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ORIGINAL PAPER

# LIN28A, a sensitive immunohistochemical marker for Embryonal Tumor with Multilayered Rosettes (ETMR), is also positive in a subset of Atypical Teratoid/Rhabdoid Tumor (AT/RT)

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**Abstract**

*Introduction* CNS embryonal tumors comprise a group of highly malignant neoplasms with a wide spectrum of histomorphological entities that includes Medulloblastoma (MB), Atypical Teratoid/Rhabdoid Tumor (AT/RT), Neuroblastoma (NB), Ganglioneuroblastoma (GNB), Embryonal Tumor with Multilayered Rosettes (ETMR), and the embryonal tumor—Not Otherwise Specified (NOS). The entity ETMR includes previously described histopathologic patterns—Embryonal Tumor with Abundant Neuropil and True Rosettes (ETANTR), Ependymoblastoma (EBL), and Medulloepithelioma (MEPL). Based on the histopathological similarities (multilayered rosettes) among ETANTR, EBL, and MEPL, as well as uniform clinical behavior and common molecular genetic characteristics, the WHO revision has created a new entity, "ETMR." Immunoreactivity of LIN28A has been identified as a sensitive tool for the diagnosis of this entity. Since there is a paucity of literature regarding immunoreactivity of LIN28A across all embryonal CNS tumors, the present study was undertaken.

*Materials and methods* During the 5-year study period (2012 to 2016), all the embryonal tumors (MB, AT/RT, other embryonal tumors—ETANTR, MEPL, PNET) that had been earlier diagnosed in the department of neuropathology (cases operated in our institute as well as received as referral) were reviewed. The archived Hematoxylin and Eosin (H&E) and the available immunohistochemistry (IHC) sections were studied. Further, for the other embryonal tumors where the paraffin blocks were available, an extended panel of IHC was performed for confirming the diagnosis of embryonal tumor and only confirmed cases were included in the study. The demographic details of the study cohort were noted. IHC for LIN28A was performed on conventional sections.

*Results* A total of 396 cases of embryonal tumors including 302 MB, 72 AT/RT, and 22 other embryonal tumors were diagnosed during the study period. Among these, 80 MB, 35 AT/RT, 4 ETANTR, 1 MEPL, 4 NB, 2 GNB, and 1 CNS embryonal tumor-NOS (total—127) were included for the study. LIN28A immunoreactivity was absent in all MB, GNB, NB, and CNS embryonal tumors-NOS whereas all cases of ETMR (4 ETANTR, 1 MEPL) and 8/35 (23%) of AT/RT showed immunopositivity for LIN28A, which was patchy and distinct in most of the cases of ETMR.

*Conclusion* Our study reiterates that LIN28A is a sensitive IHC marker for the diagnosis of ETMR. We also show that among CNS embryonal tumors, LIN28A is not specific to ETMRs and such immunoreactivity can also be seen in a proportion of AT/RTs.

**Keywords** ETMR · ATRT · LIN28A · Sensitive marker

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## Introduction

Embryonal tumors of the Central Nervous System (CNS) include a wide spectrum of histomorphological entities featuring poorly differentiated neuroepithelial cells that are capable of divergent differentiation. In the WHO 2007 classification of CNS tumors, the group of embryonal tumors included the following entities: Medulloblastoma (MB), CNS "Primitive Neuroectodermal Tumors (PNET)," and Atypical Teratoid/Rhabdoid Tumor (AT/RT) [1]. The CNS "PNET" group has since then been ever expanding and has included several embryonal tumor entities. These tumors are known to have a dismal clinical outcome which is mainly attributed to the tendency for these neoplasms to disseminate through cerebrospinal fluid (CSF) pathways. However, advancement in treatment strategies has improved the patient outcome for some of these tumors [2, 3]. Studies over the past decade suggest that "PNETs" are a heterogeneous group of neoplasms and not just one homogenous group, as earlier believed [4, 5]. Integrated genomic studies using various high throughput platforms have now confirmed that embryonal tumors are indeed defined by their classic histopathological descriptions, tumor location, molecular alterations, and capability for divergent differentiation [6].

Currently, the broad spectrum of embryonal tumors include the following histomorphological entities; MB, AT/RT, Neuroblastoma (NB), Ganglioneuroblastoma (GNB), Embryonal Tumor with Multilayered Rosettes (ETMR), and embryonal tumor—Not Otherwise Specified (NOS) [7]. The entity ETMR includes previously described histopathologic patterns—Embryonal Tumor with Abundant Neuropil and True Rosettes (ETANTR), Ependymoblastoma (EBL), and Medulloepithelioma (MEPL) which are known to exhibit a uniform clinical behavior. The entity ETMR has been introduced because many neuropathologists believed that EBL is not a distinct histological entity, rather ependymoblastic rosettes are most frequently encountered in embryonal tumors with abundant neuropil and less frequently in other CNS embryonal neoplasms. A study in this direction was carried out by Judkins and Ellison, where they observed that EBL as a diagnosis was neither precise nor specific and strongly urged discontinuation of the use of this diagnostic terminology [8]. Interestingly, Li et al., in 2009 showed for the first time that these three histologically defined tumors, ETANTR, MEPL, and EBL along with PNET with atypical features, shared a common molecular alteration, C19MC amplification, and suggested that these conventional histologic sub-classes could represent closely related molecular entities [9]. This was further validated by Korshunov et al. [10]. Later, Paulus and Kleihues, in 2010 proposed the term "Embryonal Tumor with Multilayered Rosettes (ETMR)" combining the three histological patterns—ETANTR, EBL, and MEPL [11]. Thus, in the WHO 2016 update classification of CNS embryonal tumors, the term "PNET" has been removed from the diagnostic lexicon and the C19MC amplified tumors (ETANTR, EBL, and MEPL) have been categorized into a new entity termed "*Embryonal tumor with multilayered rosettes (ETMR), C19MC-altered*" [7]. While the WHO continues to recognize medulloepithelioma as a distinctive histological entity, both ETANTR and EBL have ceased to have such recognition except perhaps as "patterns."

Importantly, immunoreactivity of LIN28A has been identified as a sensitive tool for the diagnosis of ETMR [12]. Since this entity is known to behave aggressively and the specific molecular alterations that have been identified could in future drive towards targeted therapy, there is a need to identify this group of tumors. Moreover, there is a paucity of literature regarding immunoreactivity of LIN28A across all embryonal CNS tumors.

With this background and since literature on the incidence of the group of embryonal tumors is lacking in our country, we assessed the frequency of embryonal tumors diagnosed at our institute, evaluated their demographic profile and subclassified these embryonal tumors based on histological and immunohistochemical parameters. Subsequently, we evaluated the immunopositivity of LIN28A across all the embryonal tumor entities. We show here that LIN28A immunopositivity is seen in all the ETMRs and a subset of AT/RT.

## Materials and methods

Ours is a retrospective study carried out over a period of 5 years (2012–2016), on all the embryonal tumors (MB, AT/RT, ETANTR, MEPL, "PNET") that had been earlier diagnosed in our department. This included tumor samples of cases operated at our institute as well as those received for diagnosis from various other hospitals, since ours is a tertiary referral diagnostic center.

A total of 396 embryonal tumors with the diagnosis of varied histologic subtypes were examined in this study. These included 302 MB, 72 AT/RT, and 22 other embryonal tumors (ETANTR, MEPL, "PNET"). AT/RTs had been diagnosed based on histology, and loss of INI-1 immunoreactivity (1:100, BAF47, BD BIOSCIENCE, USA), BRG1 immunostaining was not performed. The demographic and relevant clinical details were obtained from the clinical records. The archived Hematoxylin and Eosin (H&E) and the available immunohistochemistry (IHC) sections were studied by two pathologists (SR and VS). Of these 396 cases, 166 were operated at our institute and 230 were received from other centers for diagnosis. We had material [formalin-fixed paraffin-embedded blocks (FFPE)] available for 135 cases (80 MB, 35 AT/RT, and 20 other embryonal tumors). In 20 other embryonal tumors, IHC markers were employed to exclude other undifferentiated pediatric tumor entities such as anaplastic ependymoma and glioblastoma. Monoclonal or polyclonal antibodies were used against the following markers: synaptophysin (1:100 Clone SNP88, BIOGENEX, USA),

GFAP (1:200, 6F2, DAKO, GERMANY), EMA (1:100, Clone E29, DAKO, GERMANY), L1CAM (1:500, UJ127, SIGMA, USA), P53 (1:100, D07, DAKO, GERMANY), ATRX (1:100, SIGMA, USA), and H3.3K27M (1:300, MILLIPORE, USA). Following this, we had six cases with IHC profile of anaplastic ependymomas (GFAP and EMA positive, synaptophysin negative) and two glioblastomas (GFAP positive, synaptophysin negative). None of the cases were L1CAM or H3.3K27M immunopositive, and all the cases showed retained immunoreactivity for ATRX. Hence, out of the 20 cases, 12 were confirmed to be embryonal tumors. The final study cohort included 80 MB, 35 AT/RT, and 12 other CNS embryonal tumors.

IHC for LIN28A immunoreactivity was performed on this study cohort. Four micron thick sections were cut from FFPE blocks. IHC was performed using the Ventana Benchmark automated staining system (Ventana Benchmark-XT). Subsequent to the initial processing steps, the sections were incubated with the primary anti-LIN28A antibody (14E6-4E6, THERMO FISCHER, USA, diluted to 1:100) for 56 minutes followed by incubation with the secondary antibody for 8 minutes. The negative controls were treated identically except that the primary antibody was omitted. The pattern, extent, and intensity of immunoreactivity were assessed. Cytoplasmic immunopositivity of LIN28A was considered significant in accordance with previous report [12].

## Results

During the study period, out of the total of 396 embryonal tumors, 166 cases were operated at our institute. This accounted for 19% (166/886) of all the pediatric brain tumors diagnosed at our institute. MBs were the commonest, accounting for 76% of the embryonal tumors followed by AT/RTs (18%). ETANTRs and MEPL were rare tumors accounting for 1% of cases, whilst embryonal tumor-NOS accounted for the remaining 5%. The final cohort included in the present study after histological review and based on availability of FFPE blocks were as follows: 80 MB, 35 AT/RT, 4 ETANTR, 1 MEPL, 4 NB, 2 GNB, and 1 CNS embryonal tumor-NOS ($n$ = 127). We did not encounter any case of EBL in the study period.

### Demographic data

In the whole cohort ($n$ = 396), the median age at diagnosis was 13 years in MB and below 5 years across the other embryonal tumors. In the study cohort ($n$ = 127), the demographic data was in line with the whole cohort. The details of the study cohort are shown in Table 1. As known, MBs were cerebellar tumors. Most AT/RTs were also observed to involve the cerebellum. GNB and NB were seen in the supratentorial location, involving both cerebral hemispheric and intaventricular regions. Location of ETANTRs was varied, ranging from supratentorial intraparenchymal to intraventricular as well as the cerebellum.

### Histopathological characteristics

MBs had characteristic features of a cellular tumor composed of sheets of undifferentiated cells, exhibiting significant nuclear pleomorphism, high mitotic rate, and apoptotic rate (Fig. 1a). Various histological subtypes [classic ($n$ = 48), desmoplastic ($n$ = 22), large cell/anaplastic ($n$ = 8), medulloblastoma with extensive nodularity ($n$ = 2)] of MBs were noted. The tumor cells were immunoreactive for synaptophysin and exhibited a high proliferative index. NBs were characterized by undifferentiated islands, foci of neurocytic islands, and synaptophysin immunopositivity, whereas GNB had ganglionic differentiation in addition to the NB areas (Fig. 1b and c). AT/RTs had a broad spectrum of architectural and cytological features including the classical rhabdoid, rhabdoid-like cells, and undifferentiated round cells as well as spindled mesenchymal and epithelial-like tumor cells (Fig. 2a and b). In the present study, ATRTs were diagnosed based on loss of INI-1 immunopositivity only (Fig. 2c) since BRG1 staining was not performed. ETANTRs had biphasic architecture with clusters of undifferentiated cells and large neuropil areas with neurocytic cells. These tumors exhibited characteristic multilayered rosettes (Fig. 3a). The cells forming the rosettes were immunonegative for synaptophysin, whereas the differentiated neurocytic cells and the neuropil zones were immunopositive for synaptophysin (Fig. 3b). The tumor cells lining the rosettes were observed to be mitotically highly active with a high MIB-1 labeling (Fig. 3c). MEPL was characterized by papillary and tubular structures (Fig. 3e) surrounded by an external limiting membrane highlighted by periodic acid Schiff stain (Fig. 3f). The lining cells were composed of pseudostratified neuroepithelium, resembling the primitive neural tube. These tumor cells were immunonegative for synaptophysin (Fig. 3g). In addition, sheets of poorly differentiated cells with high nuclear:cytoplasmic ratio, hyperchromatic nuclei, and scant cytoplasm exhibiting brisk mitotic activity were also noted. We did not encounter any case of EBL. One tumor was composed of poorly differentiated cells and lacked specific histopathological features, hence, labeled as embryonal tumor-NOS.

LIN28A immunoreactivity: There were four tumors which would formerly have been classified as ETANTR and one that had the pattern of MEPL. All these tumors were immunopositive for LIN28A. The pattern of staining was quite distinct in the ETMRs. The LIN28A immunoreactivity was strong but the distribution of such staining

**Table 1** Demographic details of the study cohort of CNS embryonal tumors ($n$ = 127) across various embryonal tumors

| | MB ($n$ = 80) | AT/RT ($n$ = 35) | ETMR ($n$ = 5) | NB ($n$ = 4) | GNB ($n$ = 2) | NOS ($n$ = 1) |
|---|---|---|---|---|---|---|
| Median age at presentation (age range) | 13 years (3 months–49 years) | 3 years (2 months–46 years) | 2 years (1–5 years) | 3 years (1–5 years) | 1.75 years (1 and 2.5 years) | 6 years |
| Site of involvement | Cerebellum | Cerebellum, fourth ventricle | Supra and infratentorial | Intraventricular (lateral), temporal lobe | Frontal, parietal | Frontal |
| Males ($n$) | 54 | 19 | 3 | 3 | 1 | 1 |
| Females ($n$) | 26 | 16 | 2 | 1 | 1 | 0 |

*MB* medulloblastoma, *AT/RT* atypical teratoid/rhabdoid tumor, *ETMR* embryonal tumor with multilayered rosettes, *NB* neuroblastoma, *GNB* ganglioneuroblastoma, *NOS* not otherwise specified

was patchy. Among ETANTRs, intense LIN28A staining was observed mainly in the poorly differentiated cells that formed the multilayered rosettes, whilst there was absence of immunopositivity in the paucicellular neuropil zones within the tumor (Fig. 3d). Among the MEPLs, the cells lining the tubules had strong LIN28A immunoreactivity (Fig. 3h). Interestingly, 23% (8/35) cases of AT/RT also were immunopositive for LIN28A (Fig. 2d). LIN28A immunopositivity was absent in all MB, GNB, NB, and CNS embryonal tumor-NOS.

## Discussion

CNS embryonal tumors account for about 13% of pediatric brain tumors as per the published CBTRUS statistical data [13]. In our study, they comprised 19% of the pediatric brain tumors diagnosed at our institute during the study period. This group includes a specific set of neuroepithelial tumors composed predominantly of undifferentiated tumor cells and are known to occur both in the supratentorial and infratentorial compartments and rarely as primary intramedullary spinal cord tumors. MBs are tumors of the cerebellum, whereas the other embryonal tumors have been reported in supratentorial site, brainstem, cerebellum, and spinal cord. In our cohort, the localization of these tumors correlated with these previously published data [14]. Further, in accordance with the published literature, these tumors were commonly seen to affect young children in our study [15].

In the current era that heralds a histomolecular approach towards diagnosis of most neoplasms, there is a major shift towards incorporation of molecular data in the diagnosis of several CNS tumors. In this direction, major changes have been incorporated in the WHO 2016 update classification of CNS tumors, in particular, with respect to gliomas, MBs, and other embryonal tumors [7].

The recently described entity among the embryonal tumors is "ETMR, C19MC amplified." ETMRs are rare embryonal tumors usually found in children under the age of 3–4 years. These tumors are associated with a highly aggressive disease course with reported overall survival times ranging from 5 to 30 months, averaging 12 months. In our cohort, the median age of presentation was 2 years, similar to the published literature [11].

Eberhart et al. in 2000 first described the entity of ETANTR which is characterized histologically by undifferentiated neuroepithelial cells, areas of well-differentiated neuropil, and ependymoblastic rosettes [16]. Introduction of the entity of "ETMR, C19MC amplified" in the WHO 2016 CNS tumor classification was driven by major studies which demonstrated a common molecular alteration in the three histologically distinct tumors (ETANTR, EBL, and MEPL). Pfister et al. reported amplification of a chromosome band at

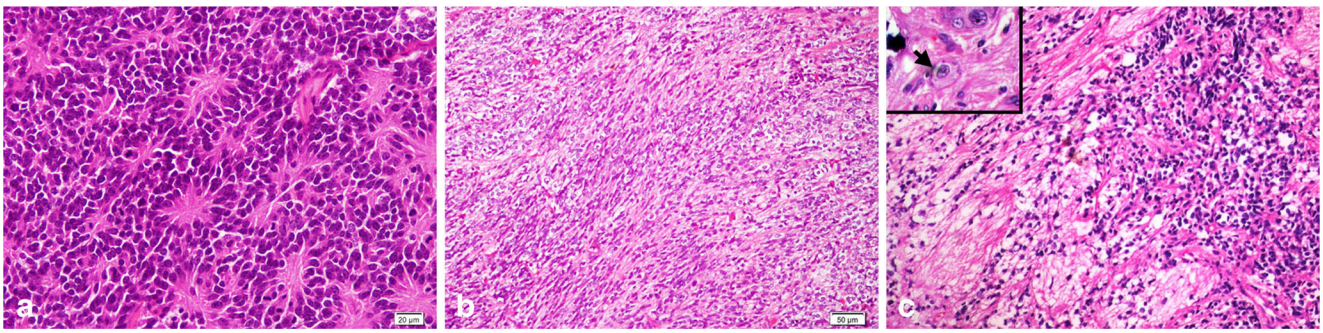

**Fig. 1** Photomicrographs of a classic medulloblastoma with Homer Wright rosettes formed by mitotically active small round cells (**a**, H&E, ×160), a neuroblastoma characterized by tumor cells dispersed in streams over a neuropil stroma (**b**, H&E, ×80), and a ganglioneuroblastoma composed of neuroblastoma component (**c**, H&E, ×80) with foci of clustered neoplastic ganglion cells (**c**, inset, *arrow*, H&E, ×160). All these cases were negative for LIN28A on immunohistochemistry (not shown)

19q13.42, which harbors a cluster of microRNAs (C19MC) [17]. Further, Li et al. observed amplification of C19MC in 11/45 cases of supratentorial "PNETs" and hypothesized that this amplification might be associated with ependymoblastic differentiation [9]. Later, Korshunov et al. carried out fluorescence in situ hybridization (FISH) analysis on 41 histologically diagnosed ETMRs and showed amplifications at 19q13.42 involving the C19MC cluster in 93% of ETMR tumors but not in any other pediatric brain tumors [18]. Subsequently, the same group of authors also proposed LIN28A to be a surrogate marker for C19MC amplification in ETMRs [12].

LIN28A, a RNA binding protein, was first identified in Caenorhabditis elegans and is known to play an important role in its development. LIN28A expression is high in embryonic stem cells and maintains pluripotency by inhibiting let-7-induced differentiation. Besides this, it is also known to be involved in several cellular processes such as proliferation, oncogenesis, development, and physiology, as well as metabolism [19]. LIN28A, a conserved cytoplasmic protein when translocated to the nucleus regulates mRNA, and along with its homolog LIN28B encodes proteins that bind to and repress the let-7 family of miRNAs, thereby upregulating cell cycle regulators targeted by let-7, such as cyclin D1/2 and cyclin-dependent kinases as well as proto-oncogenes such as MYC and RAS. LIN28A/LIN28B may also directly bind mRNAs to increase production of cell cycle regulators. High expression of LIN28A has been associated with an unfavorable clinical course in cancers of the ovary, colon, esophagus, and sympathetic nervous system (neuroblastoma) by promoting cellular proliferation, metastasis, metabolism reprogramming, cell

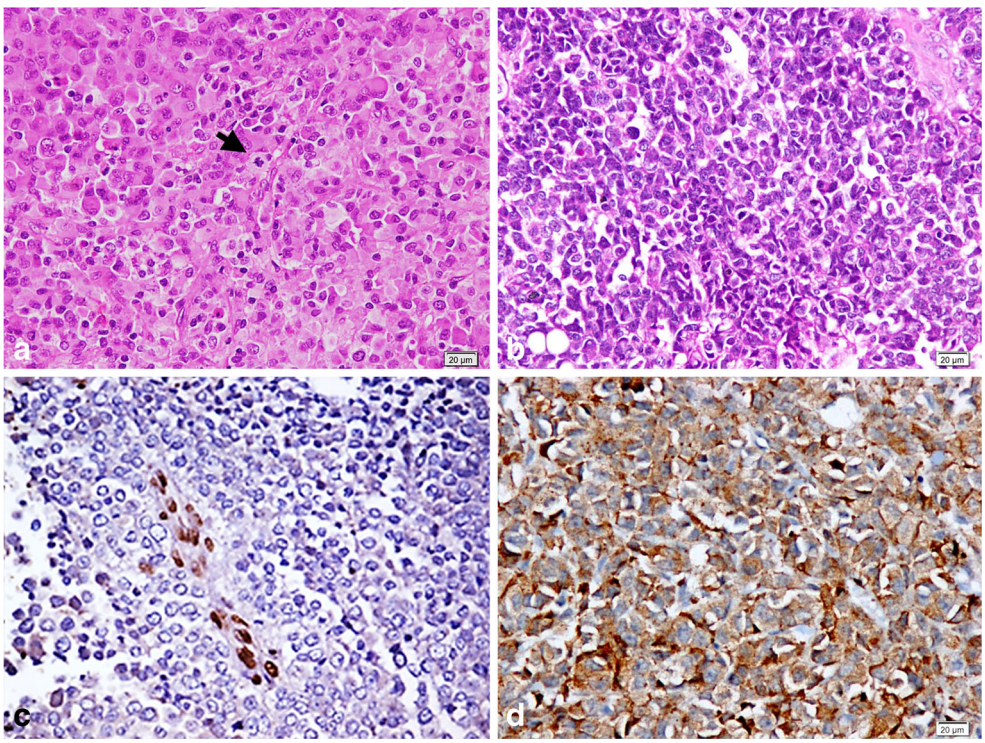

**Fig. 2** Photomicrographs of AT/RT composed of rhabdoid and rhabdoid-like cells (**a**, H&E, ×160) which are mitotically active (**a**, *arrow*) with interspersed foci of undifferentiated round cells (**b**, H&E, ×160). The tumor cells exhibit loss of INI-1 (**c**, immunoperoxidase, ×160) and cytoplasmic immunopositivity of LIN28A (**d**, immunoperoxidase, ×160)

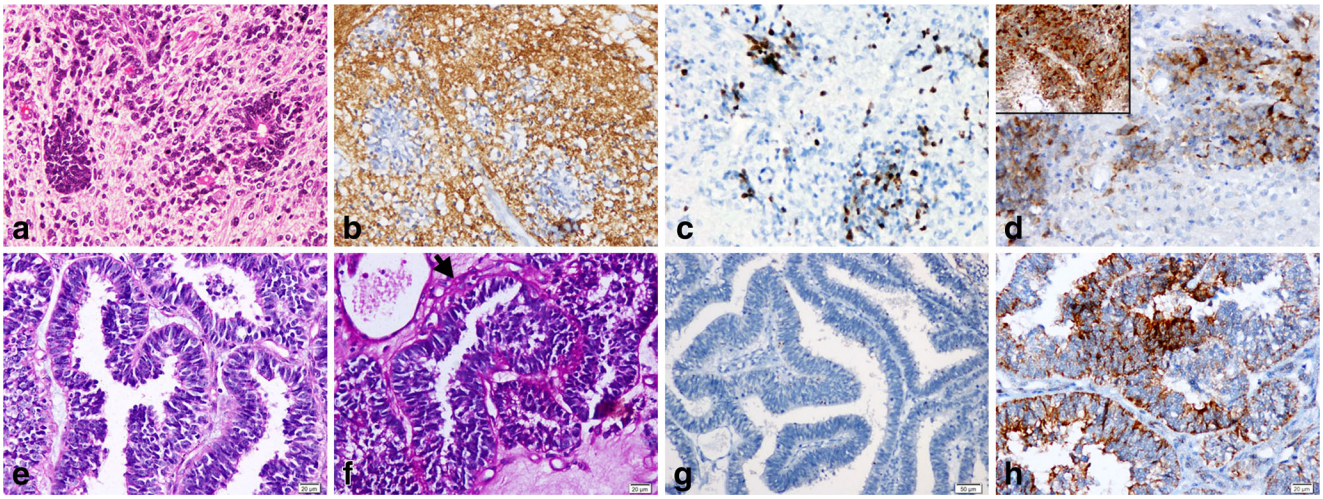


**Fig. 3** Photomicrographs of an ETANTR with characteristic multilayered rosettes set in a neuropil background (**a**, H&E, ×80). The neuropil is synaptophysin immunopositive but the cells forming rosettes are not (**b**, immunoperoxidase, ×80). The tumor cells forming the rosettes have a high proliferative index (**c**, MIB-1, ×80) and exhibit variable cytoplasmic LIN28A immunopositivity while the background neuropil is negative (**d**, immunoperoxidase, ×160). Inset shows a case of ETANTR with strong LIN28A immunopositivity in the rosettes (**d**, inset, immunoperoxidase, ×160). A case of medulloepithelioma composed of tumor cells arranged in tubules (**e**, H&E, ×160) lined by an external limiting membrane highlighted on periodic acid Schiff stain (**f**, PAS, *arrow*, ×160). The tumor cells lining the tubules are immunonegative for synaptophysin (**g**, immunoperoxidase, ×80) and immunopositive for LIN28A (**h**, immunoperoxidase, ×160)

death resistance, angiogenesis, tumor-associated inflammation, and genomic instability of cancer cells [19, 20].

Korshunov et al. observed that 100% of the ETMRs were LIN28A immunopositive compared to 12% (6/50) of the AT/RTs and none of the 41 other CNS "PNETs," 334 MBs, 223 anaplastic ependymomas, and 131 pediatric glioblastomas [12]. The authors state that LIN28A is a highly specific and sensitive marker for ETMR and recommend IHC for LIN28A as a rapid and reliable tool for the routine diagnosis of these tumors. However, in another study, by FISH analysis, the authors found amplifications at C19MC cluster in 93% of ETMR (not in any other pediatric brain tumors including AT/RTs) [18]. On the other hand, Spence et al. studied 128 MBs, 45 AT/RTs, 105 ependymomas, 50 high-grade gliomas (HGGs), 20 choroid plexus carcinomas, and 103 CNS "PNETs," and noted C19MC amplification in 24.3% of CNS "PNETs," whereas LIN28A immunoreactivity was observed in 21.4% of the CNS "PNETs," 19.5% of HGGs, and 24.4% of AT/RTs [15]. In our study, LIN28A immunopositivity was observed in 100% (5/5) of ETMRs, 23% (8/35) of AT/RTs, and none of the MBs, NB, or GNB, in line with the previous studies [12, 15], the comparison of which is shown in Table 2. Although the number of cases of ETMR is very low in this single institutional study, we show that it is a sensitive marker for ETMR. While the immunopositivity of LIN28A in AR/RTs in the present study is higher than that of the frequency noted by Korshunov et al., it seemed to match that noted by Spence et al. [11, 14]. The results of LIN28A immunopositivity in AT/RTs as shown in our study and by Korshunov et al., Spence et al. highlight the fact that LIN28A immunopositivity and C19MC amplification do not always occur concurrently. Indeed, there are non-ETMR CNS tumors with LIN28A immunoreactivity.

## Conclusion

This is the first study detailing the immunoreactivity of LIN28A on a large cohort of embryonal tumors of CNS in our country. Strong LIN28A immunopositivity was noted in ETMRs. Interestingly 23% of AT/RTs showed focal LIN28A immunopositivity, and this was not noted in any of the MBs or other embryonal tumors. Our study reiterates the sensitivity of

**Table 2** Comparison of results of LIN28A immunopositivity in the present study with the published literature

| | Korshunov et al. [12] | Spence et al. [15] | Korshunov et al. [18] | Present study 2017 |
|---|---|---|---|---|
| ETMR | 100% (37/37) | 100% (30/30) | 100% (97/97) | 100% (5/5) |
| AT/RT | 12% (6/50) | 24.4% (11/45) | ND | 23% (8/35) |
| MB | 0% (0/334) | 0% (0/128) | ND | 0% (0/80) |
| HGG | 0% (0/354) | 19.5% (8/41) | ND | ND |

*ND* not done, *ETMR* embryonal tumor with multilayered rosettes, *AT/RT* atypical teratoid/rhabdoid tumor, *MB* medulloblastoma, *HGG* high-grade glioma

LIN28A immunopositivity for diagnosing ETMRs. We also show that among CNS embryonal tumors, LIN28A is not specific to ETMRs and such immunoreactivity can also be seen in a proportion of AT/RTs.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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