INTRODUCTION

Medulloblastoma is a highly malignant embryonal neuroepithelial tumor of the cerebellum with a tendency to metastasize within the central nervous system. For clinical average risk patients, survival rates of 70–80% after craniospinal radiotherapy and chemotherapy have been reported, but lower rates were obtained if clinical risk factors such as young age or metastases were present [1–4]. Efforts have been made to identify histopathological factors for improved stratification of patients to risk-adapted treatment strategies. It has been shown that the presence of desmoplastic or extensive nodular histology of medulloblastoma is a strong predictor for low risk disease in early childhood [5]. On the other hand, a large cell variant of medulloblastoma with highly aggressive behavior has been described, and a close relation of the large cell variant and anaplasia was identified [6,7]. The large cell/anaplastic (LC/A) medulloblastoma phenotype has been associated with higher clinical risk factors as metastatic disease and inferior survival rates [7–11]. However, different definitions for anaplastic and large cell medulloblastoma variants have complicated comparisons of frequencies and clinical results of these variants in the past. Although the histological features of large cell medulloblastoma are distinct from the other variants, some cases show overlapping features with anaplastic medulloblastoma. For these reasons, anaplastic medulloblastoma and large cell medulloblastoma have recently been defined as two distinct entities in the 2007 WHO classification of CNS tumors [12]. On a molecular level, amplification of the protooncogene c-myc has been described as a negative prognostic marker for medulloblastoma [11,13–16]. Amplification as well as expression of c-myc have been associated with LC/A histology [10], and causative role in c-myc gain for anaplastic histological features, aggressive tumor behavior, and metastatic spread has been indicated [17]. Consequently, intensified treatment regimens are currently considered for patients with anaplastic and/or large cell histology. Most previous studies have identified relevant subsets of children with LC/A medulloblastoma and a normal c-myc status, but the prognosis of these children in relation to their clinical risk factors has not been reported in detail.

To assess the significance of histological subtype and biological markers on the outcome of patients with LC/A medulloblastoma, we analyzed clinical data from all patients who had a diagnosis of LC/A medulloblastoma by central histopathological review, and who were registered to one of three consecutive prospective multicenter HIT-trials of the German Society of Pediatric Oncology and Hematology (GPOH). Available tumor samples were analyzed for the presence or absence of c-myc and n-myc amplification, and mRNA expression of c-myc. Additionally mRNA expression of trkC, a favorable prognostic factor in medulloblastoma [14,18–20], and immunohistochemical staining of Ki-67 (MIB1), a proliferation marker, were analyzed.

MATERIALS AND METHODS

Patients

Children and young adolescents with medulloblastoma were treated within three consecutive prospective, multicenter trials as...
Central histopathological review was recommended in all trials. For the trials HIT 91 and SKK 92 histopathology was centrally re-evaluated recently for 175 patients [5,14]. Within the HIT 2000 trial, central histopathological review was done in 396 patients with medulloblastoma until February 2006, when the last patient with LC/A medulloblastoma was included into this analysis. From all studies, 28 patients with centrally confirmed LC/A medulloblastoma were identified.

Staging within the studies was done according to Chang criteria [23]. Evaluation of cerebrospinal fluid (CSF) for microscopic metastases was recommended for all patients. In two patients without clinical or radiological signs for dissemination at presentation, no result of initial CSF-analysis was reported, and both have been classified as M0 stage for further analyses. Twenty patients had central review of CSF cytology. Residual tumor was measured on the postoperative MRIs and classified as none, smaller than 1.5 cm³, or larger than 1.5 cm³. Twenty-two patients had central review of the initial MRI imaging.

Treatment was performed within the above-mentioned trials as described [5,21,22]. There were five different treatment strategies applied: infant: systemic chemotherapy with intraventricular methotrexate, administration of radiotherapy in case of residual tumor; infant intensified: systemic chemotherapy with intraventricular methotrexate followed by high dose chemotherapy, administration of radiotherapy in case of residual tumor; sandwich: systemic chemotherapy followed by radiotherapy; sandwich intensified: additional intraventricular methotrexate and intensified radiotherapy; maintenance: postoperative radiotherapy followed by maintenance chemotherapy.

### Histopathological Reassessment

The histological diagnosis of large cell or anaplastic medulloblastoma was confirmed by central review in all patients essentially based on the World Health Organisation classification of brain tumors 2000 [24]. Large cell component was graded as absent, single cells, focal, significant (<50% of tumor area) or predominant (≥50%). Cytological features of cytological anaplasia were increased nuclear size, marked nuclear pleomorphism, increased mitotic and/or apoptotic rate; severe anaplasia was graded as absent, focal or diffuse. Tumors were considered as LC/A medulloblastoma, and were included in this study, if they either displayed severe anaplasia or a significant or predominant large cell component. As there is a considerable overlap of large cell and anaplastic features, the term LC/A was chosen to avoid an arbitrary designation of individual cases to either subgroup as defined by the WHO 2007 [12], and to allow comparability with previously published series.

#### c-myc and n-myc DNA Amplification

DNA from 28 paraffin-embedded tumor samples was extracted with the QIAamp DNA mini kit (Qiagen). Five normal blood DNA samples were included as a control group. c-myc and n-myc gene copy numbers were analyzed in a competitive PCR approach essentially as described before using APRT as reference gene [14]. The threshold for gene amplification was based on the analysis of the group of normal control samples and was set as the respective mean value of this set of samples plus two standard deviations.

### mRNA Expression of c-myc and trkC

Isolation of total RNA from formalin-fixed, paraffin-embedded tumor tissue and real-time quantitative RT-PCR for analyses of c-myc and trkC mRNA have been performed from 20 samples with sufficient tumor material available as described previously [14,25]. The Optimum FFPE, Ambion Diagnostics for paraffin block RNA isolation kit (Ambion; Huntington, UK) was used to isolate RNA from formalin-fixed, paraffin-embedded tumor tissue. cDNA synthesis and kinetic real-time PCR quantification of c-myc and trkC mRNA was performed as described previously [14,25]. The amount of c-myc and trkC mRNA, normalized to the endogenous control 18S rRNA, was related to c-myc and trkC mRNA levels of normal human cerebellum (Becton Dickinson; Allschwil, Switzerland).

#### Statistical Analyses

Event-free survival (EFS) and overall survival (OS) were estimated by Kaplan–Meier analysis, and standard errors are given as ± values. The log-rank test was used for comparisons. Continuous variables were categorized for analysis. Age cut-off for categorization was chosen at 4 years due to clinical relevance. Categorization for c-myc mRNA expression was chosen ≤/≥1 as comparison with controls of normal human cerebellar tissue. Disease progression, recurrent disease and death of any cause were considered as events, and EFS was measured from the time of diagnosis to the time of the event or last follow-up, respectively. Associations of categorical clinical and molecular variables were analyzed by Chi-square test. Continuous nonparametric data were compared using the Mann–Whitney U-test. All P-values are considered as explorative, no significance level is fixed. All measurements were done by SPSS Software 15.0 (SPSS Inc., Chicago, Illinois).

### RESULTS

#### Patient Characteristics

Overall, 28 of 571 (5%) medulloblastoma specimens were classified as LC/A medulloblastoma by central histopathologic review. Median follow-up of the surviving patients was 4.5 years (1.2–11.5 years). Clinical and histological characteristics as well as information on therapy and outcome are shown in Table I.

#### Outcome According to Clinical Parameters

Four-year EFS and OS of all patients with LC/A medulloblastoma were 58% ± 10% and 67% ± 9% respectively (Fig. 1).
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<th>Sex</th>
<th>M stage</th>
<th>Residual tumor</th>
<th>Anaplasia</th>
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<td>Relapse</td>
<td>0.2</td>
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n.e., not evaluated due to lack of tumor tissue; n.i., not informative; NED, no evidence of disease; AWD, alive with disease; DOD, dead of disease; A, moderately anaplastic; SA, severely anaplastic.

Therapy regimen: see Materials and Methods Section. aThis patient had subsequent radiotherapy followed by high dose chemotherapy due to persisting vital tumor after radiotherapy; bThis patient had an intensified induction chemotherapy.
Four-year EFS of 10 patients below 4 years of age was 30% ± 15% compared to 73% ± 12% for 18 older patients (P = 0.01). Four-year EFS for 9 patients with metastases was 33% ± 16% compared to 70% ± 12% for 19 patients with M0 stage (P = 0.02). Younger children were more often diagnosed with metastatic disease: 6 of 10 patients younger versus 3 of 18 patients older than 4 years of age (P = 0.02).

Rates for 4-year EFS and OS of 15 patients without metastases (M0) older than 4 years were 82% ± 12% and 93% ± 6%, respectively. In contrast, 4-year survival rates of 13 patients who were younger than 4 years or who had metastatic disease were lower (EFS and OS 31% ± 13%; P = 0.001, Fig. 2).

Presence of postoperative residual tumor had no impact on prognosis in the complete group as well as in the subgroup of M0 patients. Four-year EFS was 86% ± 13% in seven M0 patients with postoperative residual tumor, and 63% ± 16% in 12 patients with complete tumor resection (P = 0.446).

**Histology**

Severe anaplasia was graded as focal (n = 4) or diffuse (n = 18). Large cell component was graded as single (n = 1), focal (n = 8), significant (n = 3), and predominant (n = 6). Severe anaplasia without a predominant or significant large cell component was present in specimen of 19 patients. Four-year EFS of these patients was 75% ± 12%. Event-free survival of 9 patients with presence of a significant or predominant large cell component was lower (4-year EFS 22% ± 14%, P = 0.005, Fig. 3). Three of these patients showed severe anaplasia as well as a significant or predominant large cell component (4-year EFS 0%). Solely a significant or predominant large cell component without presence of severe anaplasia was shown in 6 patients (4-year EFS 33% ± 19%).

MIB1 scores were between 30% and 70% in 22 patients. Two patients with low MIB1 scores (less than 30%) had no event, and three of four patients with high MIB1 scores (>70%) had tumor progression or relapse. Four-year EFS rates for patients with MIB1 ≤30%, 30–70%, and ≥70% were 100%, 61% ± 11%, and 25% ± 22%, respectively (P = 0.07).
Amplification of c-myc and n-myc Genes

Material for analysis of c-myc amplification was available for tumors of 24 patients. Nine patients with c-myc amplification positive tumors had a worse outcome compared to 15 patients whose tumors showed no c-myc amplification (4-year EFS 22% ± 14% and 89% ± 11%, P < 0.0001, Fig. 4).

Patients with c-myc amplification positive tumors were more likely to have metastases: Six of 9 patients with c-myc amplification positive tumors had metastases versus 1 of 15 with c-myc negative tumors (Chi-square \( P = 0.002 \), Table II). Patients with c-myc amplification positive tumors were also younger: Seven of 9 patients with c-myc amplification positive tumors were younger than 4 years of age at diagnosis versus 1 of 15 patients whose tumors showed no c-myc amplification (Chi-square \( P < 0.001 \), Table II). Presence of c-myc amplification was strongly correlated to large cell histology. A large cell component was present in all 9 tumors with c-myc amplification, and was significant or predominant in 5 of them, and all 5 children with this combination had an early relapse or progression. In contrast, within the group of c-myc amplification negative tumors only one of 15 showed a significant or predominant large cell component (Chi-square \( P = 0.007 \), Table II). The patient with this tumor had no clinical risk factors and is alive without disease 4 years after diagnosis.

n-myc amplification was positive in tumors of 2 of 21 analyzed patients. Seven patients had insufficient material for analysis. Both patients with n-myc amplification positive tumors were older than 4 years at diagnosis, had no metastases, and had no event with a follow-up of 1.2 and 7.8 years.

mRNA Expression of c-myc and trkC

c-myc mRNA expression was evaluated in tumors of 20 patients. For 7 patients there was not enough material available for analysis, and in one patient the endogenous control was not reliably measurable. Compared to normal cerebellum, relative c-myc mRNA expression was elevated in 10 of 20 patients (3.7- to 145-fold increase, median 66). The level of c-myc mRNA expression did not correlate with c-myc DNA amplification (Mann–Whitney U-test: \( P = 0.902 \)). Prognosis of patients with increased c-myc mRNA (>1) expression was not worse than for patients without elevated c-myc mRNA expression (4-year EFS 60% ± 15 vs. 67% ± 16, \( P = 0.5 \)). Patients with clinical risk factors tended to have higher c-myc mRNA expression levels (Chi-square \( P = 0.07 \)). In all 20 samples, trkC mRNA expression was lower than the values measured in healthy cerebellum (controls).

DISCUSSION

Several studies have shown an impaired prognosis of patients with LC/A medulloblastoma compared to children with classic medulloblastoma or other medulloblastoma variants [6,7,9,11,26]. However, given the low incidence of LC/A medulloblastoma, only limited numbers of patients with this medulloblastoma variant have been described in the literature. Our results newly indicate that a subset of patients with anaplastic medulloblastoma may be defined by clinical, histological and molecular markers, who may not have a worse prognosis compared to children with standard risk, classic medulloblastoma.

After screening of 571 patients with medulloblastoma who were enrolled in three consecutive trials and had a central histopathological review, we identified 28 patients (5%) with LC/A medulloblastoma. Rates for 4-year EFS and OS were 58% and 67%, respectively. Considering the fact that this group includes very young patients as well as patients with metastases, the outcome is not significantly different from children with classic medulloblastoma [2,3,5,21]. Other studies of LC/A medulloblastoma reported higher frequencies of metastases [7–9], and a trend for younger age [26], but patients without clinical risk factors were not analyzed separately. Our results for children with LC/A medulloblastoma who presented with clinical risk factors confirm the poor survival rates which have been reported (4-year EFS 31% for children with metastases or young age). In contrast, our study shows that patients with LC/A medulloblastoma can have favorable survival rates, if clinical risk factors as metastases and young age are absent: The survival rates of 15 patients older than 4 years at diagnosis with localized disease (4-year EFS 82%; 4-year OS 93%) were in the range of classic medulloblastoma patients without clinical risk factors [1,4,27]. Interestingly, these survival rates were obtained without intensifying treatment, as LC/A histology was not used for

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**TABLE II. Characteristics of 24 Analyzed Patients According to c-myc Amplification**

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<th></th>
<th>c-myc +</th>
<th>c-myc−</th>
<th>Total</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastases</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Localized disease</td>
<td>3</td>
<td>14</td>
<td>17</td>
<td>0.002</td>
</tr>
<tr>
<td>Age &lt;4 years</td>
<td>7</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Age &gt;4 years</td>
<td>2</td>
<td>14</td>
<td>16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Significant/predominant LC comp.</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Focal/no LC comp.</td>
<td>4</td>
<td>14</td>
<td>18</td>
<td>0.007</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>15</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

LC comp., large cell component.

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**Fig. 4.** EFS according to c-myc amplification. Patients with c-myc amplification had a worse EFS (22 ± 14%) compared to patients without c-myc amplification (89 ± 11%, \( P < 0.0001 \)).

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treatment stratification in the respective trials. Similarly, in a cohort of clinically average risk medulloblastoma patients anaplastic histology had no influence on EFS in a study by Packer et al. [4].

Since in our study the presence or absence of postoperative residual tumor had no influence on outcome, the extent of resection was not considered as a clinical risk factor in our analyses.

Historically, large cell and anaplastic variants of medulloblastoma have often been subsumed, because both variants have been related to unfavorable outcome, and because some tumors have overlapping histological features. In the past, the extent and grade of cytological anaplasia and large cell areas that qualify for the diagnosis of anaplastic or large cell medulloblastoma have not been exactly defined. This explains the broad range of frequencies of LC/A medulloblastoma (between 4% and 21% of medulloblastoma) reported in the literature [7–9]. Studies which described a high frequency of LC/A had assigned also less than severely anaplastic cases to this subgroup [28]. Based on the recent identification of severe anaplasia only as prognostically relevant [10], slightly or moderately anaplastic cases and tumors with only a minor large cell component were excluded in our analysis. This explains the relatively low frequency of 5% large cell/anaplastic tumors in our series.

While diagnosis was based on the 2000 WHO classification, we analyzed and described the extent of the anaplastic and large cell component separately for better comparability with previously published series. Recently, anaplastic medulloblastoma and large-cell medulloblastoma have been defined as separate entities in the current 2007 WHO classification of CNS tumors [12].

In our study the presence of a significant or predominant large cell component was found in nine samples, and was identified as an adverse prognostic factor. McManamy et al. [26] also described an inferior prognosis of patients with large cell compared to anaplastic medulloblastoma.

Two large studies showed a negative prognostic relevance of increasing anaplasia in medulloblastoma [28,29], while there is no information on the frequency and overlap with large cell histology given. The impact of the extent of anaplasia on outcome cannot be judged within our study, since patients were only included in our cohort if the tumors showed severely anaplasia or a significant or predominant large cell component.

According to current understanding, the pathogenesis of classic and LC/A phenotype differs from the nodular desmoplastic variants, which have been shown to have a more favorable prognosis in early childhood: Loss of heterozygosity (LOH)17p, isochromosome 17q, and c-myc amplification are predominantly described in association to the former variants, while chromosome 9q loss and activation of the sonic hedgehog (SHH) pathway are related with the latter [30,31].

The frequency of c-myc amplification has been described as about 5% of medulloblastoma in mixed cohorts [11,15]. Much higher proportions of c-myc amplification have been reported in children with LC/A medulloblastomas [7,28]. Our analysis supports this finding, as a c-myc amplification was detected in 9 of 24 children (38%). In our series, the presence of c-myc amplification was highly associated with an adverse clinical outcome among children with LC/A medulloblastoma: LC/A medulloblastoma patients with c-myc amplification had a 4-year EFS of 22% compared to 89% for LC/A medulloblastoma patients without c-myc amplification (P < 0.0001). In addition to the negative impact of c-myc amplification on survival shown by others [13,15], our data indicate that c-myc amplification is strongly connected to large-cell histology, and to the presence of adverse clinical risk factors such as young age and metastases. These clinical and histological characteristics may be the correlates of the more aggressive tumor biology induced by amplification of c-myc. Due to the limited number of patients, a multivariable analysis was not appropriate in our series. The analysis of risk factors is further limited by the wide age range and different treatment of patients.

C-myc mRNA expression was elevated in 10 of 20 patients in the present study. As previously described in another series [19], there was no clear correlation between the level of c-myc mRNA expression and c-myc amplification. The prognostic relevance of c-myc mRNA expression has been described as a single marker [16,19], and in combination with other parameters [14]. A correlation of c-myc RNA expression and anaplasia was also described [10,17]. The fact that c-myc RNA expression was not a significant prognostic factor in our analysis might be explained by the preselection of patients. Since the sample was limited to medulloblastoma patients whose tumors showed either significant anaplasia or a significant/predominant large cell component.

N-myc amplification was present in 2 of 21 patients in our study: Both patients had no adverse clinical risk factors, no significant large cell component, and remained without evidence of disease 1 and 7 years after diagnosis, respectively. In a study of Eberhart et al., n-myc amplification was detected in 5 of 18 LC/A medulloblastoma patients. Three of 5 were alive at the last follow-up, while 4 of 4 patients with c-myc amplification died [32]. The same group also analyzed the influence of n-myc expression. Three of four patients with high levels of n-myc mRNA expression (two of them with anaplastic histology) were long-term survivors [10]. As n-myc also represents a downstream target of the SHH pathway and as its expression was found to be associated with the desmoplastic medulloblastoma variant [17,31], it is unlikely to represent a specific marker for high risk disease.

TrkC mRNA expression has been shown to be associated with a favorable prognosis in medulloblastoma patients [10,14,19,33]. As in our cohort all tumors showed histological features of higher risk disease, it is not surprising that an increased trkC expression was detected in none of the 20 analyzed medulloblastoma samples.

In conclusion, our study shows that prognosis of patients with LC/A medulloblastoma may not be unfavorable in all cases. C-myc amplification and predominant large-cell histology were confirmed to be negative prognostic parameters. Both parameters were frequently associated and related to established clinical adverse risk factors. Consequently, intensified treatment strategies should be considered for patients displaying these criteria. In contrast, survival rates of patients with anaplastic medulloblastoma without c-myc amplification or predominant large cell component were not impaired. Therefore, it remains to be clarified whether a treatment intensification solely based on the histological finding of severe anaplasia in absence of molecular or clinical risk factors is justified.

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