Medulloblastoma in childhood: a clinical and biological study
Michiels, E.M.C.

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Genetic alterations in childhood medulloblastoma analyzed by Comparative Genomic Hybridization


to be submitted in condensed form
GENETIC ALTERATIONS IN CHILDHOOD MEDULLOBLASTOMA ANALYZED BY COMPARATIVE GENOMIC HYBRIDIZATION

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* both authors contributed equally to this study

to be submitted in condensed form
Introduction

Tumors of the central nervous system (CNS) are the second most frequent type of cancer in children and form the most common type of solid malignancies, comprising 20% of all childhood neoplasms. CNS tumors are very heterogeneous however. The gliomas account for about 60% of the patients 1, while medulloblastomas come second with approximately 20% of all CNS tumors 2-4.

Medulloblastomas preferably arise in the midline of the cerebellum and they have the propensity to spread via the cerebrospinal fluid to the spinal canal. Despite intensive therapy, including surgical resection, radiotherapy and sometimes chemotherapy, 5-year survival does not surpass 39 - 70% 2,5-8. This therapy, and not only the tumor itself, is responsible for disastrous long-term effects if the children survive 9-12.

In search of chromosomal aberrations to identify the loci of genes involved in oncogenic transformation, several genetic abnormalities have been found in medulloblastomas in children. Most frequently, alterations on chromosome 17, more specifically isochromosome 17q [i(17q)], are observed in approximately 30% of cases. Loss of heterozygosity of 17p is also often found. Other frequently involved chromosomes are chromosomes 1, 6, 7, 10,11 and 16 13-24. Alterations on chromosome 17 have been correlated to a poor prognosis 16,25-27.

Classical cytogenetic analysis (karyotyping) is hampered by the fact that tumor cells need to be cultured prior to analysis. Another disadvantage is the fact that rare and possibly irrelevant clonal chromosomal abnormalities can be identified. This shortcoming can be bypassed by using Comparative Genomic Hybridization, a method that only shows aberrations that are present in most of the tumor cells. This technique was first described by Kallioniemi et al. 28 and du Manoir et al. 29. Normal reference DNA and tumor DNA are labeled with different fluorochromes and cohybridized to normal metaphase spreads. After hybridization, fluorescence intensity of the different fluorochromes is measured and the ratio of intensities is calculated along the individual chromosomes. A ratio differing from 1 represents genetic imbalances in the tumor cells compared to the normal cells. Thus, in one hybridization reaction an overview is obtained of all imbalances in the whole genome. Another major advantage of this technique is the fact that - in contrast to "loss of heterozygosity" studies - normal DNA that serves as a control can be derived from another person, which is especially useful for analysis of archival tumor material. This method has already been applied to a variety of solid tumors.

In this study we analyzed 17 medulloblastomas in children in search for chromosomal alterations by means of CGH.
Materials and Methods

Patient and normal material
Clinical data of the patients are listed in Table 1. Most recent data were obtained from the treating physician. Tissue of the brain tumors was obtained during surgery and flash-frozen in liquid nitrogen. Histopathological diagnosis was performed according to the WHO classification. From this frozen material DNA was extracted according to standard procedures. Normal DNA was obtained from the lymphocytes of a healthy female.

Comparative Genomic Hybridization
Normal metaphase spreads were obtained by culturing lymphocytes from a healthy male donor. Tumor DNA was labeled with biotin-16-dUTP (Boehringer Mannheim) and the reference DNA with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation. The concentration of DNase I was adjusted so that the size of the labeled DNA fragments was 0.5 - 1.5 kb. Labeled DNA was size separated on agarose gel to confirm fragment length. For the first 10 medulloblastomas, the hybridization of the DNA on metaphase spreads was performed as described by de Meulemeester et al. with the exception that the denaturation of the chromosomes was done on a heating plate (3 minutes at 70 °C). Hybridized probe and normal DNA were detected using FITC-conjugated avidin and TRITC-conjugated sheep-anti-digoxigenin-antibody respectively. Chromosomes were counterstained with DAPI.

In the second series of experiments, the hybridization of the DNA on metaphase spreads was performed as follows: 300 ng of biotin-labeled tumor and 300 ng of digoxigenin-labeled normal DNA, together with 40 μg unlabeled Cot-1 DNA were ethanol-precipitated and the pellet was dissolved in 6 μl 50% formamide, 10% dextrane sulphate in 2 x SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0). This probe mixture was denatured at 80 °C for 10 minutes. Normal metaphase slides were denatured at 72 °C for 6 minutes in a Coplin jar containing 70% formamide in 2 x SSC, pH 7.0. The probe mixture was mounted onto the slides, sealed with a cover slip and hybridized for 3 days in a humid incubator at 40 °C. The slides were washed for 5 minutes in 2 x SSC at room temperature, three times 5 minutes in 0.1 x SSC at 45 °C, and 5 minutes in 0.05% Tween-20 in TN (100 mM Tris-HCl, pH 7.6, 150 mM sodium chloride). The slides were then preincubated for 10 minutes at 40 °C in blocking solution (0.5% blocking reagent (Boehringer) in TN), followed by a 60 minutes incubation at 40 °C with 12.5 μg/ml FITC-conjugated avidin (Sigma) and 4 μg/ml TRITC-conjugated sheep-anti-digoxigenin (Boehringer) in blocking solution.
The slides were washed three times 5 minutes at room temperature in 0.05% Tween-20 in TN and dehydrated through an ethanol series. Finally, the slides are mounted with 20 µl antifade solution (Vectashield, Vector laboratories, Burlingame, USA) containing 0.35 µg/µl DAPI.

**Image Analysis**

The slides were analyzed on a Zeiss axioscope microscope equipped with filtersets for DAPI, FITC and TRITC. The images were captured with a monochrome CCD camera and processed by Cytovision CGH-software of Applied Imaging (version 3.0). The average green to red fluorescence ratios of chromosomes of 10 to 15 selected metaphases were plotted along ideograms of the corresponding chromosomes, together with the CI.

In the first series of 10 medulloblastomas we used 3 different criteria to define a genomic imbalance: 1) a ratio less than 0.9 or greater than 1.1 with a 95% confidence interval (CI) that completely crossed the midline, 2) the 99% CI above or below the ratio of 1.0 and 3) a ratio value below 0.75 or exceeding 1.25 without calculating a 95% CI.

In the second series of experiments, the slides were analyzed on a Leica DM-RA microscope equipped with filtersets for DAPI, FITC and TRITC. The images were captured with a monochrome CCD camera and processed by Cytovision CGH-software of Applied Imaging (version 3.5). The average green to red fluorescence ratios were plotted along ideograms of the corresponding chromosomes, together with the 95% CI. Deviations from normal were interpreted as gains and losses when the 95% CI did not contain 1.0.

Regions known to be unreliable were excluded from evaluation: heterochromatic and centromeric regions, telomeric regions and chromosomes 1p32-pter, 16p, 19 and 22 showing deletions. Control experiments consisted of performing the procedure on 2 differently labeled aliquots of normal DNA and reversing the labeling on normal and tumor DNA that showed genomic imbalances.

**Southern Blotting**

Southern blotting was performed according to standard procedures. We used pNB 1 located on 2p24 as N-myc probe and pCHT 2/2.7 on 8q24.2-3 as a control probe for DNA loading.
### Results

We first analyzed 10 childhood medulloblastomas, according to the method of de Meulemeester et al. One drawback of this approach was that frequently granular signals were obtained. During this analysis it became clear that by using a combination of both temperature and formamide for denaturation of the chromosomes, more evenly distributed signals could be obtained. Therefore, the eight tumors from which DNA was still available and 7 new tumors were analyzed using temperature/formamide denaturation. Patient and tumor characteristics, as well as the results of the two experiments, are shown in Table 1.

<table>
<thead>
<tr>
<th>Lab no</th>
<th>Age(y)/sex</th>
<th>Localization</th>
<th>Clinical course</th>
<th>CGH results</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PN 1.11,2</td>
<td>4/Male</td>
<td>cerebellum left hemisphere</td>
<td>no recurrence; alive 5 years after surgery</td>
<td>7q-</td>
<td>1p+, 3q+, 9q+, 11-, 14q+, 17q+, 18p-, 18q+, 20p-</td>
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<td>PN 2.11,2</td>
<td>17/Male</td>
<td>cerebellum vermis</td>
<td>no recurrence; alive 9 years after surgery</td>
<td>2p-, 3p-, 11q-, 14q+, 21q-, Xp-, Xq-</td>
<td>4q+, 5q-, 6q+, 7q+, 8q+, 10p+, 10q+, 11p-, 12+, 13+, 17p+, 17q-, 19q+, 20q-</td>
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<td>PN 3.11,2</td>
<td>2.5/Male</td>
<td>4th ventricle and spinal canal</td>
<td>no remission; dead 3 months after surgery</td>
<td>5p+, 16q-, 17p-</td>
<td>8q, 12q-, 17p+, 17q+, 22q+</td>
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<tr>
<td>PN 5.11</td>
<td>14/Male</td>
<td>cerebellum vermis and left hemisphere</td>
<td>no recurrence; alive 8 years after surgery</td>
<td>2p+, 10p-, 10q-</td>
<td>1p+, 2p+, 4q+, 7p+, 7q+, 8p, 8q+, 9p+, 10-, 12p+, 12q+, 13q+, 16-, 17p-, 17q+, 18+, 19q-, 20q-</td>
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<td>PN 6.11,2</td>
<td>10/Male</td>
<td>cerebellum vermis</td>
<td>recurrence with multiple metastases 9 months after surgery; dead</td>
<td>1p+, 1p-, 3q+, 9q-, 11-</td>
<td>3+, 4+, 7+, 8-, 10-, 13+, 14q+, 15-, 17+, 18+, 19+, 20-</td>
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<tr>
<td>PN 7.11</td>
<td>3/Female</td>
<td>cerebellum vermis</td>
<td>no recurrence; alive &gt; 3.5 years after surgery</td>
<td>8q-, 10q-</td>
<td>6-</td>
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<tr>
<td>PN 8.11,2</td>
<td>9/Male</td>
<td>cerebellum vermis</td>
<td>recurrence with multiple metastases 1.5 years after surgery; dead</td>
<td>6p, 6q+, 7+, 8+, 9p+, 9q-, 10p-, 10q-, 11q+, 12p+, 13q+, 14q+, 15-, 17+, 18+, 19+, 20-</td>
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<tr>
<td>PN 9.11,2</td>
<td>4/Female</td>
<td>cerebellum vermis</td>
<td>no recurrence; alive &gt; 3.5 years after surgery</td>
<td>7p+, 7q+</td>
<td>5q+, 6q+, 7+, 8p-, 8q-, 10p-, 11p-, 11q-, 14q-, 17p+, 17q+, 18p+, 18q+, 19q+, 20q, X-</td>
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<td>PN 10.11,2</td>
<td>15/Male</td>
<td>fossa posterior</td>
<td>no recurrence; alive &gt; 2.5 years after surgery</td>
<td>17q+, Xq-</td>
<td>3-, 4+, 7+, 8-, 10-, 13+, 14q+, 15-, 17+, 18+, 19+, 20-</td>
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<td>PN 52.11,2</td>
<td>7/Female</td>
<td>cerebellum right hemisphere</td>
<td>no recurrence; alive 4 years after surgery</td>
<td>Xq+</td>
<td>5p+, 7q+, 8p-, 9q-, 13q+, 16p-, 17p-</td>
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<td>PN 53.12</td>
<td>6/Male</td>
<td>cerebellum vermis</td>
<td>metastases at diagnosis; recurrence 20 months after surgery; dead</td>
<td>1q+, 2p+, 3p+, 7+, 8+, 11p-, 13q-, 15q+, 16q-, 17p+, 18p+, 18q+</td>
<td>1p+, 1p-, 2p+, 2q+, 4+, 5q+, 5q-, 6q+, 7q+, 8q+, 9p+, 11p-, 11q-, 11q+, 12p+, 13q+, 16q-, 17p-, 18q+, 19-, 22-</td>
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<td>PN 54.12</td>
<td>8/Male</td>
<td>cerebellum midline</td>
<td>alive 3 years after surgery</td>
<td>1p+, 1p-, 2p+, 2q+, 4+, 5q+, 5q-, 6q+, 7q+, 8q+, 9p+, 11p-, 11q-, 11q+, 12p+, 13q+, 16q-, 17p-, 18q+, 19-, 22-</td>
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<tr>
<td>PN 56.12</td>
<td>9/Female</td>
<td>cerebellum left hemisphere</td>
<td>recurrence 28 months after surgery; dead</td>
<td>1p+, 1q+, 6q+, 9q+, 9q-, 17q-, 18q+, 19q-, Xp-, Xq-</td>
<td>5p+, 7q+, 8p-, 9q-, 13q+, 16p-, 17p-</td>
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Table 1 - Continued

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<th>CGH results&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>PN 61.2&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4/Male</td>
<td>relapse of medulloblastoma and myelum</td>
<td>alive &gt; 3 years after recurrence</td>
<td>1p+, 1q+, 2+, 3q+, 4p+, 4q+, 5+, 6q+, 7p-, 7q+, 8q+, 9p+, 10-, 11-, 13q+, 14+, 15-, 16-, 18p+, 18q+, 20-, 21q-, 22q-</td>
<td></td>
</tr>
<tr>
<td>PN 63.1&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5/Male</td>
<td>cerebellum midline and hemispheres and myelum</td>
<td>relapse 15 months after surgery; dead</td>
<td>1q+, 10p-, 16q-, 17q+</td>
<td></td>
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<tr>
<td>PN 64.1&lt;sup&gt;2&lt;/sup&gt;</td>
<td>11/Female</td>
<td>cerebellum midline</td>
<td>alive 2.5 years after surgery</td>
<td>3p-, 3q-, 7p+, 7q+, 17p-, 17q+, X-</td>
<td></td>
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<tr>
<td>PN 65.1&lt;sup&gt;2&lt;/sup&gt;</td>
<td>14/Male</td>
<td>cerebellum midline and hypophysis</td>
<td>alive 2.5 years after surgery</td>
<td>7q+, 17p-, 17q+, 18+</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Clinical characteristics and CGH results.

<sup>1</sup> Tumor analyzed in the first series of experiments
<sup>2</sup> Tumor analyzed in the second series of experiments (see text)

<sup>a</sup> Results of the first series of experiments, using the 99% CI as criterion for imbalances
<sup>b</sup> Results of the second series of experiments, using formamide denaturation and the 95% CI as criterion

A deletion in both arms of the chromosome but not involving the whole chromosome is described as p-, q-. The same holds for gains.

Interpretation of genomic imbalances was based on the ratio of the two fluorochromes and in the initial experiments, 3 different criteria that are also found in literature were used: 1) a ratio of less than 0.9 or greater than 1.1 with a 95% CI that completely crossed the midline<sup>35</sup>, 2) 99% CI above or below the ratio of 1.0, without a criterion for the mean ratio value<sup>23</sup> and 3) a ratio value below 0.75 or exceed-

![Figure 1](image)

Results of CGH analysis of 10 medulloblastomas, using the 95% CI as criterium.
Deletions are marked on the left side of the chromosome, amplifications are on the right of the chromosome. Some tumors may have multiple (interstitial) deletions and/or amplifications on one chromosome.
d 1.25 without calculating a 95% CI. Results of the first 10 analyses are depicted in Figures 1, 2 and 3 respectively. A 95% CI as criterium for imbalances (Fig. 1) resulted in the majority of chromosomes showing one or more gains or deletions. Most frequently affected chromosomes were chromosomes 7 (5/10 tumors), 6, 9, 10, 17, and the X chromosome (all 4/10 tumors). Surprisingly, chromosome 10 only showed deletions, while the other frequently affected chromosomes all showed gains as well as deletions. Narrowing the CI to 99% reduced the number of imbalances found considerably (Fig. 2). Chromosomes still affected were chromosomes 1, 2, 3, 5, 7, 8, 10, 11, 14, 16, 17, 21 and the X chromosome. Chromosome 2 showed an amplification on 2p in one tumor and a deletion in another. Chromosome 7 also showed a deletion in one tumor and a gain of nearly the whole chromosome in another. Chromosome 10 again only showed deletions, while chromosome 17 showed loss of 17p in one tumor and gain of 17q in another. The X chromosome showed deletions in 2 tumors and a gain in one tumor. The other chromosomes only showed genomic imbalances in a single tumor. Figure 3 shows the results, using a ratio above 1.25 or below 0.75 without calculating a CI.

![Figure 2. Results of CGH analysis of 10 medulloblastomas, using the 99% CI as criterium.](image-url)
A comparison of the 3 figures revealed 5 chromosomal regions which showed alterations using either of the 3 criteria: 1q, 2p, chromosome 7 and Xq showed gains while Xp showed a deletion.

The amplification of 2p was located in the region where N-myc is situated. To test whether N-myc was amplified in this tumor Southern blot analysis was performed on tumors of which enough DNA was available. Indeed amplification of N-myc was found in PN 6.1. Other tumors that showed no amplification of 2p on CGH were also tested. None of these tumors showed N-myc amplification (Fig. 4).

**Figure 3.**
Results of CGH analysis of 10 medulloblastomas, using the mean ratio as criterium.

**Figure 4.**
N-myc amplification in PN 6.1
EcoRI digested DNA was hybridized to N-myc probe (row A) and control probe 8q (row B). Tumor DNA of patients is in lanes 1 - 9, lanes are numbered with patient codes. PN 5.1 was not included because of lack of enough DNA to perform a Southern blot. Normal DNA (NC1 - NC3) is in lanes 10 - 12. Lane 6 shows the amplification of N-myc in patient PN 6.1.

Control experiments performing CGH on normal versus normal DNA showed a ratio of 1.0, except in the regions known to be unreliable in CGH (heterochromatic and centromeric regions, telomeres and chromosomes 1p32-pter, 16p, 19 and 22 for definition of deletions) (data not shown). These regions were excluded from the analysis.
In view of the difficulties in defining true imbalances, due to the different results using 95% or 99% CI, an alternative CGH protocol was used. In this protocol a stronger denaturation of the chromosomes was obtained (see Materials and Methods), resulting in less granular signals. Results of the analysis of 15 medulloblastomas using formamide as a denaturing agent are shown in Figure 5.

As this alternative method of denaturation improved the reliability of the results significantly, there was no major difference anymore using the 95% or 99% CI as criterion for imbalances (data not shown). Most frequently affected chromosomes in this series of experiments were chromosome 17 (13/15 tumors), chromosome 7 (10/15 tumors), chromosomes 8 and 18 (9/15 tumors) and 13 (7/15 tumors). Chromosome 17 showed an imbalance consistent with isochromosome 17q [i(17q)] in 4 tumors. Strikingly, 8 of the 10 tumors that showed imbalances of chromosome 7 also showed imbalances of chromosome 8 and vice versa, only one tumor showing imbalances on chromosome 8 did not reveal aberrations on chromosome 7. On chromosome 18 only gains were seen, except for 1 tumor that also showed a deletion of 18p. Analysis of these 15 tumors showed two more tumors with an amplification of 2p in the region where N-myc is situated.
Discussion

Several authors have described chromosomal alterations in medulloblastomas in childhood 13,14,16-20,36. Three studies concerned analysis by CGH 22-24. Comparing our results using the formamide as denaturing agent, with the results found by other authors shows that the amount of alterations found in our study (Fig. 5) is comparable to that found by Reardon et al. 23 and Russo et al. 24. Some frequently affected chromosomes in their study also showed alterations in many of our tumors, such as chromosomes 7, 8, 10, 16, 17 and 18. Other chromosomes were more frequently affected in our study, such as chromosome 13. This might be due to the small number of tumors studied in different groups.

The most frequent genomic alteration found in medulloblastoma in children is loss of 17p, alone or in combination with an overrepresentation of 17q to form an isochromosome 17q, i[17q]. The latter is observed in about 30% of cases 16,27,37. Our study showed 7/15 patients with loss of chromosome 17p, four of which had also a gain of 17q. Five others only showed overrepresentation of 17q without loss of 17p. In one of them the whole chromosome 17 was amplified. The c-erbB-2 oncogene is located on chromosome 17q. Different authors studied the expression of the c-erbB-2 protein 38,39. Although the number of medulloblastomas that was positive for this protein differed in the 2 studies (83.6% versus 13%), both authors found an inverse correlation between c-erbB-2 expression and survival.

Gain of the entire chromosome 7 was seen in 5 patients, while 2 patients showed a gain of a part of 7p and 7q, two more patients showed an overrepresentation of a part of 7q only (one pericentromeric and one distal). In one patient a deletion of 7p was associated with a gain of 7q. Alterations of chromosome 7 are seen frequently in medulloblastoma 14,22,23,37, as well as astrocytoma and glioblastoma 40-42. The receptor for Epidermal Growth Factor (EGFR) is located on chromosome 7p, and frequently amplified in glioblastoma 41,42. However, the meaning of this finding in medulloblastoma is not yet clear. As the imbalance involves approximately the whole chromosome in most cases, other genes can also play a role. Further investigation is needed to clarify this issue.

Chromosome 8 was also frequently affected in our study, as well as in the study of Reardon et al. 23 and to a lesser degree in the study of Schütz et al. 22 and Russo et al. 24. In most of our patients a loss of 8p was seen which was combined with a gain of 8q in one patient. Deletions of 8p are seen in carcinomas 43,44 suggesting the presence of a tumor suppressor gene on 8p. In medulloblastoma no tumor suppressor gene localized on 8p is known until now. The amplification on 8q may involve the c-myc oncogene, which is shown to be amplified in medulloblastoma/PNET by some authors 27 and even carries a bad prognosis in their study when combined with
loss of 17p. In our study only one patient showed the combination of 8q amplification and loss of 17p (PN6.1) and this child died of a recurrence with multiple metastases. However, the number of tumors studied in this group is too small to draw any definite conclusions.

Strikingly, all but two patients (PN 64.1 and PN 65.1) that showed an imbalance on chromosome 7 also showed aberrations on chromosome 8, and only one patient (PN3.1) with an amplification of chromosome 8 did not show imbalances on chromosome 7. The reason for this association of imbalances is not clear. Alterations on both chromosomes could be needed to induce transformation into a medulloblastoma, but definitive conclusions await the analysis of more tumors.

Four patients showed deletions of chromosome 10. This is a known finding in medulloblastoma and glioblastoma multiforme. Tumor suppressor genes have been postulated on 10q23 and 10q25-26. Mollenhauer et al. propose a new putative tumor suppressor gene, DMBT1, located on 10q25-26, contributing to the development of medulloblastoma. Both regions are affected in our study.

Three patients showed an amplification of the N-myc region. In one patient we had sufficient DNA to confirm the N-myc amplification by Southern blotting. This N-myc amplification is a well-known aberration in childhood neuroblastoma where it carries a bad prognosis. In medulloblastoma however it is seldom seen but patients carrying this amplification in their tumor seem to have a worse prognosis. This was also the case for 2 of our patients with N-myc amplification, who recurred 9 months and 20 months after surgery respectively (Table 1).

The poor prognosis seen in literature for patients with alterations on chromosome 17 was not seen in this study.

A number of problems may be encountered performing CGH, one of the most important being the degree of denaturation of the chromosomes on the metaphase spread. Too harsh denaturation results in unrecognizable chromosomes, which makes interpretation of the results impossible. Decreasing the degree of denaturation improves the recognizability of the chromosomes, but makes them less accessible to hybridization. This results in a more granular and less intensive signal which significantly reduces the quality of the image captured by the computer. Thus, a very important step in the procedure of high-quality CGH is the determination of the ideal way and degree of denaturation of the chromosomes, as this can significantly affect the final results. We performed two different experiments using two different ways of denaturation. Except for those tumors of which there was not enough DNA to perform both analyses, both tests were done on DNA samples of the same tumors, to enable comparison of the different denaturation methods. Results are shown in Figures 1 and 5. Not only did this alternative way of denaturation result in straighter profiles with narrower CI (Fig. 6), but the interpretation was facilitate by the more distinct
aberrations, and was less hampered by random variations of the ratio due to granularity of the signal.

Figure 6.
Comparison of quality of profiles using different protocols for denaturation of chromosomes.

a: profile of tumor PN6.1, denaturation performed as in the first series of experiments (see text).
b: profile of tumor PN6.1, formamide was included as denaturing agent.
Comparison of the genomic imbalances found by the two methods (Fig. 1 and 5) shows many more aberrations in Figure 5, and the imbalances found span a bigger part of the chromosome.

In literature different authors use different criteria for gains and losses, with different results. These discrepancies have already been mentioned by Reardon et al. in their study of CGH on medulloblastoma. Schütz et al. considered ratio values below 0.75 or exceeding 1.25 diagnostic for loss and gain (respectively) of genomic material, without calculating a 95% confidence interval for the values found. In contrast, Reardon et al. defined regions of significant gains and losses by 99% CI that were either above or below a ratio value of 1.0, without a criterium for the mean ratio value. In Figure 1 we defined losses or gains as a combination of a mean ratio value that was less than 0.9 or greater than 1.1 and a 95% confidence interval that was below or above the ratio value of 1.0, as was done in the study of Steenman et al. In literature other criteria for gains and losses are also used, e.g. the mean ratio ± 1 standard deviation above 1.15 or below 0.85, or ratio thresholds of 1.2 and 0.8 respectively. The differences are obvious. By not taking into account a confidence interval, results can be influenced by random variation of successive measurements. On the other hand, by using a very stringent confidence interval, some alterations can be missed. We analyzed our data based on the 3 different ways of defining gains or losses mentioned above. As could be expected, the results varied. Chromosomal regions of which the confidence interval is above or below 1.0 but the mean ratio is not reaching 1.25 or 0.75, will not be seen as amplifications or deletions in some studies. On the other hand, if the mean ratio reaches 0.75 or 1.25, but the confidence interval is very broad, these regions will be defined as genomic imbalances by Schütz et al., but not according to Reardon et al. or to our criteria. The conclusion of this observation is obvious: if we want to search for genomic imbalances and be able to compare different CGH studies, we definitely need to use similar criteria for the definition of gains and losses. This is especially important when relatively rare tumors such as medulloblastoma are studied. It is not expected that one study will contain sufficient data to reach significant conclusions.

Furthermore it has become clear that the quality of the fluorescence signal is a major determinant and significantly influences the final results. Strict control of the quality of the signal and ratio profiles is absolutely necessary to ensure reliable results. To obtain this control, confidence intervals should always be shown together with the ratio profiles.

It will be clear that CGH can only be used as a screening procedure to get a rapid overview of genomic imbalances found in certain tissues. Further analysis is warranted to verify the findings of the CGH study, e.g. by "loss of heterozygosity"
(LOH) studies or Fluorescent In Situ Hybridization (FISH). This is hampered by a number of problems. In the first place the low incidence of childhood medulloblastoma; second the new neurosurgical operation instruments such as the CUSA, by which tumor material is removed by suction together with blood and other fluids, which make it difficult to obtain pure tumor material of good quality. Third, DNA extracted from archival tumor tissue is frequently contaminated and of poor quality, which hinders the CGH. Fourth, LOH studies on this archival material are sometimes impossible because normal control DNA is lacking. Despite these difficulties, which may hamper genomic analysis of rare tumors, genetic studies on these samples should be performed in order to get more insight in the process of malignant transformation of these tumors.

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