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MicroRNA expression and its clinical implications in Ewing's sarcoma

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Abstract

Ewing's sarcoma (EWS) is the second most common primary bone cancer, and is a predominant childhood malignant disease. Due to limited understanding of its pathogenesis and frequent occurrence of resistance to conventional types of treatment, its management remains difficult, and mortality is frequent. Development of EWS is a multistep process involving genetic and epigenetic alterations of protein-coding proto-oncogenes and tumour-suppressor genes. MicroRNAs (miRNAs) have recently been discovered as a new category of non-protein coding; small RNA molecules that regulate gene expression at the post-transcriptional level. Substantial numbers of deregulated miRNAs have been documented in EWS and their biological significance has been confirmed in multiple functional experiments. Several studies have confirmed involvement of miRNAs in various steps of EWS pathogenesis, from occurrence to metastasis. Functionally, miRNA dysregulation may promote cell-cycle progression, confer resistance to apoptosis, and enhance invasiveness and metastasis. These miRNAs have opened a novel field in cancer research with potential clinical utilization for screening, diagnosis, prognostics and prediction of response to treatment. Elucidating biological aspects of miRNA dysregulation may help better understand pathogenesis of EWS and promote development of miRNA directed-therapeutics against it.

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Introduction

Ewing's sarcoma (EWS) is the second most common primary bone cancer, and is a predominant childhood malignancy, histologically characterized by small round cells (1). It has been estimated that 80% of EWS cases occur in patients younger than 20 years of age (2). EWS is aggressive and is associated with the most unfavourable prognosis of all primary musculoskeletal tumours (3,4). Over the last few decades, despite great efforts to advance treatment strategies (such as chemotherapy, irradiation, and surgery), very little improvement in EWS patient long-term survival has been achieved (5). With metastases, their 5-year survival is less than 30%, and this has not improved over the last 30 years (6,7). Thus, better understanding of its pathological mechanisms is critical to development of novel prognostic biomarkers and therapies.

MicroRNAs (miRNAs), are a recently discovered category of short, non-coding RNAs, which act as post-transcriptional regulators of gene expression; it is estimated that they regulate up to 30% of protein-coding genes of the human genome (8,9). Accumulating evidence indicates that miRNAs play crucial roles in diverse biological processes, such as development, differentiation, apoptosis, proliferation, metastasis, angiogenesis and the immune response (deregulation of which is crucial in cancer initiation), progression and treatment outcome (10–14). This review focuses on recent discoveries related to miRNAs involved in development of EWS and discusses their potential use as diagnostic and prognostic biomarkers and modes of treatment strategy.

MiRNA biogenesis and function

MiRNAs were initially discovered in *Caenorhabditis elegans* in 1993, but their existence was not known in mammals until the 2000s (15,16). To date, approximately 1500 human miRNAs have been registered, each of which can regulate hundreds of mRNA targets. It is

estimated that miRNAs regulate expression of at least 20–30% of all human genes (17,18).

Mature miRNAs are products of longer precursor transcripts or pri-miRNAs, typically transcribed from intragenic or intergenic regions, by RNA polymerase II (19,20). The initial cleavage step, mediated by ribonuclease Drosha and the DGCR8 complex, occurs in the nucleus and produces one or more approximately 70-nucleotide stem-loop structures from pri-miRNA, to form miRNA precursors (pre-miRNAs) (21–23). Pre-miRNAs are then transported into the cytoplasm by Exportin 5, which is followed with a second cleavage by Dicer ribonuclease, that produces a double-stranded, 18–25-nucleotide-long mature miRNA (24,25). One miRNA strand then combines with Argonaute 2 (AGO2) protein to synthesize an RNA-induced silencing complex (RISC), mediating directed pairing with target mRNAs (26).

Increasing evidence has demonstrated the importance of miRNAs as essential cornerstones of the genetic system (27,28). They induce mRNA degradation or translational repression, by selectively binding to complementary 3′ untranslated regions (3′UTR) of target mRNAs, through complementary base pairing (29,30). Recent findings have revealed that miRNAs regulate a number of biological processes, such as development, embryogenesis, lineage determination, cell maintenance and normal cell physiology (12,28,31–34); however, they are dysregulated in most, if not all, types of cancer including that of the lung, stomach, breast, bladder, liver and osteosarcoma (13,35–40). Thus, miRNAs have great potential to become a research focus for prevention and treatment of EWS too.

Alteration of miRNAs in Ewing's sarcoma

Numbers of expression profiling studies have demonstrated miRNA dysregulation in EWS. Zhang *et al.* demonstrated that let-7a was significantly underexpressed in EWS cell lines compared to human mesenchymal stem cells (MSCs) (the presumed original ES cells) using qRT-PCR (41). Karnuth *et al.* also determined and compared expression of 377 microRNAs in 40 Ewing sarcoma biopsies, 6 Ewing sarcoma cell lines and mesenchymal stem cells (putative cellular origin of Ewing sarcoma), from 6 healthy donors (42). They identified 35 differentially expressed microRNAs in EWS, including 19 up-regulated 16 down-regulated. Other research has identified differences in miRNA expression between EWS xenografts and control samples, using microarray analysis (43). It was found that 5 miRNAs (miR-106b, miR-93, miR-181b, miR-101, miR-30b) were significantly over-expressed, while 6 miRNAs (miR-145, miR-193a-3p, miR-100, miR-22, miR-21, miR-574-3p) were significantly underexpressed across the xenograft passages in relation to controls. These miRNAs were also predicted to regulate many ES-associated genes, such as those of the insulin-like growth factor-1 (*IGF1*) pathway, *EWSR1*, *FLI1* and their fusion gene (*EWS-FLI1*).

These studies on miRNA expression profiles in EWS also found significant deregulated miRNA expression in cancer cells as oppose to normal tissues, as listed in Table 1. However, only a small number of miRNAs were shared among signatures identified by different studies and several miRNAs even exhibit discordant expression patterns among studies. These discrepancies are probably due to quality of clinical samples, indistinct change and specificity of profiling platforms, different protocols for sample collection and processing, preceding cytotoxic treatments, tumour heterogeneity and underestimated hypoxia and infection. Thus, it is important to re-evaluate our current strategies in miRNA profiling and be cautious concerning interpretation of those whose signatures already exist (Table 1).

Table 1. miRNA expression profiles in Ewing's sarcoma

Num	Method	Sample	Up-regulated	Down-regulated	References
1	Microarray	Primary EWS	miR-130b, miR-92a	miR-34a, miR-23a, miR-490-3p	(4)
2	qRT-PCR	Human EWS cells, human mesenchymal stem cells		let-7a	(20)
3	Microarray	Primary EWS, EWS cell lines, human mesenchymal stem cells	miR-126, miR-146b-5p, miR-501-5p, miR-598, miR-20b, miR-10b, miR-491-5p, miR-652, miR-532-5p, miR-340, miR-331-5p, miR-422a, miR-192, miR-362-5p, miR-128, miR-342-3p, miR-301a, miR-19b, miR-324-5p	miR-31, miR-137, miR-138, miR-431, miR-708, miR-100, miR-193a-5p, miR-99a, miR-221, miR-671-3p, miR-222, miR-193a-3p, miR-493, miR-196b, miR-125b	(7)
4	Microarray	EWS xenograft	miR-106b, miR-93, miR-181b, miR-101, miR-30b	miR-145, miR-193a-3p, miR-100, miR-22, miR-21, miR-574-3p	(11)

MiRNAs as oncogene signalling meditators in EWS – for example *EWS-FLI1*

EWS-FLI1, the most common fusion gene, is expressed in 85–90% Ewing sarcoma family of tumours (ESFT) (7). It has been shown to induce miRNA expression profile changes in EWS, including miR-22, miR-30a-5p, and most notably miR-145 and let-7a (44). Riggi *et al.* showed that *EWS-FLI1* induces expression of embryonic stem cell (ESC) genes *OCT4*, *SOX2*, and *NANOG* in human paediatric MSCs (hpMSCs) (45). Importantly, EWS cancer stem cell (CSCs) phenotype is the result of combined effect of *EWS-FLI1* on its target gene expression, and repression of miR-145 promoter activity (45). These authors also provided evidence that *EWS-FLI1* and miR-145 function in a mutually repressive feedback loop and identified their common target gene, *SOX2*, in addition to miR-145 itself, as key players in ESFT cell differentiation and tumourigenicity. In line with this result, Ban *et al.* found that miR-145 was the most probable candidate for regulation by *EWS-FLI1* (46). There are 29 miRNAs regulated by *EWS-FLI1*. Reporter gene assays have revealed that modulation of EWS-FLI1 protein is mediated by microRNAs targeting the FLI1 3′-untranslated region. Mutual regulation of EWS-FLI1 and miR-145 were mirrored by inverse correlation between their expression levels in four EWS cell lines tested. Consistent with the role of EWS-FLI1 in Ewing's sarcoma growth regulation, enforced miR-145 expression halts EWS cell line population growth. One further study has found that two ESFT cell lines revealed a similar miRNA expression profile, characterized by repression of the entire let-7 family, miR-100, miR-125b and miR-31, and over-expression of miRNA 17–92 cluster and its paralogs miR-106a and miR-106b. Direct repression of let-7a by EWS-FLI1 participates in tumorigenic potential of ESFT cells *in vivo*. Regulation of ESFT population expansion by let-7a is mediated by its target gene *HMGA2*, whose repression mimics action of let-7a by blocking ESFT cell tumourigenicity (47). Consistent with these observations, systemic delivery of synthetic let-7a into ESFT-bearing mice has restored its expression in tumour cells, reduced *HMGA2* expression levels and resulted in ESFT growth inhibition *in vivo*. McKinsey *et al.* achieved stable and specific knockdown of EWS-FLI1 in EWS cells and performed miRNA microarray screening for miRNAs differentially expressed between A673 EWS cells with stably silenced EWS/FLI1 and controls (48). They found 30 miRNAs up-regulated on EWS/FLI1 knockdown. A group of miRNAs (100, 125b, 22, 221/222, 27a and 29a) was also strongly repressed by EWS/FLI1. All these miRNAs have predicted targets in the IGF signalling pathway, a pivotal driver of EWS oncogenesis. More evidence has shown that the miRNAs negatively regulate expression of multiple pro-oncogenic components of the IGF pathway, for example, IGF1, IGF1 receptor, mammalian/mechanistic target of rapamycin and ribosomal protein S6 kinase A1. Robin *et al.* have further shown that EWS-FLI1 mediates up-regulation of EYA3 with repression of miR-708 by binding to its 3′-untranslated region (49). Importantly, high levels of EYA3 were significantly correlated with low levels of miR-708 in EWS samples, suggesting that this miRNA-mediated regulation exists in human cancers. Consistent with the important role of EYA proteins in cell survival during development, loss of EYA3 reduces survival of EWS cells (Table 2).

Biological functions of deregulated miRNAs in EWS

Since miRNA profiling studies demonstrate much deregulated miRNA, their further functional characterization (specially their interaction with tumour suppressor

Table 2. EWS-FLI1 and miRNAs in Ewing's sarcoma

Num	Method	Sample	Up-regulated	Down-regulated	References
1	qRT-PCR	Cancer stem cells		miR-145	(34)
2	Microarray	WES family of tumors, mesenchymal progenitor cell	miR-145, miR-424, miR-21, miR-214, miR-28-5p, miR-424, miR-27a, miR-22, miR-409-3p, miR-125b, miR-708, miR-135b	miR-128, miR-126, miR-9, miR-101, miR-425, miR-592, miR-340, miR-505, miR-652, miR-150, miR-20a	(30)
3	Microarray	EWS tumor family cell lines, human mesenchymal stem cells	miR-125b, miR-100	miR-17, miR-18a, miR-19a, miR-20a, miR-19b, miR-92b, miR-106a, miR-106b	(12)
4	Microarray	EWS cells		miR-100, miR-125b, miR-22, miR-221/222, miR-27a, miR-29a	(16)
5	qRT-PCR	EWS cells		miR-708	(13)

genes, oncogenes and other cancer-related genes), is useful understanding molecular tumorigenesis of EWS. For example, functional analysis of miR-34a in EWS cell lines has indicated that when miR-34a is overexpressed, cells are less proliferative, less malignant, and more sensitive to doxorubicin and vincristine. Expression of miR-34a can be increased in p53 wild-type cells by treatment with nutlin-3a (50). Expression of miR-31 is lower in EWS cells compared to mesenchymal stem cells. Functional analyses have demonstrated that two of four miR-31 transfected EWS cell lines had significantly reduced expansion (19% and 33% reduction), due to increased apoptosis in one, and to increased length of G1-phase in the other. All three tested miR-31-transfected EWS cell lines also showed significantly reduced invasiveness (56–71% reduction) (42). MiR-125b has been reported to be down-regulated in EWS tissues while its overexpression suppressed cell proliferation, migration and invasion, arrested the cell cycle, and induced apoptosis of EWS cell line A673. Bioinformatic prediction has suggested oncogene, phosphoinositide-3-kinase catalytic subunit delta (*PIK3CD*), to be a target gene of miR-125b in EWS cells. Ectopic expression of miR-125b suppressed expression of PIK3CD mRNA and protein. PIK3CD participates in regulating the PI3K signalling pathway (51). Expression of let-7a has been shown to be down-regulated in human EWS cells compared to human MSCs. Functional analysis of miR-34a in EWS cell lines has indicated that overexpression of let-7a inhibits cell proliferation, migration, and invasion, arrests cell cycle progression, and induces apoptosis in two EWS cell lines. Using bioinformatic prediction, mRNA and protein expression analysis and luciferase assays, Zhang *et al.* have identified cyclin-dependent kinase 6 (CDK6) to be the putative target of let-7a (41). Iida *et al.* also found miR-125b to be up-regulated in two different doxorubicin-resistant EWS cell lines. Up-regulation of miR-125b has also been confirmed in EWS having survived chemotherapy regimens, including with doxorubicin. When miR-125b was knocked down in EWS cells, both doxorubicin-resistant and parental cells had an enhanced sensitivity to doxorubicin, which was associated with up-regulation of pro-apoptotic molecules, p53 and Bak. Inversely, overexpression of miR-125b in parental EWS cells resulted in enhanced drug resistance, not only to doxorubicin, but also to etoposide and vincristine (52). MiR-30a-5p is one miRNAs that demonstrates EWS-FLI1 modulation, while its re-expression affects cell proliferation and invasion. It has also been shown that miR-30a-5p interacts with 3′UTR region of CD99 and represses its expression (53). MiR-17~92a, miR-106b~25 and miR-106a~363 clusters are up-regulated in EWS. Using colony formation as a read-out, investigators found that blockade of selected individual cluster component miRNAs, using specific inhibitors, has little or no effect, whereas combinatorial inhibition using miRNA "sponge" is inhibitory to colony formation. To this end, blockade of whole clusters is generally more effective than blockade of individual miRNA families. It was also demonstrated that miRNA-blocking sponge directed against poorly characterized miR-106a~363 cluster, was a particularly potent inhibitor of clonogenic growth in a subset of EWS cell lines. Up-regulation of miR-15a was also recognized to be a downstream mechanism contributing to miR-106a~363 sponge growth-inhibitory effect (54) (Table 3).

Prognostic use of miRNAs and other clinical implications, in EWS

Nakatani *et al.* identified 5 miRNAs (miR-34a, miR-23a, miR-92a, miR-490-3p and miR-130b) as independent predictors of risk for EWS progression and survival (50). Further studies in extended samples indicated that both miR-34a and miR-490-3p achieved sufficient statistical power to predict prognosis. Patients with highest expression of miR-34a did not experience adverse events in the 5 subsequent years, while in contrast, patients with lowest expression suffered recurrence within 2 years. To date, despite overwhelming reports of

Table 3. Functional characterization of the deregulated miRNAs in Ewing's sarcoma

Name	Up- or down-regulation	Target gene	Role	References
miR-34a	Down		Tumour suppressor	(4)
miR-31	Down		Tumour suppressor	(7)
miR-125b	Down	PIK3CD	Tumour suppressor	(14)
let-7a	Down	CDK6	Tumour suppressor	(20)
miR-125b	Down	p53, Bak	Survived chemotherapy	(8)
miR-30a-5p	Down	CD99	Tumour suppressor	(41)
miR-17~92a miR-106b~25 miR-106a~363 clusters	Up		Oncogene	(9)

dysregulated miRNAs in EWS tissues, no circulating miRNA has been identified for non-invasive diagnosis of the tumour.

Conclusions and future perspectives

Ewing's sarcoma causes tremendous mortality due to its resistance to conventional treatment. Although aggressive surgical resection has improved the prognosis, treatment of EWS is still unsatisfactory due to risk of local relapse. Early diagnosis of EWS is a major challenge as lack of knowledge concerning molecular mechanisms involved in its pathogenesis. Thus, a more comprehensive understanding of its pathogenic mechanism is essential for formulating innovative therapeutic strategies. Over the past decade, there has been rapid accumulation of evidence on important roles of deregulated miRNAs in development of human malignancies. These hold great potential for new development in current diagnostic and therapeutic strategies in management of malignant disease, including EWS. Identification of miRNA as a posttranscriptional regulator of gene expression has strongly extended our understanding of biology of cancers, here specifically EWS. However, despite encouraging results, we still see only a tip of the iceberg, and further studies are needed to establish biological roles and to explore potential clinical utilization of miRNA. One challenge is that expression of miRNAs is spatial-, temporal-, and tissue-specific, and can be influenced by a variety of factors such as infection, hypoxia, pathology and cytotoxic treatment, which make it difficult to identify a consistent miRNA signature for prognosis and diagnosis. In addition, miRNAs play a functional role in cell signalling networks and regulate expression of multiple genes, giving rise to the possibility of off-target effects for miRNA-directed therapy. Nonetheless, potential therapeutic utilization of miRNAs may reshape our understanding of tumourigenesis and will definitely improve management of EWS in the future.

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