TET2 promotor methylation and TET2 protein expression in pediatric posterior fossa ependymoma

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Pediatric posterior fossa ependymoma (PF) is one of the most common brain tumors in children. Recently, two subtypes of PF were identified. PF-A has a dismal prognosis and shows a hypermethylation phenotype, whereas PF-B shows a great genomic instability. The ten-eleven translocation methylcytosine dioxygenase 2 (TET2) gene (TET2) has been linked to the regulation of DNA methylation. We analyzed TET2 promotor methylation and protein expression to assess the role of TET2 in PF. Medical records of all PF cases treated in our institution between 1993 and 2015 were evaluated regarding tumor histology, grade, tumor location, gender, age, tumor recurrence, distant metastasis, survival and time to progression. Subsequently, we analyzed TET2 promotor methylation using methylation-specific polymerase chain reaction. TET2 protein expression was assessed using immunohistochemistry. Low TET2 expression was detected in seven of 17 cases. There was an association between low TET2 expression and tumor recurrence (P = 0.049). A TET2 promotor methylation was detected in five of 10 cases. There was no association between the TET2 promotor methylation with recurrence, tumor grade or gender. TET2 promotor methylation and low TET2 expression was detected in a subgroup of PF. Our data show an association between low TET2 expression and tumor recurrence in PF.

Key words: immunohistochemistry, methylation-specific PCR, pediatric ependymoma, promotor methylation, TET2 protein.

INTRODUCTION

Ependymoma is the third most common tumor in children. Among all pediatric ependymomas, posterior fossa ependymoma (PF) comprises approximately 60%–70% in the pediatric population. In general, ependymomas are classified as grades II and III tumors, based on histopathological criteria. However, grading shows only a poor correlation to outcome and treatment response. Recently, two subtypes of PF were identified based upon gene expression and methylation profiles.1,2 PF type A (PFA) occurs in younger children whereas PF type B (PFB) tends to develop in older children and adults. PFA occurs predominantly lateral within the PF and harbors a male predominance. Distant metastasis as well as tumor recurrence are common; these patients carry a dismal prognosis in comparison to patients with PFB.3,4 PFB shows the greatest genomic instability among all ependymomas, whereas PFA is characterized by a 5′-cytosine-phosphodiester-guanine-3′ (CpG) methylation phenotype island methylator phenotype.5 The methylation of CG dinucleotide islands is well known to contribute to tumorigenesis by downregulation of certain tumor suppressor genes and DNA repair genes. The CpG island methylation phenotype (CIMP) was identified in several cancers, that is colorectal carcinoma, gliomas, leukemia and neuroblastomas.6–8 Several genes have been linked to the CIMP. Mutations in the isocitrate dehydrogenase 1 (IDH1) gene (IDH1) and the ten-eleven translocation 2 (TET2) gene (TET2) as well as TET2 promotor methylation were found to be connected to CIMP in leukemia and glioma.9–11

The TET family are enzymes, that catalyze the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and induce subsequent DNA demethylation. Reduced TET2 activity in glioblastoma was associated with DNA hypermethylation.12
So far, no molecular mechanism has been identified to be involved in the development of CpG methylator phenotype in ependymoma. As there is no mutation of IDH1/2 in ependymoma, other mechanisms might play a role, such as downregulation of TET2 protein expression. We therefore sought to evaluate TET2 promoter methylation and protein expression in pediatric PF.

METHODS

Patient selection
All pediatric patients (age < 18 years) with PF, treated at our institution between 1993 and 2015 were reviewed. All children underwent microsurgical tumor resection with subsequent adjuvant therapy according to the International Society of pediatric oncology ependymoma protocol.

Medical records were evaluated regarding tumor histology, grade, location, gender, age, number of operations, extent of resection, adjuvant therapy, tumor recurrence, distant metastasis, survival and time to progression. Patients were included if formalin-fixed, paraffin-embedded (FFPE) tissues were available.

The study was approved by the local ethics committee of the University Hospital of Essen (15-6458-BO) and was performed in accordance with the ethical standards laid down in the Declaration of Helsinki and its later amendments.

Immunohistochemical staining
Paraffin-embedded sections were deparaffinized with xylene, rehydrated through descending alcohol concentrations and distilled water. Sections were heated in citrate buffer (pH 6.0) and then treated with 3% hydrogen peroxide. Pre-block was performed for 10 min (Zytochem Kit, PolyHRP-100), followed by washing with washbuffer (DCS labline washbuffer, WL583C2500). Sections on slides were incubated for 30 min at room temperature with the primary antibody against TET2 (rabbit polyclonal, ab94580, diluted 1:200; Abcam, Cambridge, UK). Afterwards, post-block was done for 20 min (Zytochem Kit, PolyHRP-100), followed by washing with DCS. This step was followed by incubation with chromogen 3,3′-diaminobenzidine (DAB) for 10 min at room temperature. Slides were then counterstained with hematoxylin (dilution 1:25) for 1 min. Then sections were dehydrated with ascending alcohol concentrations, followed by washing with xylene. Normal cervical tissue was used as a positive control and cervical squamous cell carcinoma as a negative control, as previously described.13

Immunohistochemistry (IHC) score
The slides were evaluated by two independent observers regarding the intensity of immunostaining and the percentage of positive tumor cells. The evaluation of IHC was modified from previously reported grading systems.13 Immunostaining was scored as “0”, “1” and “2” according to no, weak and strong staining intensity, respectively. A further score of “0”, “1” and “2” was applied to tumor cells if 0%–5%, 6%–50%, and 51%–100% revealed positive immunostaining, respectively. The sum of both parameters resulted in the total score. The total score was divided into two categories. Low TET2 expression was assumed for a total score of 0 and 1, whereas high expression was assumed for a total score of 2 to 4.

DNA extraction and methylation-specific polymerase chain reaction (PCR)
Total DNA was extracted from FFPE histological sections as previously reported.14 Methylation-specific PCR was carried out in 10 pediatric PF to assess TET2 promoter methylation. The primers were previously described by Chim et al.15 The TET2 promoter region comprises 772 bp and two CpG islands. The primers by Chim et al.15 amplify 39 CpGs of the second CpG island. The following primer sequences were used: TET2_uf: 5′ TGAGATGAGGGAGGTGAGGTTGGGT (unmethylated primer, sense), TET2_ur: 5′ ATCTATTC TCATCACTCAAAAAACCA (unmethylated primer, antisense); TET2_mf: 5′ CCGAGCGGGAGAGGTCGGG GCC (methylated primer, sense), TET2_mr: 5′ GTCTATTC TCATCACTCAAGAAAAACC (methylated primer antisense). As a negative control, normal blood DNA was included. Universal methylated human DNA (Zymo Research, Irvine, CA, USA) served as a positive control. Product size was 211 bp for both methylated and unmethylated primer sets. Thermal cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 50 s at 95°C, 45 s at 61°C and 60 s at 72°C. The final step was 5 min at 72°C.

PCR products were run on an agarose gel (2%), stained with SYBR stain (Thermo Fisher Scientific, Darmstadt, Germany) and visualized using UV solo (Analytik Jena, Jena, Germany).

Statistical analysis
Statistical analysis was conducted using SPPS Statistics Version 21.0 (IBM SPSS Statistics; Armonk, NY, USA). For categorical data, Fisher’s exact test was applied. Continuous data are presented as the mean ± standard deviation and were analyzed using a two-sided t-test. A P-value < 0.05 was considered as significant. Progression-free survival (PFS) and overall survival (OS) were defined as the time from histological diagnosis to first progression (PFS) and to death (OS).

RESULTS

Clinical patient data
Thirty-seven pediatric patients met the inclusion criteria. Of these, 26 patients had a tumor localization in the posterior fossa. Seventeen (65.4%) patients were eligible for our study.
as FFPE tissues were missing for nine patients. Our cohort \((N = 17)\) comprised eight \((47.1\%)\) girls. Mean age at diagnosis was \(5.05 \pm 3.47\) years \((\text{range: } 1-12\) years). In five children \((29.4\%)\), a grade II ependymoma was diagnosed and the remaining cases were grade III tumors. Patients with grade III tumors were significantly younger than patients with grade II tumors \((8 \text{ vs. } 3.83\) years, \(P = 0.018)\). The proliferation index with Ki-67 varied among all tumors between 1% and 60%. During their clinical course, nine children experienced a progression of their disease and their PFS was \(563 \pm 632\) days. Progression was due to local recurrences \((n = 5)\) or distant spinal metastasis without local progression \((n = 1)\). In three cases, both a local recurrence and distant spinal metastasis was diagnosed. Two patients were treated because of a meningeosis carcinomatosa. Eight children did not experience a recurrence until their last follow-up. In our cohort, five children died. Three children died due to their local tumor progression. One girl died of a Burkitt lymphoma 6 years after PF diagnosis. One boy died due to central respiratory regulation disorder and recurrent pulmonary infections 10 years after the initial diagnosis of posterior fossa ependymoma. Mean survival in these patients were \(2104 \pm 1402\) days. One patient was lost during follow-up. Follow-up periods ranged from 242 to \(6595\) days \((\text{mean} \pm \text{SD} = 2413 \pm 1761\) days). Patients’ characteristics are shown in Table 1.

### IHC results

IHC was performed in 17 tumors. Localization for TET2 protein was found to be nuclear and also cytoplasmic (Fig. 1).

Seven \((41.2\%)\) tumors had a low expression resulting in a total score of \(“0”\). High expression was detected in all other tumors \((58.8\%)\), with a sum score of \(“2”\) in three tumors, of \(“3”\) in three tumors and \(“4”\) in four tumors. Of seven tumors with a low TET2 expression, six tumors \((85.7\%)\) showed a grade III ependymoma. There was no significant association for low TET2 expression with gender or tumor grade \((P > 0.05)\). Regarding patient age, there was no significant difference between both expression groups. There was a significant association between low TET2 protein expression and tumor recurrence \((P = 0.049)\). In patients suffering from progression, there was a tendency toward a shorter time to progression \((522 \text{ vs. } 646\) days) and a shorter OS \((1538 \text{ vs. } 2953\) days) in patients with low TET2 expression. In Figure 1, representative slides with a high and low TET2 protein expression are shown.

### Methylation-specific PCR results

For 12 tumor samples, DNA could be extracted from FFPE tissue. In 10 tumor samples, the bisulfite conversion and amplification of DNA was possible, whereas two samples suffered from too low DNA quality. A methylation of the TET2 promotor was found in five tumors \((50\%)\). Figure 2 shows the results for methylation-specific PCR for the TET2 promotor. There was no association of TET2 promotor methylation with recurrence, tumor grade or gender \((P > 0.05)\). Moreover, for patient age, time to progression and OS time, there was no significant difference between TET2 promotor methylated tumors and unmethylated tumors \((P > 0.05)\). Four cases of TET2 promotor methylation occurred in the younger age group of 6 years and younger. In six patients \((60\%)\) TET2 methylation seems to represent the mechanism for regulation of TET2 protein expression. So, three patients with TET2 promotor methylation revealed consecutive down-regulation of TET2 expression. In three further cases, tumors

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**Table 1** Patients’ characteristics and TET2 promotor methylation and TET2 protein expression in pediatric posterior fossa ependymoma

| ID | Age (years) | Gender | Histology (grade) | Location | Local recurrence | Spinal metastasis | Death | TET2 promotor methylation | TET2 protein expression |
|----|-------------|--------|-------------------|----------|-----------------|-----------------|-------|--------------------------|------------------------|
| 1  | 1           | M      | 3                 | Midline  | No              | No              | No    | Yes                      | High                   |
| 2  | 4           | M      | 2                 | Midline  | No              | No              | No    | —                        | High                   |
| 3  | 3           | M      | 3                 | Midline  | Yes             | Yes             | Yes   | No                       | Low                    |
| 4  | 7           | M      | 3                 | Midline  | Yes             | No              | No    | —                        | Low                    |
| 5  | 4           | F      | 3                 | Midline  | Yes             | No              | No    | No                       | High                   |
| 6  | 4           | F      | 3                 | Midline  | Yes             | Yes             | Yes   | No                       | High                   |
| 7  | 4           | F      | 3                 | Midline  | No              | No              | No    | No                       | High                   |
| 8  | 7           | F      | 3                 | Lateral  | No              | No              | No    | No                       | Low                    |
| 9  | 9           | F      | 2                 | Lateral  | Yes             | No              | Yes   | Yes                      | Low                    |
| 10 | 12          | M      | 2                 | Midline  | No              | No              | No    | —                        | High                   |
| 11 | 12          | M      | 2                 | Midline  | No              | No              | No    | —                        | High                   |
| 12 | 3           | M      | 2                 | Midline  | Yes             | No              | Yes   | No                       | High                   |
| 13 | 1           | F      | 3                 | Midline  | Yes             | No              | No    | Yes                      | Low                    |
| 14 | 6           | F      | 3                 | Midline  | No              | Yes             | No    | Yes                      | Low                    |
| 15 | 1           | M      | 3                 | Midline  | No              | No              | No    | —                        | High                   |
| 16 | 2           | M      | 3                 | Midline  | Yes             | No              | No    | —                        | Low                    |
| 17 | 6           | F      | 3                 | Midline  | No              | No              | No    | —                        | High                   |

M, male; F, female.

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with unmethylated TET2 promotor had a high expression of TET2 protein. For the remaining four patients, there might exist an alternate mechanism for downregulation of TET2 protein expression, as they revealed low protein expression in the absence of TET2 promotor methylation ($n=2$) or showed no association between TET2 promotor methylation and protein expression ($n=2$).

**DISCUSSION**

Recent studies revealed that pediatric PF are a highly heterogeneous group of tumors and they differ significantly in terms of clinical characteristics and molecular profiles.\(^\text{16}\) Children with PFA tumors harboring CIMP phenotype, have a dismal prognosis and benefit from a more aggressive therapy with maximal safe resection followed by adjuvant radiation.\(^\text{17}\) CIMP is characterized by a high percentage of methylated CpG sites and subsequently transcriptionally silenced genes.\(^\text{3}\) As CIMP is a well-known phenomenon in different types of cancer, several studies addressed the putative driver mechanisms for the hypermethylation phenotype. Turcan and colleagues identified in glioma, that IDH1 mutation via accumulation of 2-hydroxyglutarate is essential to establish CIMP.\(^\text{9}\) In leukemia, IDH1 and 2 somatic mutations and also TET2 loss-of-function mutations were found to be associated with the hypermethylation phenotype.\(^\text{18}\) Loss of TET2 expression has been found in leukemia leading to DNA

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**Fig. 1** Microphotographs of the tumor sections stained with HE (A, left; B, left) and immunostained for TET2 (A, right; B, Right). Grade II ependymoma shows high TET2 expression (score = 4), while grade II ependymoma shows low TET2 expression (score = 0). Scale bars: 100 μm.
hypermethylation in enhancer regions with subsequent dysregulation of several genes involved in tumorigenesis. Furthermore, in low-grade gliomas a subgroup with TET2 promotor methylation was identified, which might present as an alternate mechanism for TET2 downregulation in the absence of IDH1/2 or TET2 mutations.

In our study, we found TET2 downregulation in 40% of all investigated tumors. TET2 downregulation was significantly associated with recurrence, as only one tumor with low TET2 expression had no recurrence within our follow-up. Furthermore, tumors with TET2 downregulation were associated with characteristics of PFA tumors, like younger age, shortened PFS and OS. Due to the small samples size, these differences did not reach significance.

As a possible mechanism for TET2 downregulation, we assessed TET2 promotor methylation status in a subgroup of our cohort. Here, we identified a methylation of TET2 promotor in five out of 10 cases. Of these, 80% with TET2 promotor methylation were detected in patients aged 6 years or younger, matching characteristics of PFA tumors. Here, in the majority of PF, promotor methylation seems to represent the regulation mechanism for TET2 expression.

Recently, Pajtler and colleagues identified two major subgroups within the PFA group and nine minor subtypes. These subtypes are heterogenous regarding their molecular characteristics and their genetic driver mechanisms are not identified. Therefore, downregulation of TET2 protein expression might play a role in a subgroup of pediatric PF via DNA hypermethylation and establishment of the CpG methylation phenotype. Lately, an ependymoma cell line has been established and further functional studies are necessary to elucidate the role of TET2 in PF.

There are several shortcomings in our study design. We conducted a retrospective analysis of patients’ tissues and clinical data. Therefore, tissue samples were mostly aged and partly only small amounts of tissue were available. Additionally, methylation-specific PCR analyses could only be carried out on a part of the cohort. Furthermore, the small sample size permits only limited statistical analyses.

In conclusion, we identified a subgroup of PF with downregulated TET2 expression in combination with TET2 promotor methylation. Even in this small cohort, there was an association of recurrence and low TET2 expression.

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