Molecular mechanism and therapeutic strategies for embryonal tumors with multilayered rosettes in children (Review)

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Abstract. Embryonal tumors with multilayered rosettes (ETMR) are relatively rare but highly aggressive intracranial tumors that mainly occur in children under four years of age. Despite high-intensity and multi-modal treatment, the five-year overall survival rate of patients with ETMR remains <30%. Therefore, it is necessary to improve understanding of the molecular biological changes in ETMR. The present review presents an overview of the recent molecular and biological characteristics of ETMR in children, the current recommended treatments, and research into potential targeted strategies based on these findings. ETMR are molecularly characterized by distinct DNA methylation signatures and dysregulated expression of oncogenic miRNAs. Despite increased knowledge of the novel molecular characteristics of ETMR in children, treatment outcomes have only marginally improved. Thus, there is an urgent need to translate these new insights in ETMR biology into more effective treatment.

Contents

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Abbreviations: ETMR, embryonal tumors with multilayered rosettes; CNS, central nervous system; WHO, World Health Organization; OS, overall survival; C19MC, chromosome 19q13.42 miRNA cluster; ATO, arsenic trioxide; BRD4-NUT, BRD4 fusion oncoprotein

Key words: ETMR, C19MC, DICER1, LIN28A, treatment

1. Introduction

Brain tumors are the most common solid neoplasms and the leading cause of death from cancer in children. Tumors of the central nervous system (CNS) account for 20% of childhood cancers and are second only to leukemia in frequency. Embryonal tumors with multilayered rosettes (ETMR) are highly aggressive embryonic brain tumors classified as World Health Organization (WHO) grade IV that mainly occur in children under the age of four years (1). The five-year overall survival (OS) rate of patients with ETMR remains <30% (2). Embryonic tumors (95%), ependymomas (90%) and medullary epitheliomas (75%) are characterized by a hallmark amplification of the Chromosome 19q13.42 miRNA cluster (*C19MC*) (3). In the 2021 edition of the WHO classification of tumors of the CNS, ETMR include the most common *C19MC* type, *DICER1* alteration, not elsewhere classified or not otherwise specified (4). ETMR exhibit unique histopathological features which, alongside the detection of *C19MC* or *DICER* alterations and enrichment of LIN28A, are necessary for diagnosis. Currently, the diagnosis of ETMR relies mainly on molecular biological detection. *C19MC* amplification and *TTYH1* fusion are signature gene changes in ETMR, which are observed in ~90% of ETMR cases (5). Biallelic mutation in *DICER1* is the second most common genetic event and is present in ~5% of patients with ETMR, occurring exclusively in tumors lacking *C19MC* amplification (6). Both *C19MC* amplification and *DICER1* mutations may have common downstream mechanisms, the LIN28A/let-7 pathway, and miRNAs belonging to the *let-7* miRNA family are considered oncogenes (2). The current treatment options for ETMR include maximum surgical excision and adjuvant chemotherapy, high-dose chemotherapy with stem cell salvage, and focal or whole-brain whole-spinal radiation therapy. Most treatments for ETMR have failed under this high-intensity, multi-modal treatment. For patients receiving chemotherapy, the median OS time was 7.4 months, whereas that of those who did not receive it was 1.2 months (7). For patients with non-brainstem tumor location, the five-year progression-free survival (PFS) and OS were 35 and 47%, respectively, after treatment with intensified chemotherapy (8). With further ETMR genome and epigenetic studies, a new understanding of the ETMR molecular biological mechanism was gained and potential therapeutic targets were identified. However, current

research on ETMR remains limited. The treatment of patients with ETMR has only slightly improved, and the five-year OS rate remains <30% (2). Therefore, there is an urgent need to translate the molecular biology of ETMR into effective therapeutic measures. The present review discusses the molecular biological characteristics and treatment progress of ETMR to further elucidate the pathogenesis of ETMR and lay a foundation for molecular therapy of ETMR.

2. ETMR molecular mechanism

ETMR were distinguished from other pediatric intracranial tumors by analyzing DNA methylation and transcriptome sequencing data (9). ETMR are molecularly similar with or without *C19MC* amplification (6) and have unique transcriptional and epigenetic backgrounds (10). Besides *C19MC* amplification, few other gene changes or recurrent gene mutations occur (6,9,10), suggesting that ETMR are mainly driven by epigenetic mechanisms (10).

C19MC amplification-the signature gene change in ETMR. *C19MC*, first reported in 2009 (11), is the largest microRNA (miRNA or miR) cluster unique to primates identified thus far, with a length of >100 kb and encoding ~62 miRNAs (12,13). The specific number of functional miRNAs remains unclear owing to the conserved nature of the *C19MC* structure (12). *C19MC* is expressed only in the placenta, testis and human embryonic stem cells, and its expression decreases gradually with stem cell differentiation (14). *C19MC* amplification or fusion was found in 90% of patients with ETMR (5,12,15,16), and is considered the only major recurrent genomic alteration reported in ETMR to date (5,11,15-19). *C19MC* is also expressed in ETMR without amplification, albeit at an ~10-fold lower level, but not in the normal brain or other brain tumors, which may be related to structural variations (6). Regardless of *C19MC* amplification, ETMR exhibit high molecular similarities (6). However, *C19MC* overexpression is not unique to ETMR. *C19MC* amplification and tumor suppressor *p53* deletion are significant factors that drive undifferentiated hepatic embryonic sarcoma development (20). In estrogen receptor-positive breast cancer cells and hepatocellular carcinoma, *C19MC* overexpression promotes cell cycle progression and induces chemotherapy resistance, thereby increasing tumor cell viability. Furthermore, *C19MC* overexpression is associated with poor patient survival (21,22). Except for differences in tumor distribution, *C19MC* amplification has no significant relationship with the other clinical features of ETMR (6). Presently, it remains unclear whether the absence or presence of *C19MC* amplification influences the disease outcome (2).

ETMR are molecularly distinct entities based on the analysis of miRNA data of ETMR and other pediatric intracranial tumors (6,23). However, the differentially expressed miRNAs in ETMR are similar. Transcriptional analyses confirmed that mature miRNA expression, including *C19MC* miRNAs and *miR-17-92* miRNA clusters, increases whereas the expression of *let-7* miRNAs and *miR-15* family miRNAs decreases (23). However, *C19MC* oncomiRs promote ETMR formation by synergistically acting on the corresponding target genes. Enrichment analysis revealed that the genes affecting neural stem cell maintenance, epigenetic regulation and miRNA processes were upregulated, whereas those affecting apoptosis, mRNA stability and neurogenesis were downregulated. *C19MC* binds to the *TTYH1* promoter to facilitate tumor cell proliferation by targeting the cell cycle-related tumor suppressors p21, P27 and RBL2. *C19MC* stabilizes LIN28A and MYCN by targeting TTP, whereas LIN28A and MYCN regulate tumor cells via DNMT3 and MAZ, respectively (23).

DICER1 as a potential ETMR driver. *C19MC* amplification, *DICER1* mutation, and *MIR17HG* amplification are involved in miRNA processes (2,6). *DICER1* is an RNase III kernel enzyme involved in miRNA cytoplasmic processing. *DICER1* deletion in mice inhibits cell proliferation and differentiation, leading to early embryo death. *DICER1* mutations in human germlines, known as *DICER1* syndrome, are associated with tumor susceptibility and are characterized by early childhood tumor development (24-28). *DICER1* biallelic mutations were found in 5% of ETMR cases, which mainly occurred in cases without *C19MC* and *MIR17-92* miRNA amplification, including germline and somatic mutations, which mainly occurred in the RNASEIII region and could increase the proportion of 3p/5p miRNAs (2,6), affecting miRNA processing. Currently, *DICER1* is considered the first ETMR susceptibility gene and a potential ETMR driver (6). The *MIR-17-92* miRNA cluster is located on chromosome 13. The *MIR17HG* cluster is a carcinogenic miRNA cluster associated with cancer proliferation and increased invasiveness (29). *miR-17-92* miRNA cluster amplification was found in ~1% of ETMR cases, with one case of *C19MC* amplification and two cases of ETMR without *C19MC* amplification reported thus far. During placental development, *C19MC* is co-expressed in *MIR17HG* miRNA clusters (30), and several 'seed' sequences are identical to the mature miRNA sequences encoded by *C19MC*, suggesting that these two miRNA clusters may be co-regulated and act on the same target genes (30,31).

In addition, *CTNNB1* and *TP53* mutations were found in certain ETMR, and *CTNNB1* mutations occurred in 10% ETMR (6,32,33), which activated Wnt signaling by inhibiting β-catenin protein degradation (34). *TP53* mutations occur in 7% of ETMR (6). Generally, there are relatively few frequent gene mutations affecting the miRNA pathway, including the amplification of the *C19MC* and *miR-17-92* clusters and mutation of the miRNA-processing gene, *DICER1*. Their distribution is relatively exclusive (6).

Common downstream LIN28/let-7 pathway of C19MC amplification or DICER1 mutation. LIN28A is an RNA-binding protein, which is involved in regulating the development and self-renewal of embryonic stem cells as a post-transcriptional regulator affecting the maturation of miRNA (35-37). Nearly all *C19MC*-amplified ETMR exhibit significant enrichment of LIN28A (19,38), and LIN28A upregulation is characteristic of aggressive malignant tumors (39). However, it is not unique to ETMR and is also observed in 25% of atypical teratoid/rhabdoid tumor and 20% of high-grade gliomas, with LIN28A-positive tumor histology tending to be ependymoblastoma, medulloepithelioma, or recurrent cases, suggesting high sensitivity and low specificity of LIN28A (19,40). Therefore, LIN28A positivity supports the diagnosis of ETMR and is currently used as a marker for ETMR.

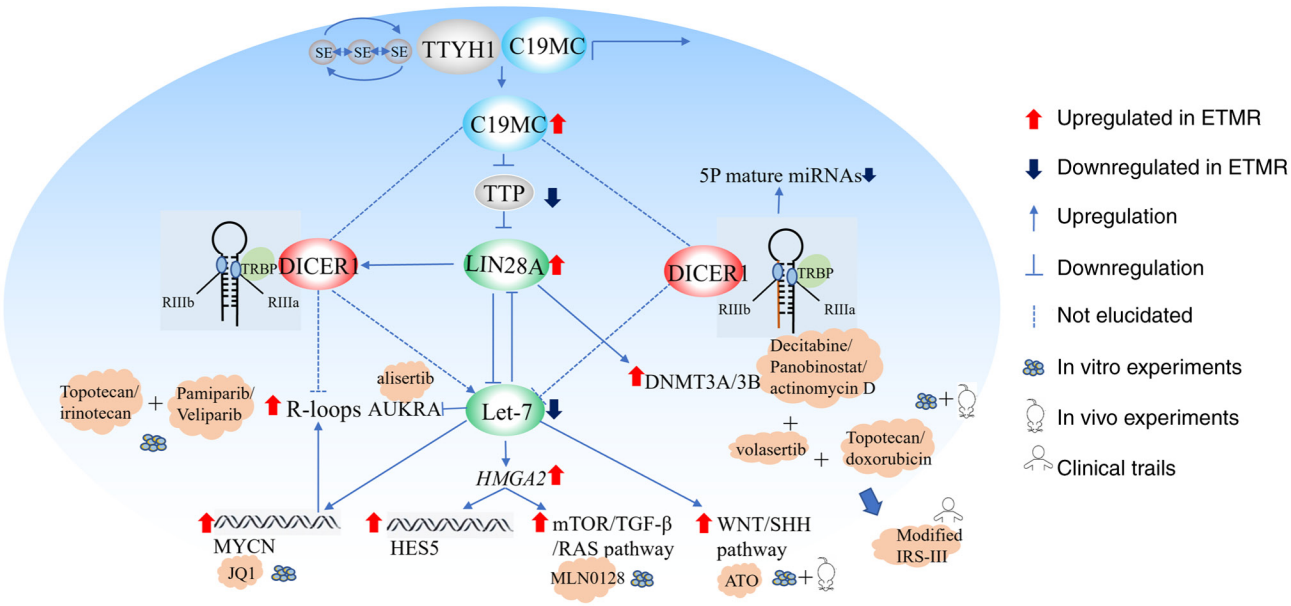

Figure 1. Active pathways in ETMR and novel therapeutic leads. The fusion *TTYH1* with *C19MC* forms a super enhancer-dependent original transcription and epigenetic state. High *C19MC*, MYCN and LIN28A expression was further promoted under the action of the super enhancer. Several drugs have been identified as treatment options according to the relevant molecular changes. ETMR, embryonal tumors with multilayered rosettes; miRNAs, microRNAs; ATO, arsenic trioxide; C19MC, chromosome 19q13.42 miRNA cluster.

LIN28A inhibits the maturation of *let-7* miRNAs by binding to the terminal loop of the *let-7* pre-miRNA and recruiting 3' terminal uridylyl transferase 4, which causes reduced levels of mature *let-7* miRNAs in the cell, and further promotes the proliferation and metabolism of ETMR cells by activating the mTOR signaling pathway (41-44). The self-renewal capacity of cells is regulated by affecting Wnt/Shh and NOTCH signaling (6,33,45). Moreover, the proliferation and transcription processes of cells are affected by promoting MYCN expression (46), whereas the DNA methylation state in ETMR is affected by directly regulating DNMT3B and DNMT3A (23). Cumulative studies discovered alternative targets for ETMR therapy (6,23,33,44,47-51).

DICER1 mutations may also affect LIN28A/let7 signaling. In ovarian Sertoli-Leydig cell tumors, Wilms tumors and Uterine Corpus Endometrial Carcinoma with *DICER1* RNase IIIb mutations, *let-7* targets are significantly upregulated compared with other tumors that lack *DICER1* RNase IIIb mutations (52-54). The phenotype resulting from *DICER1* mutations can be partially rescued by re-expressing *let-7* miRNAs (53,54). An increase in WNT and MYCN signaling was detected downstream of the *DICER1* mutations, which affected the RNase IIIb domain, suggesting that *DICER1* mutations have downstream effects similar to those of LIN28A expression (52).

Role of ubiquitination in ETMR. Proteomics is rarely studied in ETMR, but holds out the prospect to reflect functionally relevant tumor features more closely. Ubiquitination is the second most common post-translational modification of proteins following phosphorylation (55). Ubiquitination plays a crucial regulatory role in the modulation of tumors, impacting cellular survival, proliferation and differentiation. Integrated proteomics showed that histomorphology stipulates the proteome signatures of ETMR; proteasome regulatory proteins are highly abundant in ETMR, which indicates proteasome inhibition as a promising therapeutic option in ETMR (47). Generally, *C19MC* amplification is an indication of a gene change. *DICER1* mutation and *MIR17HG* amplification mainly occur in *C19MC* cases and influence the miRNA process as well as *C19MC*. *DICER1* is regarded as the first ETMR susceptibility gene and a potential ETMR driver. High LIN28A expression is a diagnostic marker of ETMR. LIN28A is involved in the activation of multiple signaling pathways and presents a potential therapeutic target. Proteasome regulatory particle abundance is a distinctive, histology independent feature of ETMR; proteasome inhibition represents a promising therapeutic vulnerability in ETMR (47).

3. ETMR treatment progress

Traditional treatment regimens have provided only limited improvement in patients with ETMR, and new treatment option development and design of new targeted therapies remain challenging. The fusion of *TTYH1* with *C19MC* forms a super enhancer-dependent original transcription and epigenetic state (23). High *C19MC*, MYCN and LIN28A expression was further promoted under the action of the super enhancer. Several drugs have been identified as treatment options in response to these molecular changes (Fig. 1).

Gualano *et al* (48) reported an ETMR patient with long-term survival (>5 years), whose post-treatment histopathology revealed maturation of undifferentiated embryonal cells into mature neuronal and ganglionic phenotypes. This finding suggests the notion of differentiation as a promising therapeutic approach toward novel drug development for the treatment of deadly pediatric brain tumors (48).

Targeting the ETMR metabolic pathway shows promise for *in vitro* assays. Small-scale drug screens of 73 small-molecule inhibitors showed that ETMR cells were particularly sensitive to mTOR, IGF1R, PI3K and topoisomerase inhibition, while showing little to no sensitivity to other receptor tyrosine kinase inhibitors (44). The BT183 ETMR cell line was screened using 35 different compounds in another study and was sensitive to a variety of drugs, including topoisomerase inhibitors such as topotecan and daunorubicin, epigenetic regulatory agents such as decitabine and Panobinostat, actinomycin D, and some targeted drugs, including the PLK1 inhibitor volasertib, auroral kinase inhibitor alisertib, and mTOR inhibitor MLN0128. *In vivo* verification indicated that topotecan, volasertib and actinomycin D alone prolonged the survival of mice and significantly inhibited tumor growth at treatment initiation; however, complete remission was not achieved. Further study of the potential therapeutic effects of topotecan and daunorubicin in a multi-drug setting, when combined with vincristine and methotrexate, respectively, demonstrated that topotecan combined with chemotherapy resulted in improved survival than daunorubicin; however, the study lacked long-term treatment or disease control (49). Cocito *et al* (50) generated two novel patient-derived ETMR cell lines from resected patient derived tumor samples and created three patient-derived xenograft models. They further conducted high-throughput drug screening utilizing 2,480 approved and investigational drugs, against the patient-derived ETMR cell lines. A total of 1,953 combinations were selected; however, the subsequent data are currently being validated *in vivo* (50). Integrated proteomics showed that ETMR and BT183 cells harbor proteasome regulatory proteins in abundance. Further, *in vitro* assays using BT183 highlighted that ETMR tumor cells are highly vulnerable toward treatment with the CNS penetrant proteasome inhibitor Marizomib. However, the study is limited by rather small case numbers. Consequently, not all molecular subtypes may be sufficiently represented (47).

Based on the aforementioned *in vitro* experimental results, the Dana-Farber Cancer Institute's modified IRS-III protocol incorporates preclinical active agents, such as doxorubicin and actinomycin D, into the treatment regimen for ETMR. Hanson *et al* (51) included five patients with ETMR, all of whom underwent complete tumor resection and were treated with IRS-III for 12-51 weeks. A total of four patients received local radiation therapy and the fifth received high-dose chemotherapy with an autologous stem cell rescue cycle. The results showed that the PFS rate of all four patients was >18 months. A total of five patients with mild sinusoidal obstructive syndrome and one patient with grade 3 peripheral neuropathy tolerated chemotherapy (51). However, the small number of cases included in the study and inconsistencies in the various additional treatments also influenced the findings.

Genetic instability has also been exploited as a potential therapeutic strategy. ETMR are highly sensitive to topoisomerase inhibitors that dissolve the R-loop (56,57). Topotecan and irinotecan act as TOP1 inhibitors via the covalent binding of TOP1 to DNA (58), and PARP1 can release TOP1 through adenosine diphosphate ribose. The combination of TOP1 and Irinotecan has been shown to increase R-loop formation and causes DNA damage. ETMR cell viability was inhibited and this synergistic effect was dependent on sufficient topoisomerase inhibition. However, this study requires *in vivo* investigation (6).

LIN28A overexpression promotes Shh and Wnt signal activation by down-regulating *let7*-miRNA in ETMR (6,33). The SHH inhibitor arsenic trioxide (ATO) inhibits ETMR cell proliferation and GLI expression in ETMR-xenografted mice, and prolongs mice survival (33). ATO also inhibits cell differentiation by acting on corresponding targets (33). Thus, ETMR growth decrease may be achieved by inducing cell differentiation, rather than by specifically inhibiting SHH.

ETMR progression and pluripotency maintenance in tumor cells depend on the high level of transcriptional activity catalyzed by MYCN. JQ1, a BET inhibitor, binds competitively to the BRD4 fusion oncoprotein (BRD4-NUT), thereby separating BRD4-NUT from chromatin and leading to cancer cell differentiation and apoptosis (59,60). JQ1 can significantly reduce the viability of ETMR cells by competitively binding to the bromine domain and inhibiting transcriptional activity while downregulating the levels of *C19MC* miRNA, LIN28A and DNTMT3B6 (23). However, further *in vivo* studies are required to confirm this hypothesis.

Despite the availability of various therapeutic strategies, ETMR remains a highly lethal disease. With the development of molecular biology, ETMR diagnosis is no longer ambiguous and further validation of these drugs through *in vitro* experiments and *in vivo* models is necessary. However, some difficulties remain. First, there is a limited number of ETMR cases and few long-term survivors. Second, large-scale drug screening is hampered by a lack of suitable *in vitro* and murine models. There are currently two cell lines containing the amplification of *C19MC* (BT183 and NCH3602); however, the establishment of suitable xenotransplantation models is complicated, possibly because of the unique microenvironment in which ETMR originate, making them different when replicated in murine xenotransplantation models. In addition, *C19MC* is unique to primates, and the resulting murine knockout/overexpression model may fail to replicate the unique epigenomic complexity of ETMR.

4. Conclusions

ETMR is a highly aggressive intracranial tumor in children. With recent studies on *C19MC* amplification, genomic and epigenetic studies of ETMR in children have improved our understanding of its biological nature. ETMR molecular diagnosis, genetic alteration identification, and possible therapeutic target exploration are also progressing. Despite preclinical and clinical studies, the survival rates of patients with ETMR have only slightly improved. These results highlight the limitations of these studies and the lack of prospective drug combination experiments. Second, because of the relatively rare disease itself, few long-term survivors, and unclear prognostic factors, the two ETMR cell lines found so far contain *C19MC* amplification but lack other ETMR molecular characteristics and drug targets, which complicates the exploration of new therapeutic strategies as preclinical models. Therefore, it is necessary to improve understanding of the molecular biological changes in ETMR; further explore the relationship between *C19MC* amplification, *DICER1* mutations, and the LIN28/let-7 pathway; determine the role of ubiquitination in ETMR as well

as establish reasonable animal models; and provide possibilities for further treatment through reasonably designed targeted therapy and comprehensive preclinical trials.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

WL conceived the study and conducted majority of the literature search and drafting the initial text. JG and XG contributed critical revision during the development of the manuscript. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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