Combined Histopathological and Molecular Cytogenetic Stratification of Medulloblastoma Patients

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ABSTRACT
This study examined the utility of stratifying children with medulloblastomas by a combination of refined histopathological classification and molecular cytogenetic evaluation.

Detailed histopathological classification of tumors from a cohort of patients (n = 87) composed mainly of children entered into the International Society of Pediatric Oncology (SIOP)/United Kingdom Children’s Cancer Study Group PNET3 trial (n = 65), included identification of the large cell/anaplastic phenotype. Fluorescence in situ hybridization was used to detect chromosome 17 abnormalities, losses of 9q22 and 10q24, and amplification of the MYCC and MYCN oncogenes.

The large cell/anaplastic phenotype, which was present in 20% of medulloblastomas, emerged as an independent prognostic indicator. Loss of 17p13.3 (38% of medulloblastomas) was found across all of the histopathological variants, whereas MYCC/MYCN amplification (6%/8% of medulloblastomas) was significantly associated with the large cell/anaplastic phenotype. Both of these genetic abnormalities emerged as prognostic indicators. Loss of 9q22 was associated with the nodular/desmoplastic medulloblastoma variant, whereas loss of 10q24 was found in all of the variants. Together with metastatic tumor at presentation, the large cell/anaplastic phenotype, 17p13.3 loss, or high-frequency MYC amplification defined a high-risk group of children whose outcome was significantly (P = 0.0002) poorer than a low-risk group without these tumor characteristics.

INTRODUCTION
Medulloblastoma is an embryonal neuroepithelial tumor of the cerebellum and the most common malignant central nervous system tumor in children. Modern advances in treatment regimens have improved 5-year survival rates to ~70% for standard-risk patients (1–4), but survival rates for high-risk patients are as low as 25% (5, 6). This clinical dichotomy, in which metastatic disease at presentation, age <3 years, or residual tumor >1.5 cm³ on magnetic resonance imaging categorize patients as high-risk, is the only stratification of medulloblastoma patients currently undertaken for therapeutic purposes. However, differences in the biological behavior of tumors within these two groups clearly exist, and additional refinement of medulloblastoma classification would be beneficial, helping to reduce long-term cognitive and endocrine side effects, whereas optimizing treatment for children with aggressive disease.

The current World Health Organization classification of nervous system tumors divides medulloblastomas into two broad categories: nondesmoplastic and desmoplastic (7). Nondesmoplastic medulloblastomas are more frequent (~85% of medulloblastomas) and are additionally classified as classic, large cell (~4% of all medulloblastomas), and the rare medulomyoblastoma and melanotic medulloblastoma (~1% of all medulloblastomas). In addition, recent histopathological studies have identified an anaplastic phenotype among medulloblastomas, which is distinguished by conspicuous nuclear pleomorphism and high mitotic and apoptotic indices (8–10). It is always present alongside the large cell morphophenotype in large cell medulloblastomas and characterizes a variable proportion of cells in classic medulloblastomas and occasionally in desmoplastic medulloblastomas (11, 12). Like large cell medulloblastomas, nondesmoplastic medulloblastomas with widespread anaplasia have a significantly poorer prognosis than other classic medulloblastomas (9, 10). Desmoplastic medulloblastomas (~15% of medulloblastomas) also show a range of histopathologic features, but typical examples demonstrate scattered nodules surrounded by desmoplastic interstitial regions. At one extreme is the medulloblastoma with extensive nodularity, which is associated both with young age and a favorable prognosis (13). Controversy surrounds whether the biological behavior of other desmoplastic medulloblastomas is distinct from that of classic medulloblastomas (12, 14–16).

To complement the emergence of pathological medulloblastoma variants with distinctive biological behaviors, several
molecular markers have been shown to correlate with a particular outcome or treatment response in patients with medulloblastoma. Thus, MYCC/MYCN amplification or overexpression (17–21), ErbB2 overexpression, and loss of chromosome 17p have been linked to aggressive biological behavior (22–24), whereas TrkC overexpression has been linked to a favorable outcome (25). However, no study has yet determined in a large number of tumors how molecular markers of medulloblastoma biology relate to recent developments in histopathological classification or how a combination of these might relate to outcome. For example, MYCC or MYCN amplification appears to occur more frequently in the large cell/anaplastic variant than in other types of medulloblastoma, yet it is unclear which characteristic might better reflect tumor behavior or response to therapy.

Detailed histopathological and molecular evaluation of medulloblastomas could supplement and refine the current clinical stratification of patients with these tumors, facilitating the objective of matching therapy to tumor biology. In principle, molecular markers that are strongly associated with particular histopathological variants will help to promote diagnostic accuracy, whereas those that are distributed across histopathological variants would allow subclassification. The aim of this study was to gather data with which to inform this paradigm. We first examined relationships between histopathological variation in medulloblastomas and the following genetic defects: chromosome 17 abnormalities and MYCC/MYCN (MYC) amplification, which have been linked previously to the large cell/anaplastic variant (8, 26–28), and loss of 9q22 or 10q24, loci for the PTCH and SUFU tumor suppressor genes, respectively, mutations of which sonic hedgehog pathway components have been demonstrated in medulloblastoma, particularly the desmoplastic variant in the case of PTCH mutations (29–32). We then examined the interplay between histopathological variation and genetic abnormalities with respect to biological behavior in a series of medulloblastomas from children entered into a pan-European therapeutic trial.

MATERIALS AND METHODS

Clinicopathologic Details. Formalin-fixed, paraffin wax-embedded tumors from 87 patients were provided for this study by several International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group centers. Most medulloblastomas (n = 65) were from patients randomized into the International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 trial of “sandwich” chemotherapy followed by radiotherapy versus radiotherapy alone (33). Other medulloblastomas (n = 22) were from patients treated either concurrently or just before the time course of this trial (1992–2003) and by combination radiotherapy/chemotherapy. All of the PNET3 trial patients in the present study formed part of the larger cohort of children whose tumors were analyzed in our study of the prognostic significance of morphophenotypic variation in medulloblastomas (10). Clinical data for all of the International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group patients, including outcome, were available from the United Kingdom Children’s Cancer Study Group data center or for the remaining patients from databases in two United Kingdom Children’s Cancer Study Group centers. Median age at diagnosis was 8.3 years, and there was a preponderance of males (1.6:1). Overall, a greater proportion of patients (60%) had received radiotherapy/chemotherapy than radiotherapy alone. Metastatic disease (all Chang stage M3) was revealed at presentation in 11 patients. Median follow-up for surviving patients was 5.3 years.

Using published criteria, an International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group central review neuropathologist (D. W. E.) first classified the medulloblastomas as classic, desmoplastic, or large cell/anaplastic variants. Medulloblastomas were classified as large cell variants if groups of large cells with round nuclei and a single nucleolus were encountered and as anaplastic if groups of cells with conspicuous nuclear pleomorphism, a high nuclear:cytoplasmic ratio, nuclear molding, and plentiful mitoses and apoptotic bodies were widespread in the tumor (Fig. 1). A nodular architecture and/or desmoplasia unrelated to invasion of the leptomeninges by tumor cells defined a group of desmoplastic medulloblastomas. Other medulloblastomas were labeled classic, a small focus of anaplasia or a mild degree of nuclear pleomorphism being regarded as insufficient for a diagnosis of anaplastic medulloblastoma.

On the basis that classic medulloblastomas demonstrate degrees of cytologic pleomorphism and may show morphological signs of differentiation, these tumors were additionally divided into two cytologically distinct types, termed small cell and intermediate cell (Fig. 1). The former term is proposed for medulloblastomas characterized by sheets of small, round uniform tumor nuclei set in a fibrillary background. Focal architectural features regarded as signs of neuronal differentiation, such as (Homer-Wright) nuclear rosettes or groups of ganglion cells, are most often encountered in this variant of medulloblastoma. Intermediate-cell classic medulloblastomas are characterized by sheets of cells with relatively uniform oval, elongated, or polyhedral tumor nuclei of medium size. These tumors show only mild nuclear pleomorphism, falling short of criteria for the anaplastic variant, yet are clearly distinct from “textbook” small-cell classic medulloblastomas. Small foci of convincing anaplasia were occasionally recorded in both small-cell and intermediate-cell tumors.

Conforming to the World Health Organization definition of desmoplastic medulloblastoma, six nodular/desmoplastic medulloblastomas were characterized by scattered nodules of neurocytic cells among desmoplastic regions containing larger and more pleomorphic nuclei. Additional desmoplastic medulloblastomas included two medulloblastomas with extensive nodularity, three “non-nodular” desmoplastic medulloblastomas characterized throughout by pericellular collagen without apparent reticulin-bound nodule formation, and two “paucinodular” medulloblastomas with scant desmoplasia and a few scattered nodules in which cells did not differ significantly from extranodular cells (Fig. 1).

Fluorescence In situ Hybridization. Specific probes for regions 2p24.3 (MYCN, bA355H10), 8q24.21 (MYCC, dJ968N11), 9q22 (bA172F4), 10q24.31 (bA310C11), 17p13.3 (bA356I18), and 17q12 (bA249G4) were generated from DNA isolated from pBACE3.6 (except dJ968N11, which was pCYPAC2) using the NucleoBond BAC100 DNA extraction kit.
Probes to the centromeric regions of chromosomes 2 (pBS4D), 8 (pZ8.4), 9 (pMR9A), 10 (pZ101.3), and 17 (D17Z1) were generated from DNA isolated from plasmids (Cytogenetics Unit, University of Bari, Bari, Italy) using the Qiagen Hi-speed DNA extraction kit (Qiagen, Sussex, United Kingdom), and used in two-color fluorescence in situ hybridization (FISH) to assess losses, gains, or amplification at loci of interest. Isolated DNAs were indirectly labeled with digoxigenin (arm) and biotin (centromere) using Vysis nick translation kit (Vysis, Richmond, United Kingdom).

Fig. 1 Classic medulloblastomas were divided into small-cell and intermediate-cell tumors. The former consists mainly of cells with round monomorphic nuclei (A, hematoxylin and eosin, original magnification, ×200), whereas the latter contains larger elongated cells with oval or polyhedral nuclei (B, hematoxylin and eosin, original magnification, ×100). Anaplastic medulloblastomas are characterized by molded cells with pleomorphic nuclei and many mitotic figures and apoptotic bodies (C, hematoxylin and eosin, original magnification, ×100). The large cell medulloblastoma contains groups of cells with round nuclei and a single nucleolus and other regions with an anaplastic phenotype (D, hematoxylin and eosin, original magnification, ×400). A typical desmoplastic medulloblastoma contains round nodules of varying size, in which cells have a lower nuclear:cytoplasmic ratio than cells in internodular regions (E, hematoxylin and eosin, original magnification, ×60). Nodules of varying shape dominate the medulloblastomas with extensive nodularity (F, hematoxylin and eosin, original magnification, ××40), whereas nodules are inconspicuous in some “pauci-nodular” desmoplastic medulloblastomas (G, hematoxylin and eosin, original magnification, ××60). A few desmoplastic medulloblastomas evince no nodules but are characterized by pericellular collagen (H, hematoxylin and eosin, original magnification, ××300).
For FISH on isolated nuclei, two to four sections (15–20 μm) were cut and prepared according to the protocol of Nicholson et al. (34), with some changes. After dewaxing in xylene, the tissue was resuspended in absolute ethanol and centrifuged for 5 min at 4000 × g (three times). This cycle was repeated with 50% ethanol and PBS. The final pellet was resuspended in 500 μl of 0.5% pepsin (Sigma, Dorset, United Kingdom) in 0.01 M HCl, disaggregated manually, and incubated at 37°C for 1–2 h. Tissue digestion was stopped with fetal calf serum. The tissue was finally resuspended in PBS and passed through a 70-μm mesh cell strainer (Fisher) at 250 × g. Isolated nuclei were resuspended in PBS and spun onto glass microscope slides. Slides were then stored at −20°C until required.

Before FISH, slides were thawed and dried at room temperature and then placed in distilled water (37°C) for 5 min. Slides were subjected to a second pepsin digestion step (4 mg/ml pepsin in 0.2 M HCl) at 37°C for 0–15 min. After digestion, slides were washed in prewarmed dH2O and PBS and dehydrated in graded alcohols. Probe mixtures to regions of interest and corresponding centromeres were preheated to 37°C dehydrated through a graded series of alcohols. After microwave hybridization was carried out on 7-μm tissue sections previously (34).

Chromogenic In situ Hybridization. Chromogenic in situ hybridization was carried out on 7-μm tissue sections baked at 60°C for 5 min. Sections were dewaxed in xylene and rehydrated through a graded series of alcohols. After microwaving in 0.01 M citrate buffer for 3 × 5 min, sections were cooled and washed in dH2O. Slides were treated with pepsin (0.5% in 0.01 M HCl) for 10 min, then washed in dH2O at 37°C for 3 min each before dehydration in alcohol. Hybridization and stringency washes were carried out according to the FISH protocol above. Antibody detection was carried out using the Zymed chromogenic in situ hybridization detection kit (Cambridge Bioscience UK, Cambridge, United Kingdom) according to the manufacturer’s protocol. Sections were counterstained with hematoxylin.

Analysis of Clinopathologic Variables. Clinical and study data were combined in several statistical analyses of overall or event-free survival. An event was defined as relapse or death from any cause. Statistical analysis involved the use of χ² tests and log-rank tests for comparisons of survival between prognostic groups and the Cox proportional hazards model for multivariate survival analysis.

RESULTS

Pathological Variants and Clinical Features

The series of tumors (n = 87) was composed of 74 non-dedifferentiated and 13 desmoplastic medulloblastomas. Of the nondesmoplastic medulloblastomas, 59 (80%) were classic, and 15 (20%) were large-cell/anaplastic variants. It was possible to divide 58 of the classic tumors into 39 (66%) small-cell and 19 (32%) intermediate-cell medulloblastomas. A single remaining idiosyncratic classic medulloblastoma demonstrated a biphasic architecture composed of two populations of tumor cells, without immunohistochemical or morphological signs of differentiation, which corresponded approximately to small-cell and intermediate-cell phenotypes. There were 10 diffusely anaplastic tumors among the nondesmoplastic medulloblastomas, and 5 large cell medulloblastomas, which all contained extensive regions with an anaplastic phenotype. Small foci of cells with an anaplastic phenotype were found in 18% of small-cell classic medulloblastomas and 47% of intermediate-cell classic medulloblastomas, a difference in frequencies that was statistically significant (P = 0.027).

The mean age of patients with classic medulloblastomas (9.4 years) was slightly higher than the mean age of those with large-cell/anaplastic medulloblastomas (8.3 years). All but one of the 11 patients who had metastatic disease at presentation (all Chang stage M3) have now died. Seven of these had classic medulloblastomas (4 intermediate-cell/3 small-cell), and 3 had large cell/anaplastic medulloblastomas (χ² test, P > 0.1). One non-nodular desmoplastic medulloblastoma, but none of the other desmoplastic medulloblastomas, was associated with metastatic disease. All but 1 patient with metastatic disease died within 2 years. The remaining patient, with a small-cell classic medulloblastoma, had an event-free survival of just over 5 years but has now relapsed.

Dividing nondesmoplastic medulloblastomas into classic and large-cell/anaplastic variants (Fig. 2A) produced significantly different overall survival curves (log rank test, P = 0.0005). Similar results were found when these pathological groups were compared for event-free survival (log rank test, P = 0.0007) and when patients from the International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 cohort alone (n = 60) were analyzed for outcome differences related to these pathological variants (log rank test, P = 0.0001). Overall survival curves were also significantly different (log rank test, P = 0.0002) when nondesmoplastic medulloblastomas were additionally classified into small-cell classic, intermediate-cell classic, anaplastic, and large cell variants (Fig. 2B).
Molecular Cytogenetic Abnormalities

DNA Ploidy. FISH with the entire range of probes to chromosomal regions of interest was successful in 84 of 87 tumors. In the remaining 3, a reliable and informative result was still possible with some probes. FISH signals from centromeric probes frequently revealed an abnormal chromosome number in neoplastic nuclei, aneuploidy being a feature of 36 of 84 (42%) medulloblastomas. The frequency of aneuploidy varied across medulloblastoma variants: large cell/anaplastic medulloblastomas, 60%; classic medulloblastomas, 45%; and desmoplastic medulloblastomas, 15%. This variability was not quite statistically significant ($\chi^2$ test, $P = 0.053$), unless desmoplastic medulloblastomas were compared directly with all of the non-desmoplastic medulloblastomas ($\chi^2$ test, $P = 0.029$). Once pathological classification had been taken into account, aneuploidy was not significantly related to prognosis or other clinical variables.

Chromosome 17 Abnormalities. Abnormalities of chromosome 17 were most common (62% of medulloblastomas) among the tested regions of interest. Signal ratios suggested that $i(17q)$ was present in 23 (27%) medulloblastomas (Fig. 3). There were also isolated losses of 17p ($n = 6$; 7%), isolated gains of 17q ($n = 9$; 11%), and red: green signal ratios in some tumors suggesting a combination of copy number alteration with 17p loss or 17q gain. In 3 medulloblastomas, admixed populations of tumor cells showed either an $i(17q)$ pattern or isolated 17p loss. To investigate the clinicopathologic significance of loss at 17p13.3 (target of the subtelomeric probe), medulloblastomas with isolated loss of 17p, monosomy 17 ($n = 2$), or typical $i(17q)$ were placed in a group termed “17p13.3 loss” ($n = 31$; 38% of total). Loss of 17p13.3 or typical $i(17q)$ occurred at a higher frequency in tumors presenting with metastatic disease; 6 of 11 and 4 of 11 patients with metastatic disease had 17p13.3 loss and $i(17q)$ respectively. However, this association did not reach statistical significance ($\chi^2$ tests, 17p13.3 loss: $P = 0.237$; $i(17q): P = 0.517$). In contrast, patients with medulloblastomas that showed either 17p13.3 loss or typical $i(17q)$ had a significantly (log rank test, $P = 0.0031$; $P = 0.0485$, respectively) worse outcome than those with medulloblastomas not demonstrating these molecular genetic abnormalities (Fig. 4). Similarly significant results were found when only the International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 cohort of patients was analyzed [log rank test, 17p13.3 loss versus no 17p13.3 loss: $P = 0.0101$; $i(17q)$ versus no $i(17q): P = 0.0184$].

Abnormalities of Chromosomes 9q22 and 10q24. Copy number alterations of chromosome 9 were present in 39% of medulloblastomas, but 9q22 loss, including 2 examples of monosomy 9, was evident in 8 medulloblastomas (9% of medulloblastomas). Gain of 9q22 occurred in 5 medulloblastomas. Loss of 10q24 occurred in 13 medulloblastomas (15% of medulloblastomas). There was no relationship between abnormalities of chromosome 9q or 10q and any clinical variable.

MYC Oncogene Amplification. Amplification of MYCC or MYCN was found in a variable proportion of nuclei from 5 of 84 (6%) and 7 of 84 (8%) medulloblastomas, respectively (Table 1). Different patterns of MYC amplification were evident in tumor nuclei (Fig. 3), ranging from a speckling of fluorescent signals (double-minute pattern) to clumps of varying size (pattern of homogeneously staining region). High-level amplification defined as large masses of amplified signal occupying much of the nucleus tended to occur in tumors with a high proportion of amplification-positive nuclei (high-frequency amplification). Over half the cells in 4 medulloblastomas contained MYCC or MYCN amplified signals (Table 1), but just a few nuclei (<5%) showed MYCC or MYCN amplification in FISH preparations from 7 medulloblastomas (low-frequency amplification).
No tumor showed coexistent amplification of *MYCC* and *MYCN*.

No patients with *MYC* amplification presented with metastatic disease. However, all 5 of the patients whose tumors showed *MYCC* or *MYCN* amplification in $\geq 25\%$ of nuclei died within 5 years (Fig. 5). There was a trend for *MYC* amplification and 17p13.3 loss to coexist; 8 medulloblastomas with *MYCC* or *MYCN* amplification also showed 17p13.3 loss, and 4 of these were large-cell/anaplastic medulloblastomas (Fig. 6).

**Chromosomal Abnormalities Across Pathological Variants**

Genetic abnormalities in nondesmoplastic and desmoplastic medulloblastomas differed in several respects. Loss at 17p13.3 or i(17q) was not detected in desmoplastic medulloblastomas with a nodular architecture, but a typical i(17q) profile was found in 2 of 3 non-nodular desmoplastic medulloblastomas (Table 2). Although i(17q) occurred at a similar frequency in classic and large-cell/anaplastic medulloblastomas, 17p13.3 loss was slightly more frequent in large-cell/anaplastic medulloblastomas (Table 2; Fig. 6).

Whereas the prevalence of 10q24 loss was about equal across the principal medulloblastoma variants, loss of 9q22 appeared more common in desmoplastic medulloblastomas than in large-cell/anaplastic or classic medulloblastomas (Table 2). All 3 of the medulloblastomas with a 1:2 profile of 9q22:c9 signals were nodular/desmoplastic variants. In 5 medulloblastomas, 9q22 loss occurred in the context of an abnormal copy number (3:4 profile of 9q22:c9 signals or monosomy 9), and all but 1 of these, a medulloblastoma with extensive nodularity showing monosomy 9, were nondesmoplastic variants.

**MYC Amplification and Morphophenotype**

All 12 of the medulloblastomas with *MYCC* or *MYCN* amplification, except 1 medulloblastoma with extensive nodularity, were classified as nondesmoplastic (Table 1). Across the series of medulloblastomas, *MYC* amplification was more frequent in large-cell/anaplastic tumors (33%) than in classic tumors (11%), which is a significant difference ($\chi^2$ test, $P = 0.034$). In addition, all 4 of the tumors with amplification signals in $\geq 50\%$ of nuclei were large-cell/anaplastic (3 large cell and 1 anaplastic), whereas 5 of 6 nondesmoplastic tumors with low-frequency amplification were classic medulloblastomas (Table 2). Chromogenic *in situ* hybridization allowed several observations on the relationship between *MYC* amplification and morphophenotype. In the medulloblastoma with extensive nodularity with low frequency *MYCN* amplification, two distinct foci of cells were found to contain amplified signals. Both foci were primarily internodular, but a group of pleomorphic cells could be seen on a hematoxylin and eosin preparation to spill out from one focus into the adjacent nodule, and cells in this region also showed amplification (Fig. 7, A and B). Unsurprisingly, large-cell/anaplastic medulloblastomas with high frequency *MYC* amplification showed a strong correlation between large-cell and anaplastic morphophenotypes and the presence of amplification. However, one anaplastic medulloblastoma with amplified *MYCN* signals in 75% of nuclei showed these to be focally admixed with tumor cells in which a normal pair of 2p24 signals was detected (Fig. 7C). Furthermore, 1 small-cell medulloblastoma with no anaplastic focus was characterized by *MYCC* amplification in 25% of nuclei (Fig. 7D). Thus, whereas we observed an association between large-cell/anaplastic medulloblastomas and *MYC* amplification and between *MYC* amplification and the anaplastic or large-cell morphophenotype, this association was far from absolute.

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*Fig. 3* FISH preparations of isolated nuclei showing (A) i(17q) 3 red:1 green signal profile; 17p13.3 probe, green; 17q12 probe, red; (B) loss at 10q24; 10q24 probe, green; c10 probe, red; (C) a patchy distribution for *MYCC* amplification; *MYCC* probe, green; c8 probe, red; (D) amplification of *MYCN*; *MYCN* probe, green.
Multivariate Survival Analysis and Stratification of Patients

Univariate survival analysis revealed a clear relationship between the large-cell/anaplastic variant and an adverse outcome. In addition, molecular cytogenetic abnormalities that conferred a poor prognosis were MYCC or MYCN amplification in >50% of nuclei and 17p13.3 loss/i(17q). Additionally, MYCC or MYCN amplification in >50% of nuclei showed a significant association with large-cell/anaplastic tumors, and there was an association between MYC amplification and 17p13.3 loss. Using only data from International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 patients, we analyzed the independence of these potential prognostic indicators in a Cox proportional hazards model. Because MYC amplification and 17p13.3 loss were exceptional in nodular desmoplastic medulloblastomas, we excluded patients with these tumors (n = 5). We also excluded children with metastases at presentation (n = 5), because such a small group of patients would confound the analysis. Receiving radiotherapy alone, rather than combined radiotherapy and sandwich chemotherapy, was revealed as a significant hazard to survival in the International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 study (33). However, age, sex, and extent of surgery were not prognostic indicators. These variables were excluded from our analysis once similar results were confirmed in our cohort of patients (data not shown). In a Cox proportional hazards model of 55 patients, large-cell/anaplastic variant, MYC amplification, and 17p13.3 loss were all significant independent hazards to survival (Table 3).

Stratifying the same cohort of International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 patients (n = 55) on the basis of pathological variant and 17p13.3 status produced significantly different (P < 0.0001) survival curves (Fig. 8A). Only 1 of 10 patients with a large-cell/anaplastic medulloblastoma characterized by 17p13.3

Table 1 MYCC/MYCN amplification

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<th>Pathology</th>
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Abbreviations: MB, medulloblastoma; ND, not determined.

Fig. 4 Kaplan-Meier survival curves for patients with medulloblastomas showing (A) either 17p loss or no 17p loss (log rank test: P = 0.0031), and (B) for patients with medulloblastomas showing either i(17q) or no i(17q) (log rank test: P = 0.0485).

Fig. 5 Kaplan-Meier survival curves for patients with medulloblastomas showing two frequencies of MYC amplification or no MYC amplification (log rank test: P < 0.0001).
loss survives at a postdiagnosis interval of 4.5 years, whereas 5-year survival for patients with classic medulloblastomas without loss of 17p13.3 is just 90%. When International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 children with nondesmoplastic medulloblastomas (n = 60) were stratified into high-risk and low-risk groups on the basis of pathological variant (large-cell/anaplastic = high risk), MYC amplification status (high frequency amplification = high-risk), 17p13.3 status (loss = high-risk), and Chang staging (M > 0 = high risk), the difference between survival curves for the two groups was highly significant (P = 0.0002) (Fig. 8B). Of the 3 patients in the low-risk group who died, 2 had intermediate-cell classic medulloblastomas and 1 had a small-cell classic medulloblastoma, but all three of the tumors were characterized by a small focus of anaplasia. Clinical, pathological, and molecular data relating to this particular cohort of patients and their stratification can be found elsewhere.1

DISCUSSION
Our data support the paradigm that analysis of medulloblastomas should combine detailed histopathological evaluation with determination of molecular status and should supplement clinical/radiological assessment for the purposes of treatment stratification. In a cohort of 60 children with nondesmoplastic medulloblastomas from the International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET 3 trial, stratification into two groups, high- and low-risk, produced significantly different survival curves, high-risk children being those with metastatic disease at presentation, with a large cell/anaplastic medulloblastoma, or with tumors characterized by 17p13.3 loss or high-frequency MYC amplification.

This paradigm potentially identifies patients with aggressive disease for whom maximal therapy is appropriate and patients who might survive with less side effects after adjuvant therapy of reduced intensity.

Histopathological Determinants of Biological Behavior in Medulloblastomas. Despite the current emphasis on molecular classification of tumors in scientific investigation, the histopathological classification of medulloblastomas continues to evolve (11, 12). The poor prognosis associated with the new anaplastic variant, which was initially proposed by the neuropathology group from John Hopkins University Hospital, has now been demonstrated in two large cohorts of children treated in clinical trials (8–10). Formerly, the large-cell medulloblastoma was the only variant demonstrated to have an aggressive biological behavior (27). The behavior of large-cell and anaplastic medulloblastomas may reflect relatively increased proliferation in these variants (10), but our data indicate that it is not significantly associated with a propensity for early metastatic disease. All of the large-cell medulloblastomas contain extensive groups of cells with an anaplastic morphophenotype, and we would support a proposal to combine large-cell and anaplastic medulloblastomas into a single group, constituting ~20% of all medulloblastomas, despite data in the present study to show that large-cell medulloblastomas appear to have a slightly worse prognosis than anaplastic medulloblastomas (9–12).

Experience gained from the histopathological review of >400 medulloblastomas from patients entered into United Kingdom Children’s Cancer Study Group trials suggested that nonanaplastic classic medulloblastomas could be divided into two types, which have been given the labels small-cell and intermediate-cell in this study. These phenotypes can be distinguished on the basis of nuclear form: uniform round nuclei in small-cell medulloblastomas versus oval or polyhedral nuclei in intermediate-cell medulloblastomas. Our data indicate that these two proposed variants differ both in terms of prognosis and frequency of anaplastic foci. In principle, they represent an

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1 Internet address: http://www.ncl.ac.uk/nicr/research/ebtd/paed/Lamont_et_al.htm.
additional division of classic medulloblastomas according to nuclear morphology and, thus, conform to observations made during the analysis of degrees of anaplasia in previous studies, incorporating tumors described as having mild or focal anaplasia or no anaplasia at all (9).

Molecular Abnormalities in Pathological Variants.

The frequency of molecular cytogenetic abnormalities in the present series of medulloblastomas conforms to data from previous studies (17, 22, 26, 35, 36), some of which have also suggested associations between pathological variant and genetic abnormalities. Our results show an association between diploid status and desmoplastic phenotype. This finding has been reported before (37), and the lack of correlation between ploidy status and outcome in our study at least reflects the conflicting data in the literature on this issue (36, 38, 39).

Mutations of the \textit{PTCH} gene on 9q22 and loss at this locus have been linked with the desmoplastic phenotype in studies of genetic abnormalities in medulloblastoma (29, 31). We supplemented desmoplastic medulloblastomas from patients in the International Society of Pediatric Oncology/United Kingdom Cancer Research.

![Figure 7](image_url)

\textbf{Table 2} Frequency of molecular cytogenetic abnormalities in pathologic variants

<table>
<thead>
<tr>
<th>MB</th>
<th>Classic ((n = 59)^*)</th>
<th>Small-cell ((n = 39)^*)</th>
<th>Intermediate-cell ((n = 19)^*)</th>
<th>Large-cell/anaplastic ((n = 15))</th>
<th>Non-nodular desmoplastic ((n = 5))</th>
<th>Nodular desmoplastic ((n = 10))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isochromosome (17q)</td>
<td>(n = 23)</td>
<td>16 (28%)</td>
<td>12 (32%)</td>
<td>4 (22%)</td>
<td>5 (33%)</td>
<td>2 (67%)</td>
</tr>
<tr>
<td>Loss 17p13.3†</td>
<td>(n = 31)</td>
<td>21 (37%)</td>
<td>15 (39%)</td>
<td>6 (33%)</td>
<td>8 (53%)</td>
<td>2 (67%)</td>
</tr>
<tr>
<td>Loss 9q22</td>
<td>(n = 8)</td>
<td>1 (12%)</td>
<td>0</td>
<td>1 (5%)</td>
<td>3 (20%)</td>
<td>0</td>
</tr>
<tr>
<td>Loss 10q24</td>
<td>(n = 13)</td>
<td>9 (15%)</td>
<td>6 (15%)</td>
<td>3 (16%)</td>
<td>2 (13%)</td>
<td>0</td>
</tr>
<tr>
<td>\textit{MYC/MYCN} low-frequency amplification</td>
<td>(n = 7)</td>
<td>5 (8%)</td>
<td>5 (13%)</td>
<td>0</td>
<td>1 (7%)</td>
<td>0</td>
</tr>
<tr>
<td>\textit{MYC/MYCN} high-frequency amplification</td>
<td>(n = 5)</td>
<td>1 (2%)</td>
<td>1 (3%)</td>
<td>0</td>
<td>4 (27%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviation: MB, medulloblastoma.

* Chromosome 17 and MYC analyses: classic MBs, \(n = 56\); small-cell MBs, \(n = 38\); intermediate-cell MBs, \(n = 18\).

† 17p13.3 loss = isolated 17p loss + monosomy 17 + i(17q).

Fig. 7 The cells in the desmoplastic intermodal region of a medulloblastoma with extensive nodularity spill out into the adjacent nodule, increasing the nuclear:cytoplasmic ratio \((A, \text{hematoxylin and eosin, original magnification, } \times 100)\). Less than 5% of cells in this tumor showed \textit{MYCN} amplification, but a focus of amplification was detected here in the intermodal and invading cells but not in the smaller intranodal cells \((B, \text{chromogenic } \textit{in situ} \text{ hybridization, } \textit{MYCN} \text{ probe, original magnification, } \times 200)\). \(C\), the proportion of nuclei with \textit{MYCN} amplification in this anaplastic medulloblastoma is 75%; this region contains an admixture of pleomorphic cells showing amplification or a normal pair of 2p24 signals \((\text{original magnification, } \times 600)\). \(D\), this small-cell classic medulloblastoma showed \textit{MYCC} amplification in 25% of nuclei, nearly all of which lacked any anaplastic morphology \((\text{original magnification, } \times 800)\).
Children’s Cancer Study Group PNET3 with desmoplastic tumors from other United Kingdom Children’s Cancer Study Group trial patients to assess whether this pathological variant has the same frequency of molecular cytogenetic abnormalities as others. Dividing desmoplastic medulloblastomas into four histopathological types: nodular, paucinodular, medulloblastoma with extensive nodularity, and non-nodular, we discovered that loss at 9q22 was more frequent in desmoplastic medulloblastomas than in nondesmoplastic medulloblastomas and that chromosome 17 abnormalities were rare. However, 9q22 loss was not confined to the desmoplastic medulloblastoma, occurring in four nondesmoplastic tumors, and losses of 10q24, where the SUFU gene is located, were split across medulloblastoma variants. SUFU is active in the PTCH pathway, and some studies of medulloblastoma have detected mutations in this gene, albeit at low frequency (32). Overall, our findings suggest that chromosomal losses at loci containing PTCH pathway genes are not confined to desmoplastic medulloblastomas.

Examples of chromosome 17 abnormalities in desmoplastic medulloblastomas were restricted to an i(17q) in 2 of 3 non-nodular tumors. These 3 tumors showed no abnormalities at 9q22, such that the genetic profile of non-nodular desmoplastic medulloblastomas appears to be more aligned to that of the classic/anaplastic/large-cell variants. We believe that these results inform the debate about the nomenclature attached to this type of medulloblastoma, and that, rather than desmoplasia, its defining feature should be the nodule, which represents a distinct microenvironment where tumor cells come out of the cell cycle and either differentiate or undergo apoptosis (11, 12).

Apart from the occurrence of i(17q) in two non-nodular desmoplastic tumors, chromosome 17 abnormalities were confined to nondesmoplastic medulloblastomas. Abnormalities included isolated 17p loss, isolated gain of 17q, monosomy 17, and FISH signal patterns suggesting i(17q). Previous mapping studies have indicated that a consensus region of allelic loss is located at 17p13.3 (40). We chose to probe this region, although candidate tumor suppressor genes at this locus, such as HIC-1, have yet to be clearly implicated in medulloblastoma histogenesis. The results of one comparative genomic hybridization study suggest that loss of 17p is always associated with large-cell/anaplastic medulloblastomas (26). Our data do not support this conclusion, indicating that both i(17q) and losses at 17p13.3 are split across medulloblastoma variants, even showing no segregation among the two types of classic medulloblastoma. The corollary of this finding is that, because a diagnosis of large-cell/anaplastic medulloblastoma and the presence of 17p13.3 loss are independent adverse prognostic indicators, a combination of histopathological evaluation and assessment of 17p status provides superadded information about medulloblastoma biological behavior.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Cox proportional hazards to survival in unadjusted (univariate) and adjusted (multivariate) analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted hazard (coefficient)</td>
</tr>
<tr>
<td>Large-cell/anaplastic phenotype</td>
<td>4.88</td>
</tr>
<tr>
<td>17p13.3 loss</td>
<td>4.50</td>
</tr>
<tr>
<td>MYC/MYCN amplification</td>
<td>6.03</td>
</tr>
<tr>
<td>Radiotherapy alone</td>
<td>2.24</td>
</tr>
</tbody>
</table>

Fig. 8 Kaplan-Meier survival curves for International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 patients with nondesmoplastic medulloblastomas stratified (A) according to pathological variant and 17p13.3 status (log rank test: P < 0.0001) or (B) into high- and low-risk groups (high-risk being defined as either metastatic disease at presentation, or large-cell/anaplastic medulloblastoma, or medulloblastoma with 17p13.3 loss, or medulloblastoma with high-frequency MYC amplification (log rank test: P = 0.0002).
Several studies have reported an association between MYC amplification and the large-cell/anaplastic medulloblastoma, although this association does not appear to be absolute (8, 26, 28, 41). Our FISH results show that amplification of both MYCC and MYCN genes in medulloblastoma is highly variable, both in terms of the pattern of FISH signals in individual nuclei and the proportion of nuclei that demonstrates amplification signals, what we have termed the level and frequency of amplification, respectively. In our series of tumors, the association between MYC amplification and large-cell/anaplastic phenotype was seen when the frequency of amplification was high; when it was low, medulloblastomas generally had a classic phenotype. Using chromogenic in situ hybridization on tissue sections, we have ascertained that the histopathological correlate of a small population of tumor nuclei with MYC amplification in FISH cytospin preparations appears to be a clone of cells developing at one or more foci in the tumor. There was correspondence between a large-cell/anaplastic morphophenotype and MYC amplification in many areas of tumors with high frequency amplification. However, there were several examples of high-level amplification in cells with a small-cell or intermediate-cell phenotype. Although large-cell/anaplastic medulloblastomas contain larger cells and have a higher mitotic count than classic medulloblastomas and MYCC expression is known to affect cell proliferation and cell size, the association between morphophenotype or medulloblastoma variant and MYC amplification does not appear absolute. The lack of a close correlation between MYCC amplification and expression may be one explanation for this (19).

With the exception of one medulloblastoma with extensive nodularity, MYC amplification was not observed in desmoplastic medulloblastomas. Medulloblastomas with extensive nodularity have a favorable clinical outcome and a distinctive histopathology, which includes many large, well-circumscribed nodules (13). The medulloblastoma with extensive nodularity with MYCN amplification in our series came from a patient without clinical risk factors who unfortunately survived <2 years. Supplementary chromogenic in situ hybridization on this tumor demonstrated two foci of MYCN amplified cells in internodular regions. These cells were larger and more pleomorphic than the intranodular cells, although they did not qualify as anaplastic. However, they had invaded the adjacent nodule, which is a relatively unusual occurrence in a medulloblastoma with extensive nodularity.

Our findings suggest that clones of MYC amplified cells come to dominate some medulloblastomas. When predominant, MYC amplified cells generally have a large-cell/anaplastic morphophenotype, which presumably could be related to the effects of MYC amplification or other coincidental molecular abnormalities.

**Stratification of Patients with Medulloblastoma.** Several molecular prognostic indicators have been proposed for medulloblastoma in recent years, including 17p loss (22, 24, 35), ErbB2 overexpression (23, 24), TrkC overexpression, and MYC amplification or overexpression (19). Of the molecular cytogenetic abnormalities in the present study, 17p13.3 loss stands out as a prognostic indicator and as an abnormality that can occur across all of the pathological variants. Previously reported associations between an adverse outcome and both MYC amplification and the large-cell/anaplastic pathological variant were reinforced by our results. However, there was some overlap between 17p13.3 loss and high frequency MYC amplification and between the large-cell/anaplastic variant and MYC amplification, which slightly diminished the significance of these variables in a Cox analysis of proportional hazards to survival. Stratifying a cohort of 60 International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET3 patients into high- and low-risk groups (high-risk if medulloblastomas were metastatic at presentation or large-cell/anaplastic variants, or showed 17p13.3 loss or high frequency MYC amplification) produced significantly divergent survival curves. Only 3 patients in the low-risk group died, suggesting that a system of stratification that uses a combination of histopathological evaluation and molecular markers can identify not only patients with biologically aggressive tumors for whom maximal therapy is appropriate but patients with relatively benign tumors who might be cured and suffer less side effects if adjuvant therapy is reduced. However, the results of other outcome studies indicate that alternative schemes might prove equally discriminatory as ours; for example, a combination of two molecular markers, TrkC and MYC overexpression, was demonstrated to have similar prognostic power when used to stratify patients with medulloblastoma (19). However, this scheme and ours differ in terms of the ease with which molecular markers are assessed. Assessment of TrkC and MYC overexpression cannot reliably be undertaken on formalin-fixed tumors, whereas the FISH method can. An important next step is to reproduce this data in a larger cohort of uniformly treated patients. This is planned as part of the International Society of Pediatric Oncology/United Kingdom Children’s Cancer Study Group PNET4 trial, which has just opened, and similar biological studies will be incorporated into forthcoming North American trials.

**ACKNOWLEDGMENTS**

Thanks are due to Andrew Brown of the Department of Neuropathology, Newcastle General Hospital (Newcastle-upon-Tyne, UK), for technical assistance, and to Kath Robinson, data manager, and Claire Weston, statistician, at the United Kingdom Children’s Cancer Study Group data center (Leicester, UK).

Dr. M. Rochi, Cytogenetics Unit, University of Bari (Bari, Italy), kindly donated all bacterial artificial chromosomes, P1 artificial chromosomes, and plasmids, except for MYCN, which was from Dr. P. De Jong, Children’s Hospital Oakland Research Institute (Oakland, CA).

The collaboration of staff in all centers across Europe that contributed to the International Society of Pediatric Oncology PNET3 trial is gratefully acknowledged.

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Combined Histopathological and Molecular Cytogenetic Stratification of Medulloblastoma Patients


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