Biological Stratification of Human Neuroblastoma by Complex “B” Pathway Ganglioside Expression

Simone Hettmer, Carolin Malott, William Woods, Stephan Ladisch, and Karen Kaucic

Glycobiology Program, Center for Cancer Research, Children’s Research Institute and the Department of Pediatrics, George Washington University School of Medicine and Health Sciences, Washington DC 20050 [S. H., C. M., S. L., K. K.], and AFLAC Cancer Center, Emory University and Children’s Healthcare of Atlanta, Atlanta Georgia 30322 [W. W., J. W.]

ABSTRACT

Ganglioside metabolism has been linked to the clinical and biological behavior of human neuroblastoma. This study investigated the importance of differences in complex “b” ganglioside (GD1b, GT1b, and GQ1b; designated CbG) expression in this tumor. Gangliosides of 74 neuroblastomas were analyzed by high-performance TLC. Associations of CbG expression with known prognostic markers and with event-free survival (EFS) were evaluated. Higher CbG expression characterized nonprogressve versus progressive tumors (median 41% versus 18% of total gangliosides; P = 0.001) and completely accounted for the observed higher overall “b” pathway ganglioside expression (median 81% versus 68%; P = 0.003). In contrast, expression of the structurally simpler “b” pathway gangliosides (GD2 and GD3) did not differ (median 31% versus 35%; P = 0.4). Absolute CbG content differed even more (median 93 versus 29 nmol/g among nonprogressive versus progressive tumors; P = 0.02) and was most striking in the case of GQ1b content (8-fold higher in nonprogressive tumors). High CbG (>35% of total gangliosides) expression was strongly predictive of a favorable outcome in: (a) the entire study population (90% versus 60% EFS at 25 months; P = 0.001); and (b) among patients assigned a low-risk status by a either single genetic or biochemical tumor marker (MYCN, DNA, NSE, or ferritin), or by both unamplified MYCN and aneuploid DNA (22–28% difference in EFS at 25 months). These data suggest that high tumor CbG content may substantiate “good prognosis” neuroblastoma patients, identifying patients at very low risk of relapse or death, and that the biological roles of CbG in neuroblastoma will be of importance to define.

INTRODUCTION

Tumor progression is a complex multifactorial process, dependent in part on interactions between the tumor cell and the host. One process, which is known to alter the tumor cell microenvironment, is the synthesis, expression, and release of tumor cell surface gangliosides (1). Ganglioside metabolism is known to vary between neuroblastoma tumors with different malignant potential, and may ultimately affect clinical behavior and patient outcome (2–4), and recent evidence supports a role for tumor gangliosides as prognostic indicators in neuroblastoma (5).

Gangliosides are cell membrane-associated glycosphingolipids, prominent in neural tissue. They consist of a carbohydrate chain, containing one or several sialic acids, and a lipid portion (ceramide), which anchors the ganglioside molecule in the cell membrane. Ganglioside biosynthesis in human tissue (Fig. 1) occurs in a sequential order of glycosylation via two major pathways designated “a” (GM2, GM1a, and GD1a) and “b” (GD3, GD2, GD1b, GT1b, and GQ1b), with a common precursor (GM3). Analogous steps in “a” and “b” pathway ganglioside biosynthesis are catalyzed by the same glycosyltransferases (6). Ganglioside molecules are overexpressed frequently in tumor cells, and actively shed into the local microenvironment and the plasma of tumor-bearing patients (1, 4, 7, 8). Tumor-derived gangliosides are generally believed to alter the tumor microenvironment to favor tumor progression (1). However, studies published recently (9–11) suggest that the three polysialated “b” pathway gangliosides (GD1b, GT1b, and GQ1b), here designated as CbG, may suppress tumor growth.

Neuroblastoma, a malignant tumor of neural crest origin, is the most common extracranial solid neoplasm in childhood. Compared with normal brain, neuroblastoma tissues overexpress the “b” pathway disialo-ganglioside GD2 (7). Expression of GD2 is an indicator of the presence of neuroblastoma, and high levels of shed GD2 in the circulation have been correlated with more rapid disease progression among patients with advanced disease (4). At the same time, decreased or absent expression of two CbG subspecies, GD1b and GT1b, has been linked to reduced survival in neuroblastoma (2, 3). These observations are consistent with ganglioside changes observed in neural tumors other than neuroblastoma (12–14) and support the concept that differences in “b” pathway ganglioside expression may contribute to the biological behavior of neuroblastoma.

We have observed previously a predominance of total “b” pathway gangliosides in low-stage tumors and infant neuroblastoma (a disease category with generally favorable prognosis), and a strong correlation between “b” pathway ganglioside predominance and improved EFS (5). The present study establishes a link between CbG expression and prognosis, and demonstrates the potential utility of tumor ganglioside expression in the biological stratification of neuroblastoma patients.

MATERIALS AND METHODS

Neuroblastoma Tumors. A comprehensive evaluation of total and individual ganglioside content of 74 neuroblastoma samples accrued in the QNSP was undertaken. The QNSP was an international cooperative study initiated in North America to evaluate the benefits of neuroblastoma mass screening (15). Appropriate informed consent was obtained for all of the patients. QNSP patients with neuroblastoma were treated based on age, stage, and biological features on specific Pediatric Oncology Group protocols open during the study period (Pediatric Oncology Group studies 8105, 8110, 8741–8743, 8844, 9140, 9243, 9244, 9248, and 9340–9343). EFS was 78% in the whole QNSP versus 81% in the present cohort. Disease was diagnosed clinically in 49 cases and detected by screening in 25 cases. Patients were staged according to the INSS. The definition of an event in this cohort of patients was disease progression, relapse, or death from disease. The study outcome was defined as EFS (alive without disease progression or recurrence) at 36 months from diagnosis. Virtually all of the events and deaths (13 of 14) occurred by 36 months.

All 74 of the samples were obtained by QNSP before any systemic therapy was initiated. Before ganglioside analysis, tumor samples were stored at −70°C. Total “b” pathway ganglioside content of 68 tumors was the subject of a previous report (5).

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2 To whom requests for reprints should be addressed, at Center for Cancer Research, Children’s National Medical Center, 111 Michigan Avenue NW, Washington, DC 20010. Phone: (202) 884-3217; Fax: (202) 884-3929; E-mail: kkaucic@cnmc.org.

The abbreviations used are: CbG, complex “b” pathway gangliosides; HPTLC, high-performance TLC; EFS, event-free survival; QNSP, Quebec Neuroblastoma Screening Project; INSS, International Neuroblastoma Staging System; NSE, neuron-specific enolase.
Data on outcome as well as clinical and biological tumor characteristics were obtained from the QNSP database. Although there were some missing values, data on the after prognostic markers of survival were available: MYCN, DNA index, NSE, ferritin, histology by Shimada criteria, INSS stage, and age at diagnosis. The following criteria defined markers as indicating a favorable versus unfavorable prognosis: INSS stage (3, 4 versus 1, 2, 4 s), age (<1 year versus >1 year at diagnosis), Shimada histology (favorable versus unfavorable), ferritin (>142 ng/ml versus ≤142 ng/ml), NSE (<100 ng/ml versus ≥100 ng/ml), MYCN (unamplified = 1 copy versus amplified >1 copy), and DNA index (aneuploid versus nonaneuploid; Refs. 16, 17). Aneuploid (favorable) DNA status was defined by a DNA index ≥1.1 and <1.9, or ≥2.4 (hyperdiploid/near triploid). Nonaneuploid (unfavorable) DNA status was defined by a DNA index ≥1.0 and <1.1, or ≥1.9 and <2.4 (near diploid/near tetraploid; Refs. 17–19).

**Ganglioside Purification and Quantification.** Neuroblastoma tumor specimens (0.05–0.5 g) were thawed, homogenized, and lyophilized. Gangliosides were purified by solvent partitioning as described previously (20), separated by HPTLC, stained with resorcinol-HCl (7), and quantified as nmol lipid-bound sialic acid by integration of the area under the peaks after the plates were scanned (5).

Expression of individual tumor gangliosides was measured in two ways: (a) as the percentage of total tissue-derived gangliosides (74 tumors); and (b) as absolute ganglioside content (nmol lipid-bound sialic acid; 16 tumors) using known concentrations of human brain gangliosides as an internal standard on each HPTLC plate. Gangliosides were classified first by major biosynthetic pathways (“a” or “b”), and then subdivided according to structural complexity at the level of GD1b/GM1a synthase (3). The designation CbG denotes the three terminal molecules of the “b” pathway, GD1b, GT1b, and GQ1b.

**Statistical Analysis.** The study outcome was defined as EFS (alive without disease progression or recurrence) at 36 months from diagnosis. Thirty-six
months was chosen because virtually all of the deaths and events (13 of 14) occurred within this time frame.

Dotplots and two-sample Wilcoxon tests compared relative ganglioside expression in the tumors of patients remaining event-free, to that in the tumors of patients experiencing disease progression, recurrence, or death by 36 months after diagnosis. Medians, interquartile ranges, and exact Wilcoxon two-sample tests compared absolute ganglioside content for those remaining event-free to those experiencing an event by 36 months for the 16 tumors for which absolute ganglioside content was measured.

The receiver operating characteristic curve using 36-month EFS illustrated trade-offs in the predictive performance of relative CbG expression (21). A threshold of 35% provided 85% sensitivity and 62% specificity to detect an event. Subsequently, CbG content <35% defined low CbG levels. Kaplan-Meier curves illustrated survival in patients whose tumors presented with or without low CbG. A log-rank test provided the P for this comparison.

Frequencies and Fisher’s exact tests evaluated associations between known prognostic markers (DNA index, MYCN status, NSE, ferritin, Shimada classification, INSS stage, age at diagnosis, and total “b” pathway ganglioside expression) and low CbG expression.

To evaluate the potential importance of low CbG expression as an independent predictor of outcome, frequencies and Fisher’s exact tests compared EFS for patients whose tumors presented with low (<35%) or high (≥35%) CbG content within subcohorts defined by favorable status for each of the known prognostic markers. Wilcoxon two-sample tests and dotplots compared relative CbG levels among those with and without an event, within subsets defined by stage and DNA index. The log rank test compared survival between patients whose tumors presented with or without low CbG expression, within a subset of patients in whom aneuploid DNA index and nonamplified MYCN suggested low-risk disease.

Throughout, all of the statistical tests were two sided, and Ps are provided as descriptors of the strength of associations of the overall patterns and consistency of findings that support the conclusions drawn.

RESULTS

High-Risk Neuroblastoma Is Characterized by Low CbG Expression. The impetus for this study was our previous finding (5) of higher survival in patients with neuroblastoma tumors whose total “b” pathway ganglioside content was ≥60% of total gangliosides. To determine whether consistent changes in the expression of individual “b” pathway components (as a percentage of total gangliosides) might reveal a more specific feature of ganglioside metabolism characterizing neuroblastomas of different degrees of clinical aggressiveness and outcome, we determined the expression of the sum of all gangliosides of the “b” pathway, as well as each “b” pathway component individually and in subgroups according to structurally complexity, using densitometric scans of the HPTLCs of the total gangliosides we had purified from each tumor.

Three specific observations derive from the present study. First, consistent with results reported previously (5), a predominance of “b” pathway gangliosides characterized tumors of patients with good outcome (Fig. 2A). In the majority of tumors from event-free patients, relative total “b” pathway ganglioside content clustered between 75% and 90% (median 81%) in contrast to lower relative total “b” pathway ganglioside content in poor-prognosis patients (median 68%; P = 0.003). Second, there was no difference in the expression of GD2 and GD3 between event-free survivors and patients experiencing an event by 36 months. This lack of difference was apparent when these structurally simple “b” pathway gangliosides were analyzed both together (median 35% versus 31%; P = 0.4; Fig. 2B) and separately (GD3 15% versus 13%, P = 0.8 and GD2 18% versus 15%, P = 0.4). Third, there was a striking difference in CbG (GD1b, GT1b, and GQ1b) content, which was significantly higher (median 40% of total gangliosides) in tumors of patients who were event-free by 36 months compared with those patients with an unfavorable outcome (median 18%; P = 0.001; Fig. 2C). Thus, the difference in total “b” pathway ganglioside expression was wholly attributable to a difference in CbG expression. This difference was also apparent when each CbG component was analyzed separately (GD1b 10% versus 4%, P = 0.03; GT1b 22% versus 15%, P = 0.002; and GQ1b 8% versus 1%, P = 0.001).

To corroborate differences found by evaluating relative ganglioside content, we used a second approach, analysis of absolute ganglioside content, in the subset of 16 tumors for which sufficient material was available (Table 1). Total ganglioside content was similar in nonprogressive and progressive tumors (median 198 nmol/g versus 126 nmol/g; P = 0.2), as shown previously (7). There was no significant difference, either, in the content of the structurally simple “b” pathway gangliosides GD2 and GD3 (median 63 nmol/g versus 61 nmol/g; P = 1.0). However, absolute CbG content was higher in tumors from event-free patients compared with those of patients who relapsed or died (median 93 nmol/g versus 29 nmol/g; P = 0.02), mirroring the data in Fig. 2. Additional analysis of individual CbG components revealed that the most striking difference was in GQ1b content, which was almost 8-fold higher in nonprogressive versus progressive tumors (median 15 nmol/g versus 2 nmol/g; P = 0.03).

Taken together these analyses reveal that high CbG expression is characteristic of good prognosis neuroblastoma tumors. Additional
analyses evaluated the potential utility of relative CbG content as a prognostic marker in neuroblastoma.

C8G Expression Predicts Outcome in Neuroblastoma. The rigorous analysis and the eventual clinical application of ganglioside expression as a prognostic marker in neuroblastoma requires defining objectively a cut-point for ganglioside expression. We have previously defined a cut-point of 60% for total “b” pathway gangliosides (S). In the present analysis of CbG content, we established a cut-point (dichotomization of CbG expression above and below a threshold) using receiver operating curve analysis (21) at 35% to favor sensitivity over specificity, maintaining high sensitivity (85%) with moderate specificity (62%), based on 36-month EFS.

Kaplan-Meier curves illustrate the ability of CbG expression, dichotomized at 35%, to discriminate between patients surviving event-free and those experiencing relapse or death (P = 0.001 by log rank test; Fig. 3). Visual inspection of the graph reveals 30% higher survival after 25 months for patients whose tumors expressed high CbG levels. Similarly, Kaplan-Meier analysis evaluating overall survival reveals 24% higher survival for patients whose tumors expressed high CbG levels compared with tumors with low CbG content (P = 0.001 by log rank test; data not shown). These data show a striking association between high tumor CbG content and good patient outcome, and suggest a role for CbG expression in the identification of patients at very low risk of relapse or death.

Prognostic Utility of CbG Expression in Conjunction with Known Markers. To evaluate the value of CbG expression as a tumor-associated biological variable, we analyzed the prognostic impact of differences in relative CbG content, assessed in conjunction with established genetic, biochemical, and clinical prognostic factors. Table 2 summarizes associations between individual known markers and CbG content, and demonstrates that unfavorable known marker status correlates highly with low CbG expression. The proportion of tumors with low CbG levels (<35%) ranged between 53% and 100% among tumors with unfavorable status for individual markers. A number of specific observations are highly significant. First, 82% of tumors with unfavorable histology but only 32% with favorable histology were low in CbG expression (P = 0.005). Secondly, 68% of nonaneuploid neoplasms but only 33% with aneuploid DNA status presented with low CbG expression (P = 0.009). Thirdly, all 4 of the MYCN amplified tumors but only 43% of nonamplified tumors had low CbG content (P = 0.04). Overall, the high correlation with known genetic, biochemical, and clinical indicators of poor outcome leads to the conclusion that low CbG expression is associated with a poor prognosis in unfavorable neuroblastoma.

Known poor prognostic variables in neuroblastoma, for example MYCN amplification and nonaneuploid DNA, have high specificity (0.97 and 0.71, respectively), consistent with their clinically proven value as highly reliable indicators of adverse outcome in neuroblastoma (22, 23). CbG expression, on the other hand, has high sensitivity (0.85), thereby suggesting a complementary role in prognostic evaluation of patients, especially in patients in whom favorable status for known markers suggests a good prognosis. Therefore, we examined whether the CbG content of tumors adds novel prognostic information in these patients. Among 53 patients whose NB tumors were diagnosed as a low INSS stage, 4 patients subsequently experienced an event. All 4 of these NB tumors had favorable Shimada histology, MYCN, and/or DNA status, whereas not a single tumor had favorable CbG content (≥35%). Conversely, among 21 patients whose NB tumors were diagnosed as a high INSS stage, 9 patients experienced an event. Whereas 7 of these 9 poor outcome tumors (88%) had favorable MYCN status, only 2 of 9 tumors (22%) had favorable CbG content (Table 3). Thus, in both cases (low and high INSS stage), low unfavorable) CbG content was associated with a poorer outcome. Table 4 demonstrates that among the 53 patients with limited disease (low INSS stage), survival was 18% higher among the 31 patients with high tumor CbG content compared with the 22 patients with low CbG content (P = 0.03). Similarly, for each genetic (MYCN and DNA) or biochemical (NSE and Ferritin) marker studied, in each patient group characterized by favorable marker status, the percentage of event-free survivors was 22–28% higher among those with tumors with high CbG versus those with low CbG content (P < 0.05 in all of the cases). The association was especially strong for tumors characterized by either aneuploidy or unamplified MYCN. EFS among the 29 patients whose MYCN unamplified tumors had low CbG levels was 72% (21 of 29) compared with 95% (37 of 39) among 39 patients having high CbG content (P = 0.01; Table 4). Similarly, the 16

Table 1 Absolute content of individual ganglioside species

<table>
<thead>
<tr>
<th>Ganglioside</th>
<th>Event by 36 months</th>
<th>Event-free by 36 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (25–75 percentile)</td>
<td>Median (25–75 percentile)</td>
</tr>
<tr>
<td>Total “b”</td>
<td>97 (37–145)</td>
<td>149 (119–191)</td>
</tr>
<tr>
<td>GD3 + GD2</td>
<td>61 (30–89)</td>
<td>63 (40–74)</td>
</tr>
<tr>
<td>CbG</td>
<td>29 (7–63)</td>
<td>93 (58–133)</td>
</tr>
<tr>
<td>GD1b</td>
<td>5 (0–14)</td>
<td>21 (8–38)</td>
</tr>
<tr>
<td>GT1b</td>
<td>22 (7–38)</td>
<td>51 (36–62)</td>
</tr>
<tr>
<td>GQ1b</td>
<td>2 (0–10)</td>
<td>15 (11–19)</td>
</tr>
</tbody>
</table>

* mmol LBSA/g tissue.
* n = 41.
* n = 43.

Table 2 Prevalence of low CbG expression among patients with favorable versus unfavorable status by individual prognostic markers

<table>
<thead>
<tr>
<th>Prognostic marker</th>
<th>Total N</th>
<th>CbG deficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA index</td>
<td>49</td>
<td>16 (33)</td>
<td>0.009</td>
</tr>
<tr>
<td>Nonaneuploid</td>
<td>22</td>
<td>15 (68)</td>
<td>0.009</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>49</td>
<td>16 (33)</td>
<td>0.009</td>
</tr>
<tr>
<td>MYCN</td>
<td>4</td>
<td>4 (100)</td>
<td>0.04</td>
</tr>
<tr>
<td>Amplified</td>
<td>68</td>
<td>29 (43)</td>
<td>0.04</td>
</tr>
<tr>
<td>Nonamplified</td>
<td>4</td>
<td>4 (100)</td>
<td>0.04</td>
</tr>
<tr>
<td>Ferritin</td>
<td>19</td>
<td>10 (53)</td>
<td>0.4</td>
</tr>
<tr>
<td>&gt; 142 ng/ml</td>
<td>51</td>
<td>20 (39)</td>
<td>0.4</td>
</tr>
<tr>
<td>≤ 142 ng/ml</td>
<td>68</td>
<td>29 (43)</td>
<td>0.4</td>
</tr>
<tr>
<td>Histology (Shimada)</td>
<td>11</td>
<td>9 (82)</td>
<td>0.005</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>56</td>
<td>18 (32)</td>
<td>0.005</td>
</tr>
<tr>
<td>Favorable</td>
<td>3, 4</td>
<td>21</td>
<td>12 (57)</td>
</tr>
<tr>
<td>INSS stage</td>
<td>1, 2, 4s</td>
<td>53</td>
<td>22 (42)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>29</td>
<td>21 (72)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥ 1 year</td>
<td>45</td>
<td>13 (29)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* CbG < 35%.
* From Fisher’s exact tests.
simple gangliosides. Circles represent tumors with favorable status for the known variable. Squares represent tumors with unfavorable status for the known variable. Open symbols represent tumors from patients surviving event-free at 36 months. Solid symbols represent patients with an event before or at 36 months follow-up. Solid bars represent the median of each group. The shaded top left quadrants in each panel represent a range of marker values characterizing tumors with very low risk of progression. Ps were determined by two-sample Wilcoxon tests.

Fig. 5. Probability of EFS in 48 neuroblastoma patients with aneuploid and MYCN unamplified tumors, stratified on the basis of tumor CbG content. Tick marks indicate censored patients. Group A: CbG content $\geq$35% (n = 33, ---); Group B, CbG content $<35%$ (n = 15, - -).

Table 3. Marker status among patients with low (1, 2, 4) versus high (3, 4) INSS stage substratified by outcome at 36 months

<table>
<thead>
<tr>
<th>Genetic markers</th>
<th>Low INSS stage (1, 2, 4s)</th>
<th>High INSS stage (3, 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Event</td>
<td>Event-free</td>
</tr>
<tr>
<td>N$^{a}$</td>
<td>4</td>
<td>49</td>
</tr>
<tr>
<td>Age &lt; 1 year$^{b}$</td>
<td>2/4 (50%)</td>
<td>34/49 (69%)</td>
</tr>
<tr>
<td>Favorable Shimada histology$^{b}$</td>
<td>2/2 (100%)</td>
<td>4/49 (100%)</td>
</tr>
<tr>
<td>Unamplified MYCN$^{b}$</td>
<td>3/3 (100%)</td>
<td>48/49 (100%)</td>
</tr>
<tr>
<td>Aneuploid DNA$^{b}$</td>
<td>4/4 (100%)</td>
<td>35/48 (73%)</td>
</tr>
<tr>
<td>CbG $\geq$ 35%$^{b}$</td>
<td>0/0 (0%)</td>
<td>31/49 (63%)</td>
</tr>
</tbody>
</table>

$^{a}$ Total N. $^{b}$ n/N (%).

Table 4. Proportion of EFS at 36 months among patients with favorable status for known markers, stratified by CbG expression

<table>
<thead>
<tr>
<th>CbG &lt; 35%</th>
<th>CbG $\geq$ 35%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total N</td>
<td>Survivors$^{a}$</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>Genetic markers</td>
<td></td>
</tr>
<tr>
<td>Aneuplod</td>
<td>16</td>
</tr>
<tr>
<td>MYCN unamplified</td>
<td>29</td>
</tr>
<tr>
<td>Biochemical markers</td>
<td></td>
</tr>
<tr>
<td>NSE $\leq$ 100 ng/ml</td>
<td>25</td>
</tr>
<tr>
<td>Ferritin $\leq$ 142 ng/ml</td>
<td>20</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Shimada favorable</td>
<td>18</td>
</tr>
<tr>
<td>Clinical variables</td>
<td></td>
</tr>
<tr>
<td>Low INSS stage (1, 2, 4s)</td>
<td>22</td>
</tr>
<tr>
<td>Age &lt; 1 year</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>11 (85)</td>
</tr>
</tbody>
</table>

$^{a}$ Event-free. $^{b}$ Fisher’s exact test.

patients whose aneuploid (and as such favorable) tumors contained $<35%$ CbG had an EFS of 69% (11 of 16) compared with 97% (32 of 33) among the 33 patients with $\geq35%$ tumor CbG expression ($P = 0.01$; Table 4). Therefore, low CbG content, even in the face of a favorable marker status, suggests an increased risk of relapse or death. High CbG expression, on the other hand, is associated with a very low risk of disease progression or death. These data illustrate the sensitivity of CbG in the substratification of neuroblastoma, thus suggesting a complementary role for CbG in patients in whom favorable status for known markers suggests a good prognosis.

To illustrate the relationship between favorable known marker status and high CbG expression, the interaction between CbG levels and DNA index was analyzed in depth (Fig. 4A). Overall, CbG content was significantly lower in aneuploid tumors than in aneuploid tumors (median 22% versus 42%; $P < 0.001$). Subclassification of tumors on the basis of patient outcome at 36 months after diagnosis demonstrated that for patients with nonaneuploid tumors ($n = 22$), outcome was independent of CbG expression (median 29% among event-free survivors versus 17% among those with disease progression; $P = 0.3$). In contrast, in patients with aneuploid tumors ($n = 49$), EFS was associated with higher CbG content among survivors compared with those with disease progression (median 46% versus 31%; $P = 0.005$). Therefore, the simultaneous presence of an aneuploid DNA index and high CbG content (shaded top left quadrant in Fig. 4A) identified a subset of tumors at very low risk of disease progression or death.

We applied an analogous model to analyze the discriminative value of CbG expression in conjunction with INSS stage (INSS 1, 2, 4 s versus INSS 3, 4) at diagnosis (Fig. 4B). The simultaneous presence of low INSS stage and high CbG content (shaded top left quadrant in Fig. 4B) again identified a subset of tumors at very low risk. Moreover, among patients with clinically advanced disease ($n = 21$), CbG content was higher in tumors from survivors compared with patients with disease progression (median 46% versus 15%; $P = 0.01$). Therefore, low CbG content was associated with a worse outcome than that suggested by advanced clinical stage alone.
Taken together, these data suggest a role for CbG content in the prognostic substratification of neuroblastoma patients with favorable marker status by identifying patients at risk of relapse or death despite their categorization as “good prognosis” patients by established parameters.

DISCUSSION

The present study demonstrates that high CbG expression (GD1b, GT1b, and GQ1b) characterizes favorable neuroblastoma tumors and accounts completely for the previously observed increased overall “b” pathway ganglioside expression in these tumors. Similarly, absolute CbG content, determined in a subset of tumor specimens, was higher in good-prognosis tumors and was most striking with respect to GQ1b content. In contrast, low CbG expression (<35%) was predictive of adverse outcome in univariate analysis and highly correlated with unfavorable status by known indicators of poor prognosis, thus supporting the concept that low CbG content is a biological feature of the clinically most aggressive neuroblastoma phenotypes.

The study population that we used contained predominantly low-risk, good prognosis tumors. However, even within this group of patients, our results were highly significant. With respect to the potential prognostic utility of CbG expression in neuroblastoma, the major finding of this study is that high CbG content is associated with decreased risk of progression among neuroblastoma tumors with an otherwise favorable phenotype. Low-risk status as assessed by established prognostic markers generally defines a heterogeneous (with respect to outcome) group of tumors, including a number of progressive tumors. High CbG expression is strongly associated with good outcome in this neuroblastoma cohort, permitting an additional biological stratification of tumors with favorable status according to established markers by identifying those cases with the lowest risk of progression. Substratification such as this may have particular utility when favorable biological markers are present in patients with advanced-stage disease.

The potential relevance of differences in CbG expression is emphasized by earlier published findings linking low or absent expression of certain gangliosides, namely GD1b and GT1b, to reduced survival in neuroblastoma (2, 3). Furthermore, a progressive loss of CbG has been associated with higher histological grades of malignancy, and lower survival in astrocytoma and medulloblastoma (12, 13), and increased immunohistochemical staining intensity for GD1b in brain tumor tissue has been correlated with lower tumor grade and increased survival (14). Finally, expression of CbG in brain tumor andveal melanoma tissue is reduced in comparison with normal brain and chorioidia (24, 25).

A substantial body of previous experimental evidence supports a role for the “b” pathway disialoganglioside GD2 in diagnosis and treatment of neuroblastoma (4, 7, 8). Because of the widespread expression of GD2 in neuroblastoma tissue, in contrast to the more benign ganglioneuroma and ganglioneuroblastoma, GD2 is a sensitive diagnostic marker distinguishing neuroblastoma from other related tumors (7) and a target antigen in therapeutic-consolidation regimens using monoclonal anti-GD2 antibodies (26). High concentrations of GD2 in the plasma of neuroblastoma-bearing patients have been associated with more rapid disease progression and lower survival rates in advanced-stage disease (4). However, just as with total tumor ganglioside expression, clinical behavior is independent of tumor GD2 content (7) demonstrating its diagnostic but not prognostic utility. In the present study, consistent with these previous observations, GD2 was a major ganglioside in neuroblastoma tissue, but patient outcome was independent of its expression in tumor tissue.

Tumor gangliosides are generally believed to favor tumor progression, and previous findings suggest that high concentrations of certain gangliosides are associated with increased tumorigenicity (1, 27). The present study underscores the functional importance of qualitative aspects of ganglioside expression in tumor tissue (28) and serves to emphasize that the biological effects of tumor-derived gangliosides may differ depending on the complexity of carbohydrate and ceramide structures. In fact, substantial experimental evidence supports the possibility that CbG in the tumor microenvironment might act to inhibit rather than promote progression of malignant disease. Malignant transformation of murine epithelial cells in response to tumor-promoting phorbol esters is accompanied by a decrease in GT1b synthesis (9). Proliferation of, and interleukin 8 production by, human metastatic melanoma cells is inhibited by GD1b, GT1b, and GQ1b (10). These gangliosides also modulate immunoglobulin synthesis in peripheral blood mononuclear cells (29–31) and cause a shift from T-helper 2 to T-helper 1 cytokine production in phytohemagglutininstimulated T-cells (32). Platelet-derived growth factor-mediated cell growth and platelet-derived growth factor receptor activation in neuroblastoma and glioma cells is inhibited by complex ganglioside species (11). Finally, increased expression of GD1b and GT1b in rat pheochromocytoma cells transfected with GD3 synthase occurs in parallel with trk-A dimerization (33), a phenomenon associated with improved prognosis in neuroblastoma. Taking these results together with ours, we speculate that CbG may function not only as tumor markers, but may also serve a protective role by impeding tumor progression.

Several possible mechanisms may underlie the alterations in ganglioside metabolism we have described, including down-regulation of enzymes at the transcriptional level, altered substrate affinity, or induction of aberrant enzymes (34, 35). In the present study, the decrease in CbG content was most striking with respect to GQ1b, suggesting that a disruption in CbG synthesis might take place far downstream, at the level of GT1b/GD1a synthase or GQ1b/GT1a synthase.

In conclusion, our results suggest that CbG expression may be a highly sensitive prognostic variable in neuroblastoma and support the hypothesis that alterations in ganglioside metabolism contribute to the clinical behavior of neuroblastoma. Future studies, exploring the metabolic alterations underlying changes in CbG expression and the biological role of individual ganglioside molecules in tumorigenesis, will contribute to our understanding of the biology of neuroblastoma and may ultimately identify novel targets in neuroblastoma therapy.

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Biological Stratification of Human Neuroblastoma by Complex "B" Pathway Ganglioside Expression

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