Long Non-Coding RNAs in Ewing Sarcoma Pathogenesis

Subjects: Biochemistry & Molecular Biology

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Ewing sarcoma (ES) is a rare aggressive cancer of bone and soft tissue that is mainly characterized by a reciprocal chromosomal translocation. As a result, about 90% of cases express the EWS-FLI1 fusion protein that has been shown to function as an aberrant transcription factor driving sarcomagenesis. ES is the second most common malignant bone tumor in children and young adults. Current treatment modalities include dose-intensified chemo- and radiotherapy, as well as surgery. Despite these strategies, patients who present with metastasis or relapse still have dismal prognosis, warranting a better understanding of treatment resistant-disease biology in order to generate better prognostic and therapeutic tools.

Keywords: Ewing sarcoma ; long non-coding RNAs ; competing endogenous (ce) RNA ; regulatory RNA ; biomarkers ; therapeutic targets

1. LncRNAs in Ewing sarcoma (ES) and as Potential Biomarkers

LncRNA investigations in ES are still in their infancy with just a few reports describing expression of specific lncRNAs in ES with a minimal fraction characterized for their functional relevance [1]. A comprehensive literature review was conducted by Barrett et al. [2] to identify clinically relevant ncRNAs in ES. Their study reported on the oncogenic activity of lncRNAs and miRNAs and highlighted the interplay between these two classes of ncRNAs in ES. A recent study by Chen et al. employed machine learning and training models on prevailing RNA sequencing data sets from ES patients to establish a set of seven lncRNAs that could serve as a prognostic risk marker for ES. Although their data needs experimental testing for the identified lncRNAs association and role in ES, their increased expression statistically correlated with poor overall survival [3]. LncRNAs that have so far been reported in ES are tabulated below (**Table 1**) and will be discussed. It must be mentioned, though, that most of these studies on lncRNAs in ES have been done using in vitro cell line models that do not fully recapitulate the physiological conditions in vivo. As such, it would be imperative to validate most of these studies using in vivo models, as suggested in a comprehensive review by Miserocchi-G and colleagues [4]. To this end, patient derived xenografts (PDX) would provide the most suitable models, as a genetic animal model for ES is still not available.

Table 1. LncRNAs so far identified in Ewing sarcoma.

LncRNA	Expression	Method of Identification	Mechanism of Action	Targets	References
EWSAT1	Up	RNA-seq	Direct target interaction	HNRNPK	[5]
HULC	Up	qRT-PCR	Sponging miR-186-5p	TWIST1	[6]
MALAT1	Up	RNA-seq	Diverse, including Direct target interaction	EZH2, Cyclin D1, Tenascin	[7][8]
DLX6-AS1	Up	qRT-PCR	Sponging miR-124-3p	CDK4	[9]
PncCCND1_B	Up	Microarray data & RNA-seq	DHX9 & Sam68 complex formation	Cyclin D1	[10]
FOXP4-AS1	Up	Microarray data analysis	Sponging miR-298	Thymopoietin (TMPO)	[11]
SOX2OT	Up	RT-PCR	Sponging miR-363	FOXP4	[12]
HOTAIR	Up	RNA-seq	Direct target Interaction	EZH2 & LSD1	[13]

LncRNA	Expression	Method of Identification	Mechanism of Action	Targets	References
TUG1	Up	RT-qPCR	Sponging miR-199a-3p	MSI2	[14]
AK057037	Up	RNA-seq	Interaction with EZH2	PRC2 complex	[15]
DPP10-AS3	?	RNA-seq	Unclear	CD40, CD70 & CD276 molecules	[16]
Hdm365	Up upon p53 activation	Northern blot Hybridization	Hdm2 transcription & processing	P53	[17]

? = Unknown.

1.1. EWSAT1 (Ewing Sarcoma-Associated Transcript 1)

The lncRNA Ewing sarcoma-associated transcript 1 (EWSAT1) was originally identified in Ewing sarcoma [5], and has subsequently been associated with proliferation, migration and metastases as well as overall survival (OS) in several other cancers [18][19][20]. Enhanced expression of EWSAT1 has been reported to promote cell growth, invasion and EMT by sponging specific miRNAs in various cancers [21][22]. In ES, though, Marques et al. (2014) showed that inhibition of EWSAT1 expression specifically mitigated ES cell lines capability to proliferate as well as to form colonies in soft agar. Co-expression of EWS-FLI1 and EWSAT1 in primary pediatric human mesenchymal progenitor cells (hMPCs), the most likely ES cell type of origin [23], repressed gene expression with a substantial overlap of repressed targets. Though subsequent studies supported the notion that EWSAT1 promotes ES proliferation in vitro, its gene regulatory mechanisms of action are thought to be diverse due to its nuclear and cytoplasmic localizations. They concluded that EWSAT1-mediated gene repression facilitates ES oncogenesis.

1.2. HULC (Highly Upregulated in Liver Cancer)

LncRNA Highly Upregulated in Liver Cancer (lncRNA-HULC), localized on chromosome 6p24.3, is among the few lncRNAs studied in ES. Mercatelli and colleagues [6] showed that high levels of HULC correlate with ES aggressiveness and its depletion reduces ES cell growth. They provided evidence that the sponging activity of lncRNA HULC on microRNA 186 (miR-186) results in the modulation of TWIST1 oncogene expression which impacts ES cell proliferation and clonogenicity. They also reported that treatment of ES cells with the small molecule compound YK-4-279, which targets EWS-FLI1 activity [24] resulted in both reduced HULC lncRNA expression and TWIST1 protein downregulation, ultimately sensitizing ES cells to YK-4-279 treatment. LncRNA HULC has shown to be aberrantly elevated in several tumors, including human pancreatic cancer [25], osteosarcoma [26], ovarian cancer [27] and gastric cancer [28]. Of note, in chronic myeloid leukemia (CML), it was reported that lncRNA HULC dysregulates the miR-150-5p/MCL-1 axis to impact imatinib resistance [29]. Since MCL-1 is highly expressed and shown to be a therapeutic vulnerability in ES [30], it will be interesting to interrogate the contribution of HULC on MCL-1 mediated ES cell survival.

1.3. MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1)

The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as nuclear enriched abundant transcript 2 (NEAT2), has been implicated in several tumor-associated cell behaviors [31]. Its overexpression has been shown to promote tumor cell proliferation, angiogenesis, migration and metastasis through various mechanisms involving chromatin and genomic modifications, transcriptional and posttranscriptional regulation that ultimately impact protein function [32]. In ES, MALAT1 activity has been based on few investigations. An earlier report showed that EZH2 helps EWS-FLI1 to drive tumor growth and metastasis in Ewing sarcoma [33]. The lncRNA MALAT1 was subsequently shown to interact directly with EZH2 in the TC71 and TC32 ES cell lines, suggesting it could have an oncogenic role in ES biology [7]. MALAT1 was then shown to be deregulated by Spleen tyrosine kinase (SYK) mediated signaling in ES cells and found to be transcriptionally activated through SYK/c-MYC pathway. They further showed that silencing MALAT1 in ES cells robustly induced cell apoptosis and G1 cell cycle arrest with concomitant suppression of cyclin D1 and upregulation of p27kip1 and p21cip1 levels [7]. They proposed that targeting SYK-mediated signaling could potentially represent a promising therapeutic strategy for treating ES patients. A current study by He-S and colleagues highlighted the fact that EWS-FLI1 induced tenascin-C (TNC) may regulate ES tumor progression through targeting the lncRNA MALAT1, and that this may be done by integrin α5β1-mdiated YAP activation [8].

1.4. DLX6-AS1 (Distal-Less Homeobox 6 Antisense RNA 1)

It has been reported in several cancers that the lncRNA DLX6-AS1 functions as an oncogene or an onco-promoting element, thereby regulating the aggressiveness and proliferation of diverse cancers [34]. In ES tissue and cells, lncRNA DLX6-AS1 was found to be significantly upregulated relative to normal tissue and cells, pointing to a potential oncogenic role of its overexpression in ES pathogenesis [9]. In the research, they also report the lncRNA DLX6-AS1 to be located in the cytoplasm of ES cells, suggesting a potential post-transcriptional regulatory role. They established that lncRNA DLX6-AS1 functions in ES cells by sponging the microRNA miR-124-3p to modulate CDK4 mRNA activity.

1.5. PncCCND1_B (Promoter Associated Non-Coding RNA Transcribed at the Cyclin D1 Locus)

Promoter associated non-coding RNAs (pancRNAs) transcribed at the CCND1 locus were first identified in Hela cells with pncCCND1_D being the most prominently expressed species [35]. In the search for factors that regulate CCND1 expression in ES cells, Palombo-R and colleagues discovered that the lncRNA pncCCND1_B is transcribed from the CCND1 promoter region [10]. In their study, they found that in the presence of EWS-FLI1, pncCCND1_B aids DHX9 complex formation with Sam68 on the CCND1 promoter modulating CCND1 expression. Sam68 belongs to the STAR (signal transduction and activation of RNA metabolism) family of RNA-binding proteins that link signaling pathways to RNA metabolism [36]. Palombo and Paronetto have recently reported that etoposide treatment of ES cells was able to enhance pncCCND1_B expression and induce Sam68 re-localization to form a network hub on the CCND1 promoter which contributes to CCND1 downregulation [37]. In the presence of growth stimulatory signals such as IGF-1, which is known to play an important role in ES pathogenesis, the complex dissociates to allow for CCND1 promoter activation. This research highlights the complex regulation of cyclin D1 in ES cells and pinpoints the Sam68-DHX9-pncCCND1_B complex as a novel player in this pathway that could serve as a potential target for therapy.

1.6. FOXP4-AS1 (Forkhead Box P4 Antisense RNA 1)

The lncRNA forkhead box P4 antisense RNA 1 (FOXP4-AS1) has been shown to regulate proliferation, migration and invasion, as well as apoptosis in various cancers [38][39][40]. The only study in ES reported an upregulation of FOXP4-AS1 that correlated with poor prognosis in patients with the disease [11]. Knockdown of FOXP4-AS1 repressed growth, migration and invasion of ES cells in vitro and its overexpression had the exact opposite effects. They established that FOXP4-AS1 is predominantly localized in the cytoplasm of ES cells and may regulate their malignant phenotype by modulating the expression of thymopoietin (TMPO) through sponging the microRNA miR-298. TMPO, also known as laminar-associated polypeptide 2 (LAP2), can interact with lamins and BAF to regulate the organization of the nuclear structure and the dynamics of the cell cycle [41], and its role in cancer biology has been recently reported [42].

1.7. SOX2OT (SOX2 Overlapping Transcript)

LncRNA SOX2 Overlapping Transcript (Sox2OT) is highly expressed in several cancers and has been associated with unfavorable prognosis in those cancers where it was found to promote migration and invasion through diverse mechanisms [43][44][45]. Sox2OT has been mapped to 3q26.3-q27 chromosomal locus in humans, demonstrated to harbor at least eight transcript variants and is abundantly expressed in embryonic stem cells [46][47]. Sox2OT was found to be crucial for the development and maintenance of the pluripotency of cancer stem cells (CSCs) [48], while its dysregulation was reported in several cancers including osteosarcoma, glioblastoma, lung and breast cancer, and others, where it plays an oncogenic or tumor-suppressor role [49]. Ma-L and associates found that in ES clinical samples, just like in most other cancers, Sox2OT lncRNA is highly expressed and contributes to its malignant behavior by sponging miR-363, thereby causing overexpression of FOXP4 protein levels to promote several downstream malignancy-inducing pathways [12].

1.8. HOTAIR (Hox Transcript Antisense Intergenic RNA)

In a recent elaborate review on the role of lncRNAs in rare tumors, Liguori-G and colleagues reported that among numerous cancer-associated lncRNAs, HOTAIR plays a crucial role in contributing to tumor development, metastatic progression and drug resistance [50]. In breast cancer, HOTAIR has been proven to have a prognostic value and suggested to be a potential therapeutic target [51]. In ES, HOTAIR was reported to be overexpressed and to promote malignant transformation through interaction with the histone-modifying proteins EZH2 and KDM1A (LSD1) by Siddiqui-H and colleagues [13]. They suggested HOTAIR may promote survival in EWS-FLI1 mediated transformation and also represent a potential therapeutic target given its high expression in tumors and low expression in most normal tissues.

1.9. TUG1 (Taurine Upregulated Gene 1)

The lncRNA TUG1 was initially identified in a genomic screen for genes in response to the taurine treatment of developing mouse retinal cells [52] and has since been found to play important regulatory functions in several cancer-associated biological processes [53]. Li-H et al. found that TUG1 was overexpressed in ES tissues and cell lines and suggested it plays a vital role in the progression of ES since inhibition of its expression reduced ES cell proliferation, migration and invasion [14]. Mechanistically, their study showed that TUG1 sponges miR-199a-3p, whose expression level they found repressed in ES cells, to enhance MSI2 expression. MSI2 belongs to the Musashi gene family and is a well-established tumor driver in the tumorigenesis of some human cancers [54]. MSI2 upregulation was also demonstrated to contribute to proliferation, migration and invasion of ES tissues and cells [14].

1.10. AK057037 (aka FEZF1-AS1)

The FEZ family Zinc Finger 1 Antisense RNA 1 (FEZF1-AS1) is a recently discovered lncRNA that has been shown to be highly expressed in several human malignancies and associated with poor prognosis [55]. It is located on chromosome 7q31.32 and was reported to play a crucial role in the proliferation, migration, invasion and Warburg effect of various tumors. In ES, FEZF1-AS1 was identified in 2013 as AK057037 by the group of Triche-T and colleagues [15] and reported to behave as an oncogene. They established its association with the PRC2 complex in ES that allows for chromatin remodeling which in turn promotes metastasis by perturbing transcription of genes involved in migration in Ewing sarcomagenesis.

1.11. DPP10-AS3 (Dipeptidyl Peptidase 10 Antisense RNA 10)

Immune-associated lncRNAs have been shown to serve as prognostic biomarkers in some cancers including breast cancer, glioblastoma multiforme and bladder cancer [56][57][58]. Upon screening for prognosis-related lncRNAs in ES, Ren-EH et al. employed a machine learning-iterative lasso regression model to construct an 11-lncRNA signature [16]. This approach not only considers the prognostic information of each individual lncRNA, but also rejects redundant prognostic information, thereby maximizing the prognostic value of the lncRNA signature. DPP10-AS3 was one of the 11 identified differentially expressed immune-associated lncRNAs in ES. In that study, they also employed bioinformatics methods to explore relationships between the lncRNA signature and prognosis-associated immune cells, investigated the potential regulatory mechanisms involved, thereby providing novel research cues in the study of immune-related lncRNAs in ES. Although the relationships between the 11 lncRNAs and ES are currently unclear, their data suggest the 11-lncRNA signature has, so far, the highest relative prognostic value not affected by other clinical characteristics. Their study therefore reports the first immune-associated lncRNA signature related to ES prognosis.

1.12. Hdm365 (Human Double Minute 365)

The human homologue of the murine double-minute 2 (mdm2) gene, HDM2, localized on chromosome 12q13-14, has been reported to be overexpressed in soft tissue sarcomas due to amplification [59]. The research group previously described a novel nuclear RNA, named hdm365, to be the major processing product of HDM2 transcripts, whose induction was observed after ectopic expression of p53 and after DNA-damaging treatment of tumor cell lines, as well as primary fibroblasts and lymphocytes [17]. Its high stress-inducible expression levels coupled with nuclear localization and absence of a corresponding protein suggested a novel RNA-based function for hdm365. Its size of 365 bases, being in the range of other lncRNAs, comprises the first five hdm2 exons, lacks polyadenylation, and after p53 induction, is detectable at the site of hdm2 transcription and processing only. The researchers postulated that the presence of a putative 3′ terminal stem-loop structure is reminiscent of non-polyadenylated histone RNAs and U2 snRNA involved in splicing and speculated that hdm365 may contribute to p53 gene activity under stress conditions in ES.

2. LncRNAs as Potential Therapeutic Targets in ES

Since several lncRNAs exhibit tissue- and cell-type specificity in both tumors and in normal tissues, they are projected as excellent druggable candidates, as well as suitable markers for diagnosis [60]. Over the past years, several studies have established a close relationship between altered lncRNA levels and cancer cell proliferation and survival. Several reasons why lncRNAs present the most interesting therapeutic targets are elaborately outlined in a review by Slaby et al. [61]. Considering the diversity in their prospective modes of action, lncRNAs can be targeted through multiple approaches that have been comprehensively reviewed by Arun and colleagues [62] and are briefly discussed below.

2.1. Antisense Oligonucleotides (ASOs)

ASOs are single stranded DNAs that bind to RNA via Watson-Crick base pairing. Upon binding to their target RNA, ASOs can modulate gene expression via steric hindrance, splicing alterations, initiation of target degradation via RNase H or other events. ASOs targeting different RNAs have recently entered clinical trials for various diseases including cancer [63] and are emerging as a potential therapeutic tool for targeting lncRNAs [62]. Although ASOs have high efficacy in cells, there are limitations to using them in the clinic, mainly due to in vivo toxicity and the absence of proper delivery systems that ultimately hampers tissue targeting by an adequate dose of therapeutic ASOs. Chemical modifications to improve their resistance to degradation and toxicity have contributed immensely to their success in the clinic [1]. GapmeR ASOs are RNA-DNA-RNA single-stranded oligonucleotide chains in which ribonucleotides may contain 2′-O-methoxyethyl modified sugar backbone [64], or additional modifications such as locked nucleic acids (LNAs) and S-constrained ethyl residues [65]. LNAs are single-stranded DNA fragments flanked by LNA nucleotides and bind complementarily to lncRNA providing recognition and cleavage of its target by RNase H. LNAs are reported to enhance affinity toward target RNA transcripts and acquire resistance to nucleases thereby enabling these oligonucleotides to remain functionally active for long periods in vivo [66][67].

2.2. RNA Interference (RNAi)

Short double-stranded RNAs are normally recognized by the RNA-induced silencing complex (RISC), resulting in base-pairing with a lncRNA/mRNA of interest that ultimately leads to argonaute degradation of the target transcript [68]. Several studies have demonstrated the successful employment of small-interfering RNAs (siRNAs) for different pathological conditions including cancer [69]. One study reported that nuclear lncRNAs were knocked down at higher levels using antisense strands while cytoplasmic lncRNAs were better knocked down using RNAi [70].

2.3. Small Molecules

Small molecules have much more favorable pharmacokinetic properties than oligonucleotides [71]. However, targeting lncRNAs (and RNAs in general) with small molecules is still an emerging field. Compared to proteins, RNAs are less structurally diverse, highly negatively charged and possess few pockets or clefts for conventional drug binding [72]. It is challenging to design drug libraries that would potentiate RNA binding, as it is still unknown what would be the main requirements for a successful candidate. LncRNAs, like other non-coding RNAs, are able to form stable secondary and tertiary structures [73]. Small molecule inhibitors targeting these unique structural elements in lncRNAs could potentially destabilize the transcript or allosterically interfere with their protein binding to confer a therapeutic effect [62]. To date, several small molecules which selectively bind RNA motifs have been identified, mostly through high-throughput screening, despite the fact that existing drug libraries are optimized for protein binding [74]. An interesting example from the point of view of ES is targeting a triple helix encoded by lncRNA MALAT1 [75] most potently with a compound that acts through entropically driven binding deeply within the triplex. Either the RNA structure itself was disrupted to reduce MALAT1 levels, or compound binding prevented interaction of the triplex with other cofactors. There is a clear trend towards development of technologies for small molecule discovery against lncRNA targets sequestering proteins through protein-RNA interactions [71] and focusing on approaches that would deliver molecules with drug-like properties. Several groups concentrated on inhibition of the HOTAIR:PRC2 complex. In a 2015 study, promiscuous intercalator camptothecin was identified as inhibitor of HOTAIR-EZH2 complex formation, but the full potential of this strategy is yet to be proven [76]. Finally, lncRNA-HULC was demonstrated to be downregulated by YK-4-279 in ES, however, most likely as an indirect consequence of targeting EWS-FLI1 activity [6].

2.4. CRISPR-Cas System

Currently, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system, namely CRISPR/Cas, has been proven to be a very efficient gene editing tool and has predominantly been used for modifications of protein coding genes [77]. Few reports have indicated that transcriptional silencing of lncRNAs using CRISPR based approaches is feasible and will most likely be exploited in the near future for therapeutic targeting of these molecules at the transcriptional level [78][79]. Also, the recently developed RNA-targeting CRISPR-Cas13 system represents a promising approach to deplete lncRNAs with a potential for therapeutic purposes [80].

Based on their reported functions in the progression and growth of several cancers, targeting lncRNAs would be one potential approach to mitigate Ewing sarcomagenesis therapeutically.

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