MYCN amplification predicts poor outcome for patients with supratentorial primitive neuroectodermal tumors of the central nervous system

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Background. Primitive neuroectodermal tumors of the central nervous system (CNS-PNETs) are a rare group of neoplasms occurring in the CNS that includes supratentorial CNS-PNETs, medulloepitheliomas, and ependymoblastomas. While ependymoblastomas frequently carry chromosome 19q13.41 amplification and show aggressive clinical behavior, the biological mechanisms and molecular alterations contributing to the pathogenesis of supratentorial CNS-PNETs remain poorly understood. Moreover, genetic alterations suitable for molecular risk stratification are undefined to date.

Methods. In order to identify possible molecular markers, we performed multiplex ligation-dependent probe amplification (MLPA) and molecular inversion probe (MIP) analysis on DNA samples of 25 supratentorial CNS-PNETs (median age, 5.35 years; range, 2.41–17.28 years). Tumors with ependymoblastic rosettes (ependymoblastoma/ETANTR) and LIN28A positivity were excluded.

Results. MLPA and MIP analysis revealed large losses of genomic material of chromosomes 3, 4, 5, and 13, while frequent gains affected chromosomes 1, 17, 19, 20, and 22. High copy number gains (amplifications) were found in particular at chromosomes 2p24.3 (MYCN, n = 6 cases) and 4q12 (n = 2 cases). Patients with tumors harboring 2p gain or MYCN amplification showed unfavorable overall survival (P = .003 and P = .001, respectively). These markers were independent of the presence of metastases, which was indeed a clinical factor associated with poor overall survival (P = .01) in this series.

Conclusions. In the era of the personalized neuro-oncology, the identification of these molecular prognostic markers associated with patient outcome may represent a significant step towards improved patient stratification and risk-adapted therapeutic strategies for patients suffering from supratentorial CNS-PNETs.

Keywords: CNS-PNET, LIN28, molecular inversion probe analysis, MYCN amplification, multiplex ligation-dependent probe amplification, primitive neuroectodermal tumors of central nervous system, 2p gain.
collaborative international studies have led to better understanding of the biology of medulloblastomas (MBs) and atypical teratoid/rhabdoid tumors, the molecular alterations underlying supratentorial CNS-PNET pathogenesis remain poorly understood so far and are limited by their overall very low incidence.\(^2\)\(^--\)\(^3\) Expression analyses and a handful of comparative genomic hybridization studies (CGH)\(^4\)\(^,\)\(^5\) have indicated that supratentorial CNS-PNETs are genetically heterogeneous and may show a broad spectrum of copy number aberrations.\(^2\)\(^,\)\(^5\)\(^,\)\(^6\) To date, amplification of MYCN, PDGFRA and PDGFRB, as well as deletions of CDKN2A/2B and a few other sporadic alterations of different pathways (including RASSF1A promoter methylation, p14\(^ARF\)) methylation and, transcriptional silencing of DLC-1) have been also reported.\(^7\)\(^--\)\(^12\) More recently, the identification of chr19q13.41 microRNA (miRNA) cluster (C19MC amplification)\(^13\)\(^--\)\(^15\) has permitted to better define among CNS-PNETs the ependymoblastoma/ETANTR (embryonal tumor with abundant neuropil and true rosettes) subgroup, which also shows aggressive clinical behavior, specific histopathological features and expression of stem cell marker LIN28A.\(^15\)\(^,\)\(^16\)

In the era of personalized oncology, the identification of prognostic molecular markers may represent a significant step towards improved patient stratification and risk-adapted therapy for children with supratentorial CNS-PNET. In fact, despite multimodal therapy, less than half of affected patients currently survive 5 years after diagnosis, and severe late effects associated with current treatment protocols are a significant complication for survivors.\(^17\)\(^,\)\(^18\) In order to identify suitable genetic markers with predictive value for outcome, we performed a multiplex ligation-dependent probe amplification (MLPA) and molecular inversion probe (MIP)-based analysis of 25 supratentorial CNS-PNET cases enrolled in the German multicenter GPOH HIT study.

**Materials and Methods**

**Patients and Study Design**

The study included 25 patients with the histological diagnosis of supratentorial CNS-PNET according to the revised WHO classification of CNS tumors.\(^1\) The histology of tumors was reviewed by an expert neuropathologist (T.P.). Cases with histological features of ependymoblastoma/ETANTR (including presence of multilayered ependymoblast rosettes, neuropil-rich areas, and LIN28A positivity) or medulloepithelioma features were excluded from this study (Supplementary Fig. 1). Twenty patients who underwent surgery between January 2001 and December 2011 in German, Austrian, and Swiss medical centers were enrolled in the prospective HIT 2000 study. Five patients diagnosed before the start of the HIT 2000 study were treated similarly and were also included in this study. Children aged 4–21 years were treated by postoperative chemotherapy (2 cycles of HIT-SKK chemotherapy)\(^19\) followed by radiotherapy and maintenance chemotherapy. Young children (aged <4 years) were treated in a risk-adapted manner with systemic chemotherapy (intraventricular methotrexate) followed by high-dose chemotherapy and administration of radiotherapy in case of residual tumor. Clinical data including age, metastatic disease status at presentation, sex, extent of surgical resection, event-free survival (EFS), and overall survival (OS) were collected. The HIT2000 trial and the HIT2000 registry were approved by institutional review boards, and informed consent was obtained from legal representatives of all patients.

**Tumor Samples, Immunohistochemistry and DNA Extraction**

Formalin-fixed, paraffin-embedded (FFPE) tissue specimens were retrieved from the archives of the Institute of Neuropathology at the University of Bonn Medical Center and the German Brain Tumor Reference Center in Bonn. Immunohistochemical analyses (IHC) were performed on a semi-automated IHC Stainer (Tecan) or a Ventana Immunostainer (Roche-Ventana) with antibodies against Map-2 (Sigma), S-100 protein (Dako), epithelial membrane antigen (Dako), glial fibrillary acidic protein (GFAP, Dako), neurofilament protein (Dako), synaptophysin (Dako), INI-1 (Becton Dickinson), Vimentin (Dako), NeuN (Chemicon), OLG-2 (R&D Systems), LIN28A (R&D Systems), and p53 (clone DO-7, Dako). Genomic DNA from FFPE tumor tissue was extracted using the QIAamp DNA Mini Tissue Kit (Qiagen) according to the manufacturer’s instructions. Histological assessment of tissue fragments chosen for this study confirmed that all specimens consisted of at least 80% tumor cells.

**Molecular Inversion Probe Assay**

To identify copy number gains and losses, we performed a custom-designed OncoScan FFPE Express 330K MIP assay (Affymetrix) on 15 tumors, as described.\(^20\) Data were analyzed using Genomic Suite 6.6 Software (Partek). The segmentation analysis was performed according to the software manufacturer’s instructions. High-copy-gain (amplification) was defined as average copy gain higher than 10.

**Multiplex Ligation-dependent Probe Amplification**

For MLPA analysis, the SALSA MLPA p302-A1, SALSA MLPA p303-A1, and SALSA p175 (MRC-Holland) assays were used. MLPA was performed in accordance with the manufacturer’s instructions.\(^21\) In brief, 100 ng DNA was denatured for 5 minutes and cooled down to 25°C. Following addition of the probe mix, the sample was hybridized for 16 hours at 60°C. After ligation, PCR was performed in a total volume of 50 μl containing 10 μl of the ligation mix on a thermocycler (Biometra). Subsequently, a LIZ-labeled internal size standard was added to the tumor samples, and fragments were separated and quantified on an ABI 3730 capillary sequencer after denaturation (Applied Biosystems) and analysed using the Gene Mapper software (Applied Biosystems). Differences of ±3-fold SD from the mean were considered as significant gains or losses, respectively, after normalization of the assay using FFPE cerebellar tissue. A >5-fold mean value of the mean of controls was considered as genomic amplification.

**Fluorescence in Situ Hybridization Analysis for MYCN**

FFPE tumor slides were hybridized overnight with the Zyto-Light SPEC MYCN/2q11 Dual Color Probe (ZytoVision). Briefly, deparaffinization, protease treatment, and washes were performed on the half-automated VP2000 processor system (Abbott Molecular). Before pretreatment, the slides were denatured at 10°C for 5 minutes at 75°C and hybridized at 37°C overnight. Posthybridization saline-sodium citrate (SSC) washes were performed at 72°C, and the slides were stained with 4',6-diamidino-2-phenylindole before analysis. Normal tissue served as internal positive control. Cases were further evaluated only if control tissue nuclei displayed 1 or 2 clearly distinct signals of each color. Tumor tissue was scanned for amplification hot spots by using ×40 or ×63 objectives (DM5500 fluorescence microscope; Leica). As the MYCN signals were homogeneously distributed, random areas were used for counting the signals. Twenty contiguous tumor cell nuclei were individually evaluated with the ×63 objective by counting green MYCN and orange 2q11 signals, and the MYCN/2q11 ratio was calculated. According to the diagnostic guidelines for neuroblastoma, cases were considered as amplified in case of a >4-fold MYCN copy number in relation to the copy number of chromosome 2, and a gain was defined as a 1.5–4-fold MYCN copy number in relation to the copy number of chromosome 2.
Fig. 1. Principal component analysis and virtual karyotype results from the molecular inversion probe analysis of supratentorial CNS-PNET cases. The cases analyzed in this study commonly showed CNS-PNET histology with undifferentiated, small-cell cytology. (A, upper part: hematoxylin and eosin). All tumors included in the study were LIN28A negative (A, lower part: immunostaining with LIN28A antibody). The principal component analysis showed molecular heterogeneity of the 15 supratentorial CNS-PNETs examined with MIP. (B) No molecular subgroups were identifiable according to patient age (red points represent patients <4 years of age; blue points represent patients >4 years). The virtual karyotype (C) showed frequent gains of 1q, 17, 19, 20, and 22 as well as losses of 1p (in particular 1p33-1p21), 3, 4, 5, and 13. No case presented focal chromosome 19q13.41 amplification. Gains were indicated by the bars (red) on the right side of each chromosome, and losses (blue) are shown on the left side. Thicker bars indicate areas of recurrent change.
the time period between date of diagnosis to date of first progression, relapse, death from any cause, or last contact; For statistical analysis, pairwise comparisons were made using Fisher’ exact test (for categorical variables) and t tests (for continuous variables). Survival analysis was performed using the Kaplan–Meier method and log-rank test. Two-tailed P values were reported and were considered significant when < .05. The study was not powered for the multitude of statistical tests we performed; analyses should be understood as exploratory. Multivariable analysis Cox regression models were not assessable due to the low number of patients and events/deaths, respectively. Statistical analyses were performed using SAS 9.3 (SAS).

Results

The study included 15 male and 10 female patients with a median age at diagnosis of 5.35 years (range, 2.41–17.28 years). Presenting symptoms included headaches, seizures, nausea/vomiting, cranial nerve palsies, somnolence, weakness, and sensory abnormalities. No patient presented clinical features or familiar history suspicious for Li-Fraumeni syndrome or other genetic tumor-predisposing syndromes. The tumors arose in the cerebral hemispheres and in some cases involved multiple lobes, the basal ganglia, or the thalamus. Detailed neuroradiological data were available for 19 cases. Most tumors (8/19; 42%) were localized in the frontal lobe, while 6 cases affected the temporal or parietal lobe. One case affected the third ventricle region. The large majority of cases presented in homogeneous contrast enhancement (11/19; 57%) and did not appear to be sharply demarcated from the surrounding brain tissue. Intratumoral cysts were also observed frequently.

The median follow-up of surviving patients was 3.7 years (range, 0.66–11.43 years). Residual tumor was present in 15 patients, and 2 patients had metastatic disease. Among the clinical parameters examined, only the presence of metastatic disease was significantly associated with unfavorable OS (P = .011).

Histologically, all tumors were composed of undifferentiated cells with hyperchromatic nuclei (Fig. 1A) showing no definitive evidence of glial differentiation. The tumor cells did not show expression of glial markers (such GFAP) and presented variable expression of synaptophysin and constant and diffuse positivity of MAP-2. All cases were LIN28A negative (Fig. 1A). Strong expression of synaptophysin and constant and diffuse positivity of MAP-2 were observed in 11/19 (57%) and did not appear to be sharply demarcated from the surrounding brain tissue. Intratumoral cysts were also observed frequently.

In order to define chromosomal alterations in the tumors of this series, we performed molecular analysis using MLPA on the whole cohort of patients. MLPA validated the results of MIP analysis and confirmed frequent losses of chromosomes 3p and 5q as well as gains of chromosomes 1q, 2p, and 7p (Fig. 3A) in the tumors. The analysis confirmed the presence of 4q21 amplifications and the absence of EGFR amplifications. Three cases showed heterozygous loss of CDKN2A. In addition to the 4 cases already identified in the MIP analysis, we found 2 additional cases harboring MYCN amplification (Fig. 3B). One tumor showed gain at MYCC locus, but no amplification. The fluorescence in situ hybridization (FISH) analysis was informative in 19 of the 25 cases investigated and revealed 4 cases (Fig. 3A) harboring MYCN amplification. The FISH results were concordant with MLPA data in all informative cases.

Kaplan–Meier analysis revealed that patients with tumors harboring 2p gain had a less favorable OS (P = .003) compared with the remaining patients. Moreover, the patients with tumors presenting MYCN amplification revealed a poor OS (P = .001) (Fig. 3D). None of these parameters was significantly associated with shorter EFS. A trend toward significance was observed for MYCN amplification (Fig. 3C) (P = .062).

Discussion

Clinical and biological parameters have rarely been evaluated as prognostic markers in patients with supratentorial CNS-PNETs, which were often analyzed together with medulloblastoma series in the past. Their molecular heterogeneity and absence of recurrent alterations have further hindered the identification of possible molecular markers suitable for clinical risk stratification and outcome prediction. Only a few recent studies attempted to integrate clinical and molecular markers in order to identify specific prognostic subgroups. Along with these experiences, we investigated the potential predictive value of molecular markers in supratentorial CNS-PNET patients enrolled in the GPOH HIT clinical study. This study is, to our knowledge, the first to investigate a homogeneously treated series of supratentorial CNS-PNETs. EPBL patients were excluded (Supplementary Fig. 1) because they now represent a distinct molecularly defined subgroup of CNS-PNET that is characterized clinically by worse prognosis and poor response to therapy.

While extent of surgery, age at diagnosis, and residual tumor had no prognostic relevance in our cohort of supratentorial CNS-PNET patients, we found that patients with metastatic disease indeed had an unfavorable OS (P = .011). These data are consistent with previous reports indicating that metastases at diagnosis are an independent adverse prognostic factor for OS and EFS. In our series, OS was significantly shorter in patients with metastatic disease (P = .001). Moreover, strutural and biological parameters have rarely been evaluated as prognostic markers in patients with supratentorial CNS-PNETs, which were often analyzed together with medulloblastoma series in the past. Their molecular heterogeneity and absence of recurrent alterations have further hindered the identification of possible molecular markers suitable for clinical risk stratification and outcome prediction. Only a few recent studies attempted to integrate clinical and molecular markers in order to identify specific prognostic subgroups. Along with these experiences, we investigated the potential predictive value of molecular markers in supratentorial CNS-PNET patients enrolled in the GPOH HIT clinical study. This study is, to our knowledge, the first to investigate a homogeneously treated series of supratentorial CNS-PNETs. EPBL patients were excluded (Supplementary Fig. 1) because they now represent a distinct molecularly defined subgroup of CNS-PNET that is characterized clinically by worse prognosis and poor response to therapy.
similar to those observed in MB patients, where the M+ status is a main clinical adverse prognostic factor and the benchmark for all risk stratification and therapy. Recently, a molecularly homogeneous subgroup of supratentorial CNS-PNET (defined as “oligoneural” group), which showed a low incidence of metastases clinically, has been identified and characterized by an increased expression of OLIG-2, a transcription factor involved in early development of the CNS. Due to the small number of cases, we could not demonstrate significant correlations between OLIG-2 expression and the M+ status of our patients, but OLIG-2 expression was not statistically associated to OS or EFS. Also p53, whose increased expression has been associated with metastatic tumor potential and worst prognosis in MB, was statistically not associated with shorter OS or EFS in patients with supratentorial CNS-PNETs.

As M+ status, 2p gain was also found to be associated to a shorter OS. This alteration, as revealed previously, is not uncommon in supratentorial CNS-PNETs. Interestingly, 2p gain seems to be one of the most frequent aberrations maintained in primary and recurrent CNS-PNET pairs. While poorer OS in patients with tumor with 2p gain has been found to be associated to concomitant presence of chr.19q13.41 amplification in other studies, we did not include EPBL/ETANTR cases in our

Fig. 2. High copy gains at 4q12 and at 2p24.3 (MYCN locus) observed in the molecular inversion probe analysis. The plots represent (A) the 2 supratentorial CNS-PNET cases with 4q12 amplification and (B) 2 representative cases (of 4 identified) with high copy gain at 2p24.3 (MYCN locus).
Fig. 3. Multiplex ligation-dependent probe amplification (MLPA) analysis and Kaplan–Meier analysis according to MYCN status in 25 patients with supratentorial CNS-PNETs. (A) Intensity plot shows the results of MLPA (SALSA MLPA p302 and p303) analysis, which further revealed the frequent occurrence of chromosomal gains and losses (gains depicted in orange, losses in blue, amplifications/high-copy gains in red and unchanged region in white; on the x-axis, patients number; on the y-axis, chromosomal regions. (B) Representative electropherograms of MLPA analysis of a tumor showing MYCN amplification (SALSA MLPA p302-A1, MYCN probe 033327-L02466, length 436 bp. Normal cerebellar tissue was used as control). (C) In regard to event-free survival, the Kaplan–Meier analysis showed a clear trend to shorter survival, but significance was not reached ($P = .062$). (D) On the other hand, the Kaplan–Meier analysis revealed that patients with tumors harboring MYCN amplification showed shorter overall survival ($P = .001$).
study. The association between the presence of 2p gain and unfavorable OS appeared to be related to the presence of MYCN amplification.

MYCN is a member of the MYC family of proto-oncogenes and encodes a 60–63 kDa protein. MYC transcription factors (MYCC, MYCN, and MYCL) have a conserved structure that includes a transcriptional activation domain in the N-terminus and a C-terminus basic helix-loop-helix leucine zipper (HLH-zip) domain that plays a role in protein dimerization, sequence-specific DNA binding, and regulation of transcription.31

Normal MYCN expression is restricted to the central and peripheral nervous systems, kidneys, lungs, and spleen during embryonic development. MYCN is a transcription factor that controls expression of many target genes implicated in basic cellular functions including proliferation, cell growth, protein synthesis, metabolism, apoptosis, and differentiation. While genetically engineered mouse models harboring amplification of MYCN develop peripheral neuroblastoma,32 recent evidences also show that orthotopic transplantation of MYCN-transduced neural stem cells generates different tumor subtypes in which their histology is dependent on the site of origin of precursor cells.33

In addition to peripheral neuroblastoma, where amplification is described in 40% of cases, MYCN and MYCC amplifications have been reported in supratentorial CNS-PNETs5,8,15 and MB.34,35 While amplifications of MYCC (8q24) and MYCN (2p24.3) have been detected in 5%–10% of MB, often with large cell/anaplastic features, and are associated with poor prognosis,34,35 the number of cases of CNS-PNET showing MYCN amplifications have varied largely among different studies (ranging from 5% to 50%) and have not been described to date as being clinically associated with shorter OS.5,8,15 However, we found a significant association between shorter OS and MYCN amplification. Patients with MYCN-amplified tumors appear to have slightly less favorable EFS. The presence of MYCN amplification seems to be unrelated to the age of the patient. Notably, 4 cases with MYCN amplification were localized in the temporal or parietal lobes, while only one case arose in the frontal lobe. On the other hand, we did not find MYCC amplification in tumors in our study, which seems to be rare among supratentorial CNS-PNETs8: the single patient showing amplification in tumors in our study, which seems to be rare among supratentorial CNS-PNETs.

Because MYCN is already one of the most important prognostic parameters in the existing panels of molecular MB markers and it can be routinely evaluated by quantitative PCR, FISH, or MLPA-based approaches on FFPE or frozen material, routine evaluation of its status could be also performed for supratentorial CNS-PNET, but some alterations (such as gains at 1p, 2q, and 21q as well as losses at 6q, 4q, 11q, and 16q) have been described for both tumor entities.36,37 To further support this evidence, we found two supratentorial CNS-PNET cases with amplification of the 4q12 region harboring, among others, PDGFRα, PDGFRα amplification is, by far, the most common genomic event identified in pediatric glioblastomas.36,37 On the other hand, MYCN amplifications have also been observed in pediatric glioblastomas.36 In this regard, further studies are needed to definitively explore the possible molecular relationship and overlapping features between these two tumor entities. From a diagnostic point of view, supratentorial CNS-PNETs may also be difficult to distinguish from diffuse high-grade gliomas, and often represent an “exclusion-diagnosis” based on the identification of a supratentorial embryonal neoplasm with medulloblastoma-like histology that is frequently associated with expression of neuronal markers.1 However, definitive histological distinction between a supratentorial CNS-PNET and a glioblastoma sometimes remains impossible; in such cases, the final diagnosis depends on the expertise of the neuropathologist.

In conclusion, the identification of MYCN amplification as a molecular marker associated with patient outcome may represent a further significant step towards the risk stratification of patients suffering from supratentorial CNS-PNET.

Supplementary Material
Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

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