Prognostic importance of DNA ploidy in medulloblastoma of childhood

MASAHARU YASUE, M.D., TADANORI TOMITA, M.D., HERBERT ENGELHARD, M.D., FRANK GONZALEZ-CRUSSI, M.D., DAVID G. McLONE, M.D., PH.D., AND KENNETH D. BAUER, PH.D.

Division of Pediatric Neurosurgery and Department of Pathology, Children's Memorial Hospital and Northwestern University Medical School, Chicago, Illinois

The deoxyribonucleic acid (DNA) content of 53 medulloblastomas was analyzed by means of flow cytometry and compared with the clinical and histological findings in the host patients. Analysis of DNA showed that about half of the tumors were diploid and the other half were aneuploid. More diploid tumors were found among patients of a young age, but the difference was without statistical significance. Cellular differentiation of the tumor did not correlate with DNA ploidy. No correlation was found between Chang's T staging system and the DNA ploidy, whereas the M staging correlated with the ploidy; diploid medulloblastomas had a greater tendency to metastasize than aneuploid medulloblastomas (p = 0.0003). Four-year survival was compared with the extent of resection and DNA ploidy. The patients with total resection and aneuploid medulloblastoma had a better prognosis than those with subtotal resection and diploid tumor (p = 0.001). There was only one survivor among eight patients with subtotally resected diploid medulloblastomas, while all of the seven patients with totally resected aneuploid medulloblastomas survived. Comparison of the G0/G1 phase fraction and S phase fraction in the surviving group and the deceased group offered no significant information.

KEY WORDS • brain neoplasm • DNA aneuploidy • flow cytometry • medulloblastoma

MEDULLOBLASTOMAS are among the most malignant brain tumors in childhood. With current diagnostic procedures and treatment, the 5-year survival rate in these patients is about 50%. Clinical and therapeutic factors are relevant to the patients' prognosis. A young age at diagnosis and an advanced stage of tumors based on the modified grading system of Chang, et al., were associated with an unfavorable clinical outcome. The extent of surgical resection and the use of craniospinal radiation have been reported to influence the survival rate. Cellular differentiation in medulloblastomas may be a prognostic factor. Nonetheless, all of these factors are not necessarily reliable. Additional objective methods are needed to select modes of therapy and predict prognosis.

The deoxyribonucleic acid (DNA) content in the nuclei of neoplastic cells can be readily analyzed by flow cytometry, and may contribute to the management of patients with various malignancies. Originally, DNA analysis by flow cytometry required a fresh surgical specimen; however, Hedley, et al., introduced a method that can utilize formalin-fixed paraffin-embedded specimens. This method made it possible to analyze specimens retrospectively from patients whose clinical outcome was already known, and to identify the response of a given treatment. DNA aneuploid indicates the presence of an abnormal DNA stem line and reflects cells with at least a 5% change in DNA content relative to that of 46 chromosomes. Recently, we used this technique for DNA analysis of 26 medulloblastomas and found a significant correlation between DNA ploidy and patient outcome. The present study expands our previous communication by including a greater number of cases, and correlating DNA content with the clinical and histological findings in each case. The study demonstrates that cellular ploidy is an important prognostic factor in medulloblastoma.

Clinical Material and Methods

Pathological Material

All pathological specimens from previously untreated primary medulloblastomas were collected at the Chil-
dren's Memorial Hospital. Tissue was fixed in buffered 10% formalin and processed using standard histological embedding technique in paraffin. Fifty cases treated between 1965 and 1986 were reviewed. Four-micron thick sections of tissue adjacent to the material being studied by flow cytometry were stained with hematoxylin and eosin. Besides these specimens, three fresh surgical samples were included in 1987. All specimens were reexamined and classified according to the degree of histological differentiation in two categories: undifferentiated, if no obvious differentiation was apparent, the tumor being uniformly composed of small cells of basophilic nuclei; and differentiated, if any form of differentiation (such as astrocytic, oligodendroglial, mesenchymal) was found.

**Tissue Deparaffinization, Rehydration, and Dissociation**

Paraffin-embedded tissue was deparaffinized, rehydrated, and dissociated using a previously reported modification of the method of Hedley, et al. Four or five 50-μm thick sections were cut with a microtome and deparaffinized with xylene. The deparaffinized tissue was rehydrated in a graded series of ethanol and washed in distilled water. The deparaffinized tissue was minced into small pieces, suspended in 3 ml of 0.1% pepsin solution, and incubated for 30 minutes at 37°C with vortex mixing at 5-minute intervals. The pepsin proteolysis was stopped by the addition of 0.1 ml of pepstatin A. The digested material was filtered through a 37-μm nylon monofilament mesh. Hanks' balanced salt solution (HBSS) and 10 mM of N-2-hydroxy ethyl-piperazine-N'-2-methanesulfonic acid (HEPES) was added to a final volume of 15 ml. This mixture was centrifuged and the nuclear pellet was resuspended in several milliliters of 10 mM HEPES-HBSS according to the size of the pellet. The number of cells was determined with a hemocytometer.

**DNA Staining and Flow-Cytometric Analysis**

Propidium iodide (PI) staining was performed as reported previously. Following centrifugation of the suspended solution, the dissociated nuclei were suspended in a solution of 0.1% Triton X-100 in phosphate-buffered saline (PBS) at a concentration of 1 × 10^6 nuclei/ml for 3 minutes at 4°C and then were centrifuged. The pellet was incubated with ribonuclease (180 U/ml) at a concentration of 1 × 10^6 nuclei/ml for 20 minutes at 37°C. The nuclei were centrifuged and stained using a 50-μg/ml solution of PI in PBS at a concentration of 2 × 10^6 nuclei/ml. The specimens were kept light-free at 4°C overnight.

The fluorescence of the PI-stained nuclei was monitored on an EPICS 752 flow cytometer. Immediately prior to flow-cytometric analysis, the cell suspensions were filtered through a 37-μm nylon monofilament filter. The stained cells were excited by means of an argon ion laser at 488 nm (500 dalton molecular weight), and fluorescence was monitored through 515-nm interference filters and 515-nm longpass filters. Fluorescent microspheres were used to assess instrument performance at the beginning of each run and as an external standard. A minimum of 2.5 × 10^4 nuclei was evaluated.

**Cell Cycle and Aneuploidy Analysis**

The DNA histogram display and analysis were performed with a personal computer and the cytological cell analysis software. Cell cycle analysis was conducted using a simple rectangle model which allowed electronic subtraction of cellular debris from the DNA distribution and permitted consideration of multiple aneuploid peaks.

A DNA aneuploid population was considered present only if two or more distinct G0/G1 populations were present.
DNA aneuploidy in medulloblastoma

### TABLE 1
Chang's operative staging system for cerebellar medulloblastomas*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>tumor &lt; 3 cm in diameter &amp; limited to the classic midline position in the vermis, roof of the 4th ventricle, and less frequently to the cerebellar hemispheres</td>
</tr>
<tr>
<td>T2</td>
<td>tumor ≥ 3 cm in diameter &amp; further invading 1 adjacent structure or partially filling the ventricle</td>
</tr>
<tr>
<td>T3</td>
<td>stage divided into T3A &amp; T3B</td>
</tr>
<tr>
<td>T3A</td>
<td>tumor further invading 2 adjacent structures or completely filling the 4th ventricle with extension into the aqueduct of Sylvius, foramen of Magendie, or foramen of Luschka, thus producing marked internal hydrocephalus</td>
</tr>
<tr>
<td>T3B</td>
<td>tumor arising from the floor of the 4th ventricle or brain stem &amp; filling the 4th ventricle</td>
</tr>
<tr>
<td>T4</td>
<td>tumor spreading further through the aqueduct of Sylvius to involve the 3rd ventricle or midbrain, or tumor extending to the upper cervical cord</td>
</tr>
<tr>
<td>T*</td>
<td>tumor extending through the cerebellar surface and invading the adjacent structure</td>
</tr>
</tbody>
</table>

### TABLE 2
Relationship between patient age and ploidy*

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Diploid Tumors</th>
<th>Aneuploid Tumors</th>
<th>Tetraploid Tumors</th>
<th>Total Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 3 yrs</td>
<td>11</td>
<td>6</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>4-9 yrs</td>
<td>10</td>
<td>13</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>≥ 10 yrs</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>total</td>
<td>25 (47.2%)</td>
<td>25 (47.2%)</td>
<td>3 (5.7%)</td>
<td>53</td>
</tr>
</tbody>
</table>

* No significant difference was found between diploid and aneuploid types (p > 0.1).

Stage T* was added to the staging system because five cases could not be classified into the T staging system (Table 1). The Stage T* tumors protruded from the cerebellar surface and invaded adjacent structures. For example, one tumor extended up to the middle cranial fossa through the incisura tentorii.

Four-year survival was compared with the extent of resection, the dose of radiation therapy, and the DNA characteristics in 42 cases treated between 1965 and 1983. All patients underwent a posterior fossa craniotomy; visible total resection was performed in 18, subtotal resection (> 75% of tumor resected) in 22, and biopsy alone in two cases. Posterior fossa radiation of 5000 rads or more, in addition to craniospinal radiation, was considered an appropriate dose. This dose was given in 33 cases, but nine patients received 4500 rads or less, which was considered an inappropriate dose. No patients received adjuvant chemotherapy for the primary tumor.

All of the data were statistically analyzed. The chi-square test (Yates' modification) or Fisher's exact test was used to correlate statistical significance.

### Results

#### Relationship Between Patient Age and Ploidy

DNA analysis by flow cytometry of 53 medulloblastomas showed that 25 cases (47.2%) had a diploid pattern, another 25 cases (47.2%) an aneuploid pattern, and three cases (5.7%) a tetraploid pattern (Table 2). The distribution of ploidy in relation to the patient's age at diagnosis indicated that the patients aged 3 years or younger more frequently had diploid than aneuploid tumors. On the other hand, aneuploid cases were predominant over diploid cases in the older groups. The three tetraploid-type tumors were seen in the 4- to 9-year-old group. No statistically significant relationship was noted between patient's age and DNA ploidy (p > 0.1).

#### Relationship Between Extent of Differentiation and Ploidy

Thirty-four cases (66.7%) were classified into the undifferentiated type, and 17 cases (33.3%) were grouped into the differentiated type (Table 3). However, two were unclassified due to a lack of tissue. The diploid group consisted of 13 undifferentiated and 10 differ-
TABLE 3
Relationship between extent of differentiation and ploidy*

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Undifferentiated</th>
<th>Differentiated</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>diploid</td>
<td>13</td>
<td>10</td>
<td>43.5</td>
</tr>
<tr>
<td>aneuploid</td>
<td>19</td>
<td>6</td>
<td>76.0</td>
</tr>
<tr>
<td>tetraploid</td>
<td>2</td>
<td>1</td>
<td>66.7</td>
</tr>
<tr>
<td>total</td>
<td>34 (66.7%)</td>
<td>17 (33.3%)</td>
<td></td>
</tr>
</tbody>
</table>

* Two tumors were not classified due to a lack of tissue. No significant difference was found between diploid and aneuploid types (p > 0.1).

Relationship Between T and M Staging and Ploidy

The T staging system was compared with the DNA ploidy of the patient (Tables 1 and 4). No T1 case was found in this study. The 30 cases treated after the availability of CT scanning were classified into categories T2 through T4: five in T2; 14 in T3A; two in T3B; four in T4; and five in T5. Fifteen diploid and 15 aneuploid cases were grouped into T through T5, but no correlation between diploid and aneuploid types was found (p > 0.1).

According to the M staging system, 30 cases were classified into categories Mo through M3: seven in Mo; four in M1; 14 in M2; four in M3; and one in M4. No case with Stage M4 was found. Fifteen cases in the diploid group were composed of: three in M1; eight in M2; three in M3; and one in M4. No case with M0 was found in the diploid group. On the other hand, 15 cases of the aneuploid group were composed of: seven in M0; one in M1; six in M2; and one in M3. No aneuploid case with M1 was found in the aneuploid group, which suggests that in the early stage of disease tumors with a diploid pattern disseminate more than tumors with an aneuploid pattern (p = 0.003).

Relationship Between 4-Year Survival and Extent of Resection or Radiation

Among 18 patients who underwent total resection, 13 patients survived for 4 years or more, but five patients died in less than 4 years (Table 5). In the subtotal resection group, only six patients survived for 4 years or more whereas 16 patients died in less than 4 years. The patients with total removal had a significantly better survival rate than those with subtotal resection or biopsy alone (p = 0.003).

Relationship Between DNA Characteristics and 4-Year Survival

The DNA ploidy was compared with the 4-year survival rate. In order to standardize patient groups, the cases with inadequate treatment such as biopsy (two cases) or inappropriate radiation therapy (four cases) were excluded from analysis. The remaining 30 patients were analyzed (Table 6). Six patients in the diploid group survived for 4 years or more, whereas 10 patients died in less than 4 years. On the other hand, 12 patients in the aneuploid group survived for 4 years or more and only two patients died in less than 4 years. The rate of 4-year or more survival of the aneuploid group (85.7%) is much higher than that of the diploid group (35.3%) (p = 0.009). One patient with tetraploid pattern died within 4 years. Figure 2 displays cumulative survival rates for 40 patients who received adequate surgical and radiation therapies. They were grouped according to DNA ploidy. The patients with DNA aneuploid medulloblastomas also showed significantly better survival than the others.

The DNA ploidy is further compared with surgical resection and 4-year survival (Table 6). Among the patients with aneuploid medulloblastomas, all seven patients with gross total resection are alive without recurrence and five (71.4%) of seven survived four years or more in spite of a subtotal resection. Among the patients of the diploid group, one (12.4%) of eight patients with subtotal resection is alive but five (62.5%) of eight patients with total resection survived for 4 years or more.

The mean percentages of the S and G0/G1 phases were compared with 4-year survival. The mean percentage of the S phase (with standard deviation) was 7.7% ± 2.5% in 14 surviving patients and 8.48% ± 4.3% in the 12 patients who had died. Four cases were excluded due to unavailability of data caused by the broad coefficient variation. The mean percentage was a little lower in the surviving group than the fatality rate.

**TABLE 4**

Relationship between Chang’s operative staging system and ploidy*

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>T1</th>
<th>T3</th>
<th>T3A</th>
<th>T3B</th>
<th>T4</th>
<th>T5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>diploid</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>aneuploid</td>
<td>0</td>
<td>3</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>total</td>
<td>0</td>
<td>5</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

* This analysis includes 30 patients managed after computerized tomography became available (1978 to 1987). See Table 1 for definition of staging. There was no significant difference between diploid and aneuploid types (p > 0.1). The difference between M0 and M1 + M2 + M3 was significant (p = 0.003).
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The mean percentages of the $G_0/G_1$ phase were also compared with 4-year survival. Because of the broad coefficient variation, six cases were excluded. The mean percentage of the $G_0/G_1$ phase was 87.2% ± 5.1% in the group of 13 surviving patients and 85.4% ± 4.2% in the 12 patients who died. The mean percentage was a little higher in the former than the latter group; however, the difference was also statistically insignificant.

**Discussion**

Disease-free survival times among children with medulloblastomas have markedly improved during the past decade. This improvement is due to the prevailing surgical preference to resect the tumor totally, and better techniques in diagnosis and therapy. Nonetheless, certain patients with medulloblastoma still suffer from recurrences and ultimately die despite modern therapy. Several ways to evaluate prognosis have been proposed, but none are consistently reliable. In the present report the DNA ploidy of medulloblastomas was studied as an adjunct in estimating the patient's outcome.

Since the advent of flow cytometry, it has become possible to analyze the DNA content of a tumor very quickly. The new method for DNA analysis of paraffin-embedded specimens has been increasingly applied to various forms of cancer. The results of DNA analysis by this method are quite comparable to those obtained from fresh specimens. The DNA flow-cytometric study using archival paraffin-embedded tissue permits rapid retrospective analysis of large numbers of uncommon tumors (such as medulloblastoma) in patients whose outcome and response to treatment are already known. It is recognized that young patients with medulloblastoma have a poor prognosis. In this study, patients in the young age group (≤ 3 years) included more diploid cases than aneuploid cases, but this was not statistically significant. The poor prognosis of the patients may be related to those factors. For instance, the very young child cannot endure irradiation, and thus does not receive optimal therapy. The age factor for DNA ploidy is comparable to that of neuroblastomas outside the central nervous system, since diploid neuroblastomas are common in infants with a high clinical stage.

**TABLE 5**

<table>
<thead>
<tr>
<th>Extent of Surgery</th>
<th>Alive ≥ 4 Yrs</th>
<th>Alive &lt; 4 Yrs</th>
<th>Total No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>total resection</td>
<td>13</td>
<td>5</td>
<td>18</td>
<td>42.9</td>
</tr>
<tr>
<td>subtotal resection</td>
<td>6</td>
<td>16</td>
<td>22</td>
<td>52.3</td>
</tr>
<tr>
<td>biopsy</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

*Survival rate was significantly better after total resection than after subtotal resection or biopsy (p = 0.003).

Several studies have shown a correlation between ploidy and differentiation in various cancers. These studies have demonstrated that highly differentiated tumors are more frequently diploid, while primitive anaplastic tumors are often aneuploid. More undifferentiated medulloblastomas were found among the aneuploid group than in the diploid group, but the difference was not statistically significant.

When Chang's TM classification is used as a prognostic indicator, medulloblastomas in advanced stages show poor prognosis. Although this classification clarifies the extension of the tumors, no correlation between the T staging system and DNA ploidy was found in our study. Concerning tumor metastases, however, DNA ploidy correlated with Chang's M staging system (p = 0.003). The diploid medulloblastomas behaved more aggressively and tended to disseminate through the cerebrospinal fluid pathway. To our knowledge, the relationship between DNA ploidy and Chang's classification has not been previously documented.

The extent of surgical resection and the dose of radiation have been considered prognostic indicators. In the present study, both factors were correlated with a 4-year survival. Patients with total resection had
a much better prognosis than those with subtotal resection or biopsy alone (p = 0.003). Besides these factors, the ploidy of the tumors was correlated with 4-year survival. Based on this parameter, patients with aneuploid medulloblastomas had a better prognosis than those with diploid medulloblastomas (p = 0.009). In fact, the patients with total removal and an aneuploid pattern had an excellent result, whereas those with subtotal removal and a diploid pattern had the worst survival times (p = 0.001). The fact that the aneuploid type is a favorable prognostic factor has also been reported for other pediatric cancers such as neuroblastoma and lymphoblastic leukemia. In contrast, the presence of DNA aneuploidy indicates aggressive behavior and poor prognosis in adult neoplasms such as ovarian, bladder, breast, and colon cancer.

The reason for this discrepancy between pediatric and adult tumors remains unsolved. The S phase and G0/G1 fractions of tumors have been considered to be additional prognostic indicators. A high S phase fraction could be associated with greater tumor aggressiveness and worse outcome than a low S phase. The G0/G1 phase fraction may be inversely correlated with the outcome. In this study, the mean S phase fraction of the patients who died was higher than that of the survivors. The mean G0/G1 phase fraction of those who are alive was slightly higher than the mean G0/G1 phase fraction of the deceased patients. However, this difference was statistically insignificant. Furthermore, because of the broad coefficient variation and debris in the DNA histogram, the cell cycle in several cases could not be calculated by flow-cytometric DNA analysis using paraffin-embedded specimens. Therefore, the significance of cell cycle analysis as a prognostic indicator for medulloblastomas needs further investigation.

Since a highly variable distribution of ploidy in malignant glioma (including glioblastoma) and a variable DNA ploidy between different regions within the same tumor have been reported, analysis of DNA ploidy has not been considered a good prognostic indicator for malignant glioma. However, the present study proved that the DNA ploidy can be one of the important prognostic factors for medulloblastoma. Regional differences in paraffin-embedded tumors were not seen, but this was probably because of limited tumor sampling. In three recent cases, two different samples of fresh tumor were obtained from different sites in the tumor and were analyzed by flow cytometry. There were no regional differences in DNA ploidy. It is of interest to identify the heterogeneous pattern of DNA ploidy in prospective patients, and multi-institutional investigation of the prognostic value of DNA ploidy for medulloblastoma should be undertaken. Transferring the paraffin-embedded tissue to the centers that are capable of flow-cytometric DNA analysis would permit a more comprehensive survey of a greater number of cases.

We conclude that total resection continues to be the mainstay in the therapy of patients with medulloblastoma: surgeons should attempt to resect as much tumor as possible on every case. If our DNA ploidy results are further confirmed, it might be possible to recommend more diverse postoperative management approaches. Thus, patients with totally resected aneuploid medulloblastomas may require radiation therapy only, whereas those with diploid medulloblastomas (especially if incompletely resected) would need more vigorous treatment, including radiotherapy and chemotherapy.

References
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Address reprint requests to: Tadanori Tomita, M.D., Division of Pediatric Neurosurgery, Children's Memorial Hospital, 2300 Children’s Plaza, Chicago, Illinois 60614.