]. The levels of Drosha in homozygous EWS mice were lower than those in heterozygous EWS mice (Fig. 1B).

3.2. Accumulation of pre-let-7g by siRNA knockdown of EWS

It has previously been reported that EWS interacts with pre-let-7g through electrophoretic gel mobility shift assays [16]. However, the role of EWS in regulating let-7g remains unknown. In order to identify the role of EWS in let-7g processing, we used the four

different siRNAs to knockdown EWS in U2OS cells. After EWS knockdown in the U2OS cells, the miRNA expression levels were determined by quantitative RT-PCR (qRT-PCR). First, we determined the mRNA expression level of EWS by qRT-PCR (Fig. 2A). Following the knockdown of EWS, we observed that primary let-7g was not affected (data not shown). However, interestingly, pre-let-7g was upregulated with EWS siRNA #E2, #E3, and #E4 but not EWS siRNA #E1 (Fig. 2B). As a control, we used pre-miR214, which was not affected by EWS siRNA treatment. In contrast to the increase in pre-let-7g, mature let-7g was decreased in EWS-knockdown U2OS cells, whereas mature mir214 did not change (Fig. 2C). These results suggest the possibility that EWS regulates the processing of pre-let-7g to mature let-7g.

3.3. Downregulation of mature let-7g in Ewing sarcoma cells and shuttling of Dicer from the cytoplasm to the nucleus

To assess the possibility that let-7g is related to Ewing sarcoma, we examined the expression levels of let-7g in three Ewing sarcoma cell lines (TC71, CHP100, and A4573) by qRT-PCR. We used U2OS cells, an Osteosarcoma cell line as a control. These Ewing sarcoma cells have different fusion types that arise from chromosomal translocation t(11;22)(q24q;q12). The levels of primary (pri-) and pre-let-7g were not different in three Ewing sarcoma cells compared to U2OS cells; only pre let-7g increased in A4573 cells (data not shown). However, mature let 7g was downregulated in all three Ewing sarcoma cells (Figure 3A)

To address whether let-7g affects its target genes in the Ewing sarcoma cell line, we examined expression levels of *Dicer* and *CCND1* (*Cyclin D1*), which are known target genes of the let-7 family. *CCND1* is also known as a transcriptional target of EWS/FLI-1 [17–19]. In contrast to let-7g downregulation, *CCND1* and *Dicer* were upregulated in Ewing sarcoma cells compared to U2OS cells, as determined by western blotting (Fig. 3B). Interestingly, trimethylation of lysine 27 on histone H3(H3K27me3) was also upregulated in Ewing sarcoma cells Although endogenous EWS is mostly nuclear, a small portion resides in the cytoplasm [20]. Therefore, we determined whether EWS interacts with other miRNA machinery genes in RNA-induced silencing complex (RISC)-associated events in the cytoplasm and, performed IP–western blotting with AGO2 and AGO2-interacting proteins such as Dicer and PABP1. EWS interacted with PABP1 and interacted weakly with Ago2 but not with Dicer (Fig. 3C). Although we could not determine whether EWS interacts with Dicer, we performed immunostaining to determine the localization of endogenous Dicer in three Ewing sarcoma cell lines. Dicer was localized in both the nucleus and cytoplasm in some Ewing sarcoma cells (Fig. 3D). Meanwhile, in others, Dicer is localized in the cytoplasm in the CHP100 (Fig. 3E). These results imply that the nuclearcytoplasmic shuttling of Dicer may be regulated in a cell-cycle-dependent manner. Our results suggest that the inactivation of EWS may cause the transport of Dicer from the cytoplasm to the nucleus.

3.4. The decrease of mature let 7g in the Ewing sarcoma

Next, we determined the expression levels of pri-, pre-, and mature let-7g in the nine primary Osteosarcomas and eight Ewing sarcomas by qRT-PCR. The results show that pri- and pre-let-7g were elevated in Ewing sarcoma compared to osteosarcoma (Fig. 4A and B). In contrast, mature let-7g levels were moderately reduced in Ewing sarcoma (Fig. 4C).

4. Discussion

The components comprising the microprocessor complex that processes primary miRNA have been characterized extensively. EWS is reported as one of the subunits of the microprocessor complex [9], but its role is undefined. Here, we show that EWS may play a role in the transportation of let-7g.

Pri-miRNA has been processed by the Drosha/DGCR8 complex to create pre-miRNA [10]. Pre-miRNA is transported from the nucleus to the cytoplasm via a Ran GTP-dependent mechanism. Pre-miRNA binds to the nuclear export factor, exportin 5 (Exp5), which is a Ran GTP-dependent dsRNA-binding protein [21–23]. The pre-miRNA/Exp5/Ran GTP heterocomplex is exported from the nucleus

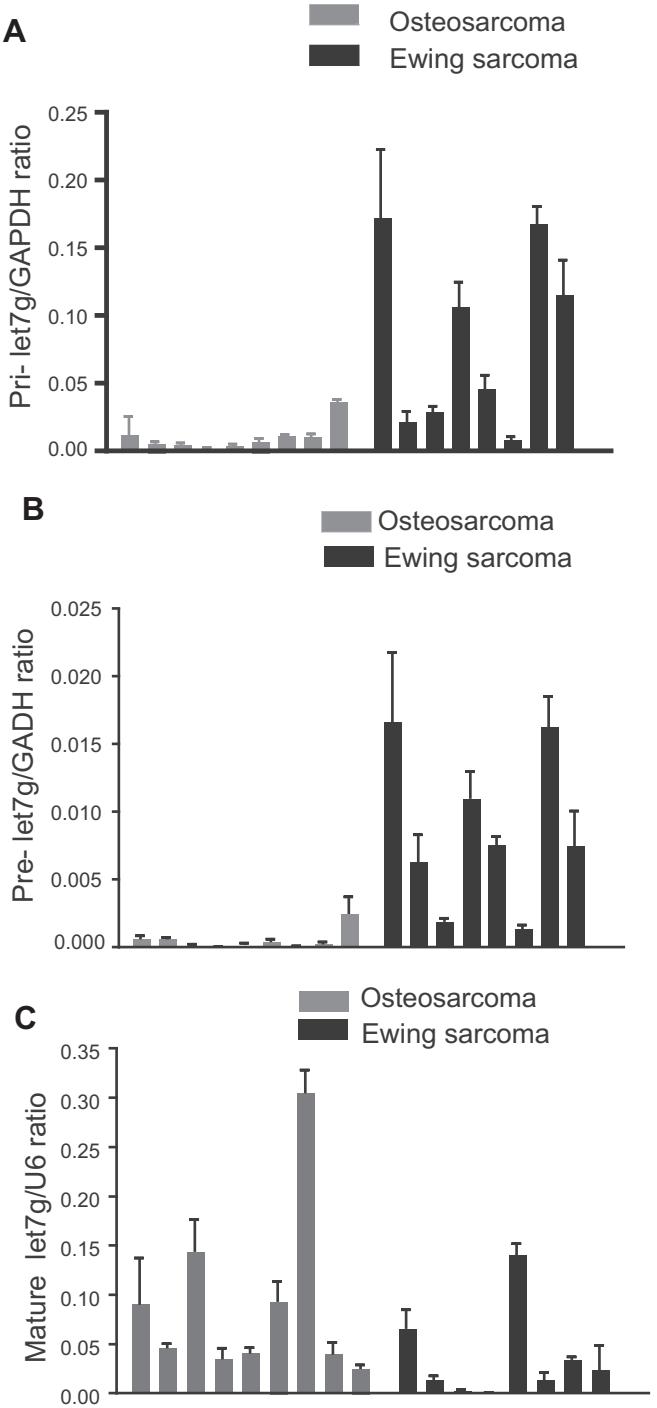

Fig. 4. Decrease in let-7g expression in primary Ewing sarcoma (A–C). Total RNA was isolated from primary eight Ewing sarcomas and nine Osteosarcomas. The Pri let-7g levels (A), Pre-let 7g (B) and mature-let7g (C) were determined by RT-qPCR. U6 was used for normalization.

and is cleaved by Dicer in the cytoplasm. The depletion of EXP5 results in the accumulation of pre-miRNA [24]. In the present study, we observed that the depletion of EWS led to increased levels of pre-let-7g and downregulated mature let-7g levels in U2OS cells. Let-7g was also reduced in the Ewing sarcoma cell lines and primary sarcoma. These results suggest that EWS may function in mediating pre-let-7g, which precedes mature let-7g. EWS might play a role as the transporter of pre-miRNA from the nucleus to the cytoplasm. Alternatively, this may explain another possibility, that EWS may affect the RISC complex to generate mature miRNA from pre-miRNA. It also needs to be investigated whether EWS affect different microRNA than let-7g.

EWS causes the chromosomal translocation t(11;22)(q24;q12) by causing the *EWS/FLI-1* fusion gene to be expressed. The EWS/FLI-1 fusion protein occurs in Ewing sarcoma, which is an aggressive undifferentiated bone tumor affecting children and adolescents. The N-terminal domain of EWS is fused to the DNA-binding domain of FLI-1, which is a member of the ETS family of transcription factors [25]. EWS/FLI-1 encodes an aberrant transcription factor by altering gene expression. Primary Ewing sarcoma deregulates the cell cycle via *p16INK4* and *p14ARF* mutation/deletion or CCND1 (cyclin D1) overexpression [26]. Recent studies show that EWS/FLI-1 alters miRNA expression. EWS/FLI-1 and Mir 145 play roles in repressive feedback loops [27], and EWS/FLI-1 is a transcriptional target of let-7a, which promotes the development of cancer stem cells [28].

Several recent studies report the nuclearcytoplasmic shuttling ability of Dicer. Dicer is known to exhibit cytoplasmic localization in humans although it is capable of localization in the nucleus as well [23,29,30]. In addition, Exp5 knockdown causes the accumulation of Dicer mRNA in the nucleus [31]. In our study, EWS siRNA knockdown resulted in the nuclearcytoplasmic shuttling of Dicer. In addition, Ewing sarcoma cells that are EWS haploinsufficient exhibit a nuclearcytoplasmic distribution of Dicer depending on the stages of cell cycles. This result suggests the possibility that EWS may be responsible for the nuclear export of Dicer. EWS was identified as the transport substrate of kayropherin beta (importin/exportin), which is responsible for the nuclearcytoplasmic transport pathway [31,32]. We assume that EWS as the transport substrate of importin or exportin may cause the nuclearcytoplasmic distribution of Dicer. However, exactly how EWS may be responsible for the nuclearcytoplasmic transport of Dicer requires further study.

Understanding how both EWS and EWS/FLI-1 mediate miRNA expression and alter miRNA machinery may provide invaluable information for therapeutic targets in Ewing sarcoma.

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