Whole Chromosome Alterations Predict Survival in High-Risk Neuroblastoma without MYCN Amplification

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Abstract  
Purpose: Patients with stage IV neuroblastoma over the age of 500 days without MYCN amplification have a survival rate of <30% and there are currently no reliable means of predicting which of these patients will survive or succumb to the disease. The goal of this study is to develop a DNA copy number–based prognostic profile for these patients.

Experimental Design: We have used comparative genomic hybridization to identify genome copy number changes that can predict outcome in patients with stage IV neuroblastoma without MYCN amplification.

Results: A strong correlation of patient survival with the presence of whole chromosome changes (WCC ≥2) was observed, even in the group of patients older than 500 days at time of diagnosis. This novel prognostic marker showed a significant dependence on the date of diagnosis; patients with WCC ≥2 diagnosed after 1998 had a significantly higher probability of survival compared with those diagnosed earlier. At the same time, no such time dependence was found among the samples with WCC ≥2, suggesting that medical progress patients in recent years has particularly benefited those patients with a stage IV non–MYCN-amplified disease if WCC ≥2 were present.

Conclusions: In this pilot study, we present a novel prognostic marker for survival of high-risk neuroblastoma patients over the age of 500 days without MYCN amplification and diagnosed after 1998. Further validation study is required to establish this risk stratification for these patients.

Neuroblastoma, the most common solid extracranial tumor of childhood, is derived from the sympathetic nervous system and is the cause for 15% of all cancers related to death of children in the United States (1). A hallmark of the disease is its heterogeneity (2); the outcome ranges from spontaneous regression to death. Treatment options vary accordingly, from observation only via a minimal therapy using surgery alone to an aggressive use of high-dose chemotherapy and radiotherapy combined with autologous bone marrow transplant, depending on the expected aggressiveness of the tumor. Correct stratification of a diagnosed tumor is thus a key in determining a patient’s treatment. In North America, the Children’s Oncology Group (COG) currently stratifies patients into three groups (low, intermediate, and high risk) based on a well-characterized panel of clinical and tumor biological factors. The survival probability for patients with low-risk disease exceeds 90%; however, it is currently only ~30% (3) in the high-risk group. Although the survival rate for high-risk patients is very low, there is some evidence of modest improvement in this rate compared with ~20 years ago when the survival rate was ~10% (4).

COG risk stratification is based on International Neuroblastoma Staging System (INSS) stage, Shimada histology, patient’s age, and MYCN amplification status (5). The lower, localized stages (INSS stage I and IIa/b) are most of the time associated with a low risk, whereas more advanced stages (INSS stages III and IV) imply intermediate or high risk. Specific combinations of the other factors modify this assessment: If all other negative factors, namely age ≥365 days, unfavorable Shimada histology, and MYCN amplification, are present simultaneously in stage II, the disease is stratified as high risk. Conversely, the aggressiveness of neuroblastoma is slightly lowered (to intermediate) if MYCN amplification is absent and the patient is either young (<365 days) or if the tumor has a favorable Shimada histology. An effort by the International Neuroblastoma Risk Group to develop a new risk stratification is under way (1), aiming to replace the currently regionally varying risk assessments. Although still in a draft stage, the new risk stratification will very likely adjust the age cutoff from 1 year to 18 months to adapt for recent observations (6) that this higher cutoff improves prognostic precision.

The advent of high-throughput molecular profiling techniques has led to increased efforts to identify additional molecular prognostic markers in neuroblastoma, probably motivated in part by the hope to further refine the existing
risk stratification, ultimately leading to better informed treatment decisions. In recent years, microarray techniques have been used to analyze neuroblastoma mRNA transcript levels, DNA copy number (7–11), and micro-RNA expression levels (12). Five studies (13–17) reported development of prognostic classifiers based on mRNA level analysis. The various gene sets overlapped only slightly, thus making a biological interpretation more difficult and slowing down a potential adaptation in the clinic. A significant part of the complexity of mRNA transcription profiles arises from the unknown dynamics of the transcription regulation network. The absence of any dynamic regulation makes classifiers derived from DNA copy number alterations much simpler in this respect. Furthermore, the “normal” state of a cell, two DNA copies (up to copy number polymorphisms), is exactly known, which further reduces the complexity of copy number–based analysis compared with expression profiles, where much less is known about the “ground state” of expression levels in the corresponding normal cell. Besides MYCN amplification, which is a major factor in the current risk stratification, other genomic alterations were also reported to correlate with outcome. Near triploidy correlates with positive outcome but only in patients younger than 1 year (18). Loss of chromosome 11q23 is frequently observed in single-copy MYCN neuroblastoma and was found to correlate with negative outcome (19, 20). Also, combinations of complex cytogenetic aberration patterns and expression profiles were recently shown to be complementary usable as predictive markers for patient survival (21).

The goal of this study was to develop a DNA copy number–based prognostic profile for high-risk neuroblastoma, which complements the existing stratification factors. Array-based comparative genomic hybridization (CGH) was used to generate DNA copy number data. Earlier work had indicated a strong correlation of the diverse factors in the current risk stratification of neuroblastoma (11, 22), such as stage and MYCN status with the pattern of recurrent genomic alterations. We focused in this report on INSS stage IV neuroblastomas without MYCN amplification for which there remains no currently available methods to predict which of them over the age of 500 days will survive on current conventional therapy.

Materials and Methods

Tumor samples. Fifty-eight pretreatment primary neuroblastoma tumor samples with outcome annotations and follow up for at least 3 years were obtained retrospectively from three sources presenting time of diagnosis between 1992 and 2002 (Table 1). Of these, 32 samples came from patients older than 500 days at the time of diagnosis. Metastatic stage IV neuroblastomas in patients older than ~18 months are considered high risk, whereas in younger patients the disease is currently stratified as an intermediate risk. All of the patients were treated according to local or national guidelines that followed similar protocols. All of the samples were anonymized, and our protocol was deemed exempt from the NIH Multiple Project Assurance. Pretreatment tumor samples were snap frozen in liquid nitrogen after removal. Tumors were diagnosed as neuroblastoma by local centers experienced in the management of these cancers. Patients were divided into two outcome groups: The “good outcome” group had event-free survival (i.e., neither relapsed nor neuroblastoma progression; n = 29) and the “poor outcome” group died due to the disease (n = 29).

Oligonucleotide and cDNA array CGH. Patient tumor genomic DNA samples were extracted from the interphase of a Trizol preparation according to the manufacturer’s instructions (Invitrogen). Genomic DNA was treated with RNase A and protease (Qiagen), and purified by phenol/chloroform extraction followed by ethanol precipitation. Control male human genomic DNA samples (Promega) were used in all hybridizations. For oligonucleotide array CGH experiments, array construction, DNA labeling, hybridization, and washing were done as described in detail by Selzer et al. (9). Briefly, the tiling-path CGH arrays were designed for whole-genome analysis with up to 385,000 oligonucleotides from unique sequence regions that are of variable length to achieve a melting temperature of 76°C. Genomic DNA was randomly fragmented by sonication to a size range of 500 to 2,000 bp. Each DNA sample is directly labeled by random primers extension labeling (Cy3 for tumor sample and Cy5 for the reference DNA). The Cy3-labeled tumor sample and Cy5-labeled reference sample were combined and applied for hybridization as described (9). Data were extracted from scanned images using NimbleScan 2.0 extraction software (NimbleGen Systems, Inc.). For cDNA array CGH experiments, cDNA microarray preparation, DNA labeling, hybridization, and washing were done as described previously (7, 22). The fluorescent ratios were normalized for each array using a pin-based normalization method.

Data normalization and identification of whole chromosome changes. DNA copy number log ratios between tumor DNA and reference DNA on each microarray were standardized by mode centering: A histogram of DNA copy numbers was created for each chromosome was identified with “whole chromosome gain (loss)” if the running average (l = 100) along the chromosome was s = 3 SDs larger (or smaller) than zero for at least 95% of all loci. The delineation of whole chromosome changes (WCC) did not overly depend on the number of SDs; varying this parameter within reasonable limits, s = 2.5 . . . 4, did not change the results of our analysis. Localized copy number alterations (P value for the presence of an aberration) were identified using the topological statistics algorithm (23).

Statistical analysis and evaluation of prognosis in survival. The frequency of localized genomic alterations was estimated using a parameter-free method (7, 22). Rather than counting the number of samples Nw of the total number N1 with gains (or losses) by selecting those samples exceeding a preset threshold, this method obtains an estimator for the frequency by calculating the average P value of gains (or losses, respectively). This quantity is proportional to the frequency, as can be seen by calculating the expectation value

\[ p \approx \frac{(0.5 \times N_{w \text{ gains}}) + (0 \times N_{w \text{ losses}})}{N_1} = (1 - v) \times 0.5 \]

The probability of survival was calculated using the Kaplan-Meier method, and the significance of the difference between Kaplan-Meier curves was calculated using the log-rank test.

Results

A particular interest of CGH studies lies in the identification of recurrent genomic alterations as those regions may code for products relevant for the biology of that cancer. Figure 1
11. Columns '1' to '22' and 'X', 'Y' indicate gain ('+'), loss ('-'), or no ('0') WCC of the corresponding chromosome.

NOTE: Fifty-eight pretreatment tumor samples from stage IV without MYC/ amplification were used. Of those, 29 survived without event for at least 3 y (denoted by a '+' in the outcome column) and 29 died of the disease ('-'). Patients were diagnosed between 1991 and 2002 (diagnosis date); the age at date of diagnosis is given in days. Column 'BP17' indicates the presence ('Yes') or absence ('No') of a breakpoint leading to a gain on chromosome 17; column '11q-BP' indicates a breakpoint leading to loss of genomic material on the q-arm of chromosome 11. Columns '1' to '22' and 'X', 'Y' indicate gain ('+'), loss ('-'), or no ('0') WCC of the corresponding chromosome.

Abbreviations: BP, chromosomal breakpoint; Surv, survived; DoD, died of disease.
Loss of chromosome 11q has been linked (19) with negative outcome. In these study samples, loss of part of the q-arm of chromosome 11 (relative to the global copy number of the entire chromosome) was identified (Table 1) and it was found that loss of 11q was more frequent in samples from the deceased patients. This is compatible with the reported negative prognostic feature of 11q loss, although the result by itself was not significant (P = 0.14; Table 2A). Similarly, for all samples, 17q gain was more frequent in the negative outcome group (P = 0.07), whereas 17q gain was slightly more frequent in samples with a positive outcome when restricted to patients older than 500 days.

We next analyzed if WCC are indicative for survival. Figure 2A shows, for each chromosome, the frequency, f, of samples with WCC number alterations, separately for good or poor outcome samples. Compared with the poor outcome group, the fraction, f, of samples with WCC was larger in the good outcome group, often by more than a factor 2. Chromosome 10, for example, was most informative (P = 0.002) with f = 0.34 (10 of 29 samples from the positive outcome group) compared with f = 0.03 (1 of 29 poor outcome samples). Not all chromosomes were equally informative partially because some chromosome copy number alterations were relatively infrequent, rendering the observed differences statistically not significant. For example, chromosome 1 was changed in only 5 of the 58 total samples, and the differences between the positive and negative outcome group, f = 0.10 and f = 0.07, respectively, were not significant. The observation that all chromosomes were affected by WCC and that changes were always more frequent in the positive outcome group suggested that survival is not associated with the change of a specific chromosome, but that the aggregate number of changes indicates survival. In Fig. 2B, the frequency of samples with at least one (column A) WCC is plotted. Roughly 80% of all samples in the study fall in this category, but the distinction of the positive and negative outcome groups was poor (P = 0.1). A much clearer distinction (P = 0.003) with 23 of the 29 positive and 12 of 29 negative outcome samples was obtained when counting only samples with at least two WCC (columns B and C). The effect of further changing the chromosome number threshold is shown in the receiving operator characteristic analysis in Fig. 3A, where each square symbol in the graph represents a different number of altered chromosomes as a copy number cutoff. The symbol in the top right corner at (1,1) includes all samples, regardless of the number of WCC. Every step further toward lower sensitivity increases the threshold by 1. This analysis also shows that the prognostic power remained strong in patients older than 500 days at time of diagnosis because the receiving operator characteristic curve was not strongly changed when we removed the samples from the 20 patients <500 days (see Fig. 3A; Table 1).

Of interest on further investigation of our data, this marker’s prognostic power sensitively depended on the date of diagnosis as shown by comparison of histogram columns B and C in Fig. 2B. Although both indicate a significantly higher frequency of WCC ≥2 in the group of surviving patients, the difference between the two groups is much more pronounced when, as in column C, the samples are restricted to those diagnosed after...

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**Table 2. Association analysis**

**A**

<table>
<thead>
<tr>
<th></th>
<th>ALL</th>
<th>11q-BP</th>
<th>17q+BP</th>
<th>WCC ≥ 1</th>
<th>WCC ≥ 2</th>
</tr>
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<tr>
<td>Survived</td>
<td>29</td>
<td>14</td>
<td>17</td>
<td>25</td>
<td>23</td>
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<tr>
<td>Deceased</td>
<td>29</td>
<td>19</td>
<td>23</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>All</td>
<td>58</td>
<td>33</td>
<td>40</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td>Pdeath</td>
<td>—</td>
<td>0.14</td>
<td>0.07</td>
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<tr>
<td>Psurvive</td>
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<td>0.940</td>
<td>0.97</td>
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</table>

**B**

<table>
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<th>17q+BP</th>
<th>WCC ≥ 1</th>
<th>WCC ≥ 2</th>
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<tr>
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<td>13</td>
<td>11</td>
<td>12</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
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<td>18</td>
<td>22</td>
<td>16</td>
<td>10</td>
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<tr>
<td>All</td>
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<tr>
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</tr>
<tr>
<td>Psurvive</td>
<td>—</td>
<td>0.33</td>
<td>0.58</td>
<td>0.06</td>
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</tbody>
</table>

**NOTE:** Association of localized loss of genomic material on 11q, localized gain on 17q, and whole chromosome gains/losses with patient survival for (A) all samples and (B) the subset of samples from patients older than 500 d at time of diagnosis. Each column shows the number of samples with the corresponding characteristic separated for deceased/survived patients. In each column, a P value for association of the observed distribution with the patient’s death Pdeath or survival Psurvive is given.
After this date, copy number alterations occurred almost exclusively in the good outcome samples ($P < 0.0001$). Thus, for patients with WCC $\geq 2$, date of diagnosis was a marker for the patient's survival. Figure 3B plots the $P$ values obtained for a “running cutoff,” asking if patients after the respective cutoff date had a survival advantage over patients diagnosed earlier. In this graph, a low $P$ value for a specific year indicates that a significant difference in the survival probability exists between patients diagnosed before and after that date. The “dip” in the curve for patients with WCC $\geq 2$ indicates that the survival rates changed significantly over time, with the clearest separation set around 1997 to 1999. The maximum likelihood estimate of the survival probability with WCC $\geq 2$ diagnosed before and in 1998 was $f_0 = 0.3$; after that, the survival rate changed to more than 90% (only 1 of 17 patients with WCC $\geq 2$ died after 1998).

In the group of patients with WCC $< 2$, no significant signal for a change in survival rates was visible at any time point. For example, using 1998 as the cutoff date, the maximum likelihood estimate of survival rates before ($P_{\text{surv}} = 0.23$) and after ($P_{\text{surv}} = 0.3$) that cutoff differed only within the margin of errors.

Kaplan-Meier survival analysis showed that the patients with or without WCC $\geq 2$ have significantly different survival probabilities (Fig. 4A). In this analysis, WCC $\geq 2$ is significantly correlated with survival for the patients diagnosed after 1998, but not for the patient diagnosed before or in 1998 (Fig. 4B–C). The difference of correlation between WCC $\geq 2$ and survival in patients diagnosed before and after 1998 also exists in the patients older than 500 days (Fig. 4D).

**Discussion**

The current COG risk stratification homogeneously classifies all stage IV neuroblastomas occurring in patients older than 18 months as a high-risk disease. Currently available clinical pathologic assays do not allow further determination of which of the high-risk patients will benefit from the standard multimodal aggressive therapies with 13-cis-retinoic acid in minimal residual disease. This study showed that gain or loss of...
two or more whole chromosomes reliably predicted survival of patients with high-risk neuroblastoma, including patients older than 500 days. The established risk factors “stage” and “MYCN amplification status” were kept constant among the samples used in this study. The resulting marker is therefore not confounded with these factors, but rather identifies a molecularly defined subgroup with a high survival probability within the current high-risk group of patients. WCC thus complements (and is independent from) existing factors used in the current risk stratification of COG. The focus of this study was on high-risk neuroblastoma samples with INSS stage IV without MYCN amplification. Although samples from patients younger than 500 days (intermediate risk) were used in parts of the analysis, the observed prognostic power of WCC was shown to remain in the high-risk group of patients older than 500 days. This finding sets WCC apart from earlier study (24), reporting that hyperdiploidy in stage IV neuroblastoma without MYCN amplification predicted survival in patients younger than 18 months. By definition of near triploidy, gain of 12 to 36 additional whole chromosome copies in humans, WCC includes near-triploid (and near tetraploid) cells. Near triploidy was shown to indicate positive outcome; however, this finding was found to be valid only in patients younger than 1 year (18). By design of microarray experiments and the need to standardize the numerical values obtained from scanning the arrays, this technology is not able to measure the global copy number (ploidy) of samples.

From the original experimental setup of this study, built around high-resolution CGH microarrays, it was not anticipated to find such a low complexity prognosis prediction marker as WCC. This observation has not been described before possibly because previous analysis took into consideration patients diagnosed before 1999 when the pattern simply did not predict outcome, at least not as clearly. Our analysis indicated that the survival probability of patients with WCC ≥2 has significantly improved recently as shown in Fig. 3B: In the group of high-risk neuroblastoma patients with WCC ≥2, time of diagnosis predicts survival. The most significant separation was observed when the cutoff for the diagnosis date was set on or around 1998. Because outcome is generally the result of interplay of tumor biology and available treatment options, the improvement in outcome reflects medical progress, assuming that the neuroblastoma biology has not changed.

The subgroup of samples with WCC <2 did not show a significant signal for medical progress, at least with the number of samples used in the current study. This observation implies that WCC is a potential pharmacogenomic marker: Within the group of stage IV high-risk neuroblastoma without MYCN amplification, it identifies a specific subgroup of patients (those with WCC) for which a certain therapy is predicted to be on benefit. Compared with markers that “only” predict survival, but do not link the predicted outcome to a treatment alternative, this aspect has the potential to add significant value in guiding treatment decisions. Unfortunately, our

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**Fig. 4.** Kaplan-Meier survival analysis. The survival probabilities of patients with WCC ≥2 or WCC<2 are compared. A, all samples with survival information (n = 51). The patients classified based on the WCC status had significantly different survival probabilities (P = 0.01). B, the patients diagnosed before or in 1998 (n = 23). C, the patients diagnosed after 1998 (n = 27). D, the patients diagnosed after 1998 with age >500 d (n = 15).
clinical annotation data did not permit to identify which specific recent protocol changes caused the increased survival rates. It is nevertheless tempting to speculate what this might be. One candidate is the combination of high-dose chemotherapy, autologous bone marrow transplantation, and subsequent treatment with 13-cis retinoic acid (4). Study 3891 of the COG had shown a statistically significant improvement in event-free survival for patients consolidated with autologous bone marrow transplantation compared with patients treated with continuation chemotherapy after initial high-dose chemotherapy. Subsequent treatment with 13-cis retinoic acid was found to be most efficient for patients with minimal residual disease. According to this study, the survival probability of patients with high-risk neuroblastoma increased from 11 ± 4% for patients receiving only chemotherapy to 29 ± 7% for patients consolidated with autologous bone marrow transplantation and retinoic acid. The resulting publication in 1999 (4), close to the “optimal” cutoff date of our data, suggested as the “basis for treatment of patients with high-risk neuroblastoma,” a group including the samples used in this study.

Chromosomes were affected with unequal frequencies as shown in Fig. 2A, where chromosome 7 was most affected and chromosome 1 was least frequently affected. This uneven distribution suggests that gene dosage effects, which are known to alter expression levels in neuroblastoma (25), affect the biology of the tumor. Specific combinations may offer advantages in the development of the tumor whereas others may cause disadvantages, leading to a certain selective pressure in the microevolution (26) of the disease. Still, patient survival was to a large degree independent from the specific set of affected chromosomes, and gain and loss of any (two or more) whole chromosome was a superior marker compared with any single chromosome. This makes it very likely that WCC themselves are not causal for the increased susceptibility to treatment but consequential of a more susceptible tumor biology. This suggests that high-risk neuroblastoma with WCC follows a different developmental path, supporting earlier speculations (10, 27) of multiple molecular subtypes of neuroblastoma.

A direct estimate of the fraction of patients presenting in WCC from the samples used in this study is difficult because the samples were not epidemiologically distributed. Nevertheless, the increase in survival rate reported previously (4) can be used to obtain a rough estimate if one uses the observation that predominantly patients demonstrating WCC have benefited. Although attributing the entire difference in survival rates, 20% to 30%, to the WCC group is somewhat too simplistic, it still indicates that a significant fraction of patients in the clinic diagnosed with stage IV high-risk neuroblastoma without MYCN amplification will show WCC.

The observed time dependence of WCC as a marker for survival is a reminder of the fact that the typical use cases of markers, namely definition, comparison, and application, always need to consider the “universe” of samples in which the marker operates. Use of pretreatment samples in molecular screenings does not necessarily isolate the study from treatment parameters, as the treatment is intimately linked to the end points used in association studies. In fact, it is quite likely that some of the earlier studies analyzing mRNA levels for survival include this convolting time-dependent factor. While finalizing this article, two studies (11, 21) reported results of cytogenetic aberrations in neuroblastoma. Both identified a correlation of characteristic patterns of copy number alterations with the known phenotypic classification, extending earlier results (22). Both studies additionally identified specific cytogenetic aberrations that correlate with patient survival in a broad set of neuroblastoma samples covering the entire spectrum of risk categories. Differently, in this study, samples were restricted to a homogeneous subset of the highest-risk category. This strategy was used to further reduce and eliminate confounding with the already well-established risk factors. However, restricting samples for a comparatively rare tumor like neuroblastoma added to the difficulty of obtaining very large sample sets, although the current study is among the largest CGH study for this high-risk subtype. The estimated significance level (P = 0.003) of our primary finding, the association of WCC >2 with survival, is by itself highly significant, but a note on multiple hypothesis testing may be in place because this study tested not only the WCC but also the alteration of the 22 autosomal chromosomes, partial loss of 11q23, and gain of 17q. Bonferroni correction for multiple comparisons ensures that the entire list of hypotheses determined as significant does not contain a single error at a predetermined level of significance. One may be concerned that using this correction might render the presented results nonsignificant. If one counts the N = 22 (autosomes) + 2 (partial loss) = 24 hypothesis, change of chromosome 10 (individual P = 0.001, P = N × P = 0.03) remains significant; but for WCC, π = 0.003 × 24 = 0.072 exceeds the generally accepted threshold of 0.05. However, this naive application of Bonferroni’s correction is overly conservative because the tested hypothesis were not independent (it is known a priori that WCC tend to affect multiple chromosomes simultaneously), leading to an overestimation of N. Even if one insists on treating the different hypothesis as independent, it would be more appropriate to consider the “false discovery” rate, which one can interpret to imply that a larger study may identify a modification of the WCC >2 rule as the best classifier.

In summary, this study presented a novel marker prognostic for survival of high-risk neuroblastoma patients (stage IV neuroblastoma without MYCN amplification), even for patients older than 500 days. For patients with WCC ≥2, the date of diagnosis was a strong predictive factor and patients diagnosed after 1998 with this marker had a survival probability 90%, comparable with patients with neuroblastoma currently stratified as low risk. Although statistically significant, the numbers in this study is relatively low and requires a large controlled study to validate and translate these potentially significant findings to the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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