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# Bone marrows from neuroblastoma patients: An excellent source for tumor genome analyses

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ABSTRACT

Neuroblastoma is the most common extra-cranial solid tumor in childhood. Presence of disseminated tumor cells (DTCs) in the bone marrow (BM) at diagnosis and at relapse is a common event in stage M neuroblastomas. Although the clinical heterogeneity of disseminated neuroblastomas is frequently associated with genomic diversity, so far, only little information exists about the genomic status of DTCs. This lack of knowledge is mainly due to the varying amount of BM infiltrating tumor cells, which is usually below 30% even at diagnosis thereby hampering systematic analyses. Thus, a valuable chance to analyze metastatic and relapse clones is, so far, completely unexploited. In this study, we show that the enrichment of tumor cells in fresh or DMSO frozen BM samples with a minimum of 0.05% or 0.1% infiltration rate, respectively, by applying magnetic bead-based technique increased the DTC content to a sufficient level to allow SNP array analyses in 49 out of 69 samples. In addition, we successfully used non-enriched BM samples with ≥30% DTCs including non-stained and immunostained cytospin and BM smear slides for SNP array analyses in 44 cases. We analyzed the genomic profile of DTCs by an ultra-high density SNP array technique with highest performance detecting all segmental chromosomal aberrations, amplified regions, acquired loss of heterozygosity events and minor aberrations affecting single genes or parts thereof.



## 1. Introduction

Neuroblastoma, an embryonic tumor arising from the sympathetic nervous system, is the most common extra-cranial solid tumor in childhood accounting for about 15% of all pediatric cancer deaths (Maris et al., 2007; Brodeur, 2003). This disease is characterized by a high degree of clinical heterogeneity, ranging from spontaneous regression

*Abbreviations:* BM, bone marrow; CGH, comparative genomic hybridization; cnLOH, copy neutral loss of heterozygosity; DMSO, dimethylsulfoxide; DTC, disseminated tumor cell; FISH, fluorescence in situ hybridization; LCM, laser capture microdissection; LOH, loss of heterozygosity; MACS, magnetic activated cell sorting; Mb, megabase; MLPA, multiplex ligation-dependent probe amplification; MNC, mono nuclear cell; NGS, next generation sequencing; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SCA, segmental chromosomal aberration; SNP, single nucleotide polymorphism; UHD-SNP array, ultra-high density SNP array.

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(D'Angio et al., 1971; Evans et al., 1976; Hero et al., 2008) or maturation (Ambros et al., 1996) to highly malignant courses with widespread metastases and poor outcome. Besides age (Brodeur et al., 1993; Cohn et al., 2009), tumor histology (Shimada et al., 1999), status of *MYCN* oncogene (Brodeur et al., 1993), and DNA index (ploidy) (Ladenstein et al., 2001), segmental chromosomal aberrations (SCAs) like 1p and 11q deletions (Attiyeh et al., 2005; Caren et al., 2010; Maris et al., 1995; Schleiermacher et al., 2011) have been shown to predict tumor behavior and patient outcome.

Until recently, gains and losses of chromosomal material affecting different chromosomes were analyzed individually by fluorescence in situ hybridization (FISH), or MLPA (multiplex ligation-dependent probe amplification) techniques. However, during the last years genome-wide techniques such as array-CGH (aCGH) and whole genome single nucleotide polymorphism (SNP) arrays have been applied in order to detect genomic aberrations in neuroblastoma (George et al., 2007). Amplifications of certain genes and gains and losses of large chromosomal segments commonly defined as "segmental chromosomal aberrations" (SCAs) can be visualized by these techniques. With the advent of ultra-high density SNP arrays (UHD-SNP arrays) also minor aberrations like deletions or gains of single genes or parts thereof can be detected (Ambros et al., 2014). As SNP array technologies provide information on the allele distribution together with the copy number status, the information that can be gained from these data is more robust as compared to conventional array platforms, which rely only on copy number information, thus providing a higher security for the correct interpretation of the data (Caren et al., 2010; George et al., 2007). Different studies have shown that the genomic changes are frequently associated with certain clinical sub-types of neuroblastoma and suggest that pan-genomic data based on microarray techniques will improve neuroblastoma risk estimation (Ambros et al., 2009; Bilke et al., 2005; Coco et al., 2012; George et al., 2007; Schleiermacher et al., 2011; Spitz et al., 2006). Based on the statistical evaluation of 8.800 neuroblastoma tumors it became clear that genomic information helps to refine current risk classification systems (Cohn et al., 2009). Recent publications demonstrate that besides SCAs, spanning a few Mb in size up to a whole chromosomal arm, some other aberrations frequently can be found in different tumor entities. These aberrations include minor changes affecting single genes or parts thereof such as deletions of or within the *ATRX* gene and chromothripsis – a peculiar form of aberration by shattering of genomic segments within single chromosomes or chromosome arms, and subsequent random reassembly of the fragments. While *ATRX* deletions/mutations only occur in a distinct neuroblastoma patient subgroup (over 18 months, no *MYCN* amplification) and in only a few other tumor entities, the phenomenon of chromothripsis is found in all age groups (Ambros et al., 2014; Cheung et al., 2012; Molenaar et al., 2012).

Bone marrow (BM), easily accessible by aspiration through the iliac crest, is a common site for disseminated tumor cells (DTCs) in neuroblastoma patients. The majority of stage M neuroblastoma patients present with tumor dissemination in the BM at diagnosis and also frequently at relapse. Nowadays, immunocytology either alone or in combination with FISH, like the AIPF (automatic immunofluorescence plus FISH) technique, is used to quantify and also to genetically analyze DTCs at diagnosis or as a response predictor during therapy (Beiske et al., 2009; Mehes et al., 2001; Seeger et al., 2000). However, as so far only FISH techniques have been applied to study the genomic aberrations in a large patient group, no complete picture on all genomic aberrations present in DTCs has been possible. Thus, only very limited information about the genomic status of DTCs in neuroblastoma patients is available (Vandewoestyne et al., 2012). A number of questions have not been answered yet: i) does the genomic status of the primary tumor correspond to that of DTCs in the BM? ii) does the genomic make up of DTCs differ at different time points? iii) do relapse tumor clones from different patients share certain genomic aberrations? and iv) is it possible to detect these clones already as a subpopulation at diagnosis, thus helping the identification of patients at higher risk for relapse?

As the fraction of DTCs in the background of normal cells is frequently below the detection limit of array-based techniques, enrichment of the DTC fraction was necessary (Schulz et al., 1984; Sun et al., 2011; Zhe et al., 2011). The combination of a magnetic bead-based enrichment technique together with a highly sensitive array technique met these requirements. We took advantage of two facts: i) the high and consistent expression of the ganglioside GD2 on virtually all neuroblastoma cells enabling magnetic bead-based enrichment, and, ii) the fact that the SNP array method enables identification of genomic aberrations also in cases with lower tumor cell fractions as compared to conventional array techniques. In addition, we examined whether the conventional BM smear and cytospin slides can be used for genomic characterization in cases with higher tumor cell content (≥30%).

## 2. Materials and methods

### 2.1. Samples

Different sources of infiltrated bone marrows were tested: DMSO frozen, native BM, GD2/CD56 stained and non-stained cytospin slides and routine BM smear slides. In case of liquid BM samples with equal or less than 50% tumor cell infiltration, tumor cells were enriched by magnetic beads followed by DNA extraction. However, in cases where no fresh or DMSO frozen BM was available, DNA was extracted directly from stained or non-stained cytospin slides in samples with ≥30% tumor cell infiltration rate. GD2/CD56 stained cytospin slides, after scanning for DTCs, were stored at +4 °C and non-stained cytospin slides at −20 °C in tightly closed boxes. In addition, BM smears were suitable for DNA extraction and SNP array analysis. Furthermore, DNA was extracted from fresh or snap frozen tumor tissues and tumor free mononuclear cell fraction of each patient as reference. Ethical approval was obtained from the local ethics committee.

### 2.2. Immunofluorescence detection

All samples were routinely analyzed by GD2/CD56 (GD2: 14.18 delta CH2 clone/CD56: eBioscience UK Ltd) double

staining for quantification of the tumor cell load and for evaluating the fluorescence profile applying the RCDetect device (Metafer, MetaSystems GmbH, Altlussheim, Germany) according to the method described by Ambros et al. (2003). Only cases with strong and uniform GD2 staining were chosen for the enrichment experiments. Evaluation of the positive and negative fraction after magnetic beads separation by GD2 staining was performed by fluorescence microscopy (Zeiss, Germany).

### 2.3. *Tumor cell enrichment*

To enrich the GD2+ neuroblastoma cell fraction a density gradient centrifugation was applied and followed by a magnetic bead-based enrichment technique (Figure 1). After density gradient centrifugation by Lymphoprep™ (AXIS-SHIELD PoC AS, Oslo, Norway) at 4 °C, mononuclear cells containing tumor cells were collected for enrichment by magnetic activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were spun down at 300 g for 10 min and resuspended in 2 ml cold MACS buffer (PBS PH 7.2, 0.5% BSA, 2 mM EDTA). The fraction was incubated at 4 °C with FITC-labeled anti-GD2 antibody for 20 min and subsequently with 75 ml anti-FITC magnetic beads solution for 15 min without any washing steps in between. The starting volume and amount of the beads was increased and the washing steps were skipped compared to the manufacturer's recommendation. Selection of GD2+ neuroblastoma cells was performed on an MS column (Miltenyi Biotec, Bergisch Gladbach, Germany). From a small portion of the GD2 positive and GD2 negative fractions cytospin slides were made to calculate the GD2+/DAPI+ in relation to the GD2-/DAPI+ cell fraction by immunofluorescence microscopic evaluation. DNA extraction was done for the samples with more than 30% tumor cell infiltration.

### 2.4. *Collecting cells from BM cytospin and smear slides*

To enable DNA extraction from cytospin and BM smear slides with ≥30% tumor cell infiltration, 200 µl and 500 µl phosphate-buffered saline (PBS) were pipetted onto the glass slides, respectively. The cells were scraped with a pipette tip from the glass surface and the suspension was poured into an Eppendorf tube. This procedure was repeated several times to ensure that all cells were removed from the slide. The cell suspension was spun down using microcentrifugation at 400 g for 10 min and the supernatant was discarded.

### 2.5. *DNA extraction*

DNA was extracted with the high salt extraction method (Miller et al., 1988). The cells were resuspended in 300 µl nuclei lysis buffer (10 mM Tris–HCl, 400 mM NaCl and 2 mM EDTA, pH 8.2) and digested overnight at 56 °C with 13 µl 20% SDS and 3 µl proteinase K (20 mg/ml) solutions. When digestion was completed, 100 µl 6 M NaCl was added and after microcentrifugation at 13,000 rpm the supernatant containing the DNA was transferred to another tube. DNA was precipitated by adding 1 ml absolute ethanol followed by microcentrifugation at 13,000 rpm for 30 min at 4 °C and used for genomic analysis without any additional amplification steps.

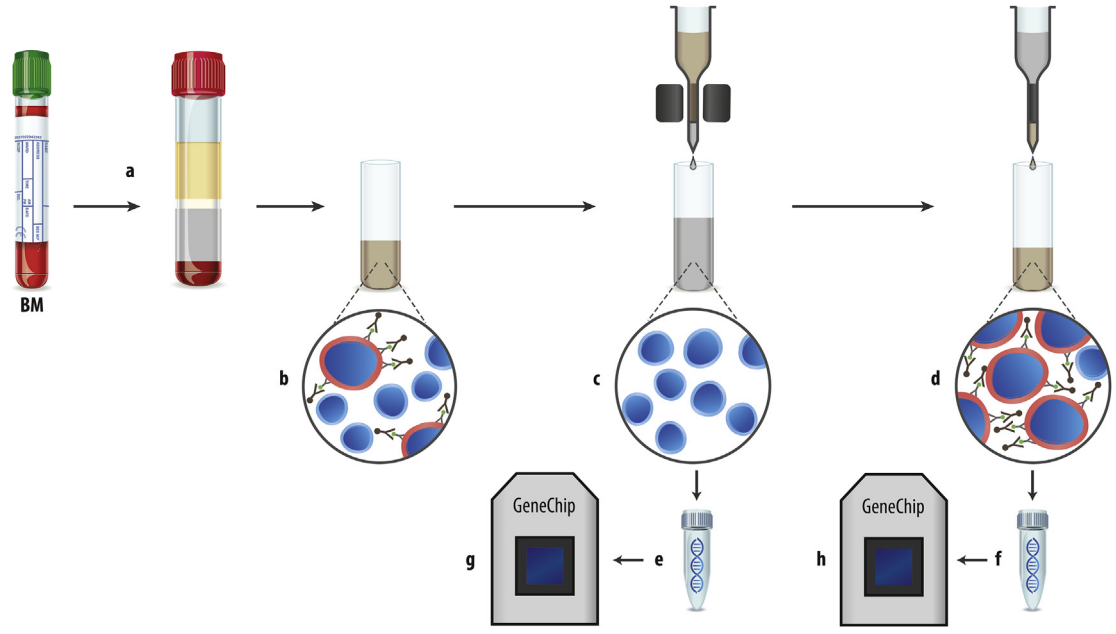


**Figure 1 – DTC enrichment, DNA extraction and SNP array analysis. After density gradient centrifugation (a), mononuclear fraction containing tumor cells was incubated with FITC-labeled anti-GD2 antibodies and subsequently anti-FITC magnetic beads (b). Selection of GD2+ neuroblastoma cells was performed in a column which was placed in a magnetic field and the negative fraction was collected (c). DTCs were recovered after removing the column from the magnet (d). DNA was extracted from the negative (e) and positive (f) fractions for SNP array analysis (g and h).**

### 2.6. Genomic analysis

Genomic profile of tumor cells was examined by CytoScan™ HD Array (Affymetrix Inc., UK Ltd), a genome-wide UHD-SNP array technique with more than 2.6 million copy number and SNP markers. All steps were done according to the detailed protocol provided by the manufacturer. After labeling and hybridization onto arrays, the arrays were washed, stained and scanned with the GeneChip scanner (Affymetrix Inc., UK Ltd) and the data was analyzed using the ChAS software (Affymetrix Inc., UK Ltd).

## 3. Results

Magnetic bead-based enrichment was applied to 69 BM samples with an original tumor cell infiltration rate ranging from 0.05% to 50%. 65 samples were DMSO frozen and four samples were fresh. Frozen samples had 0.1%–50% tumor cell infiltration rate. 44 samples had been kept frozen in the gas phase of liquid nitrogen for up to 23 years and 21 samples had been stored for less than one decade. Frozen samples were classified in four groups according to the tumor cell content before enrichment (Figure 2, Table 1, and Supplementary Figure). In group A, 0.1%–1% of mononuclear cells were GD2 positive and thus defined as tumor cells. The tumor cell infiltration before enrichment in groups B, C and D was 2%–9%, 10%–30% and 31%–50%, respectively. Although the highest increase in tumor cell content by enrichment was observed in group A, the mean tumor cell content after enrichment grew steadily in group A to D. In the first group consisting of 14 samples a final concentration of ≥30% GD2 positive cells (acceptable purity for SNP array analysis) was obtained after enrichment in six cases and in five out of these samples more than 50% GD2 positive cells were obtained. Altogether, the enrichment factor (the fold change of tumor cell content by enrichment) in this group was 82.5. In group B, in 14 out of 18 samples (77.8%) the tumor cell content was raised to a level higher than 30%. For samples with at least 10% tumor cell infiltration (groups C and D, $n = 33$), enrichment increased the tumor cell content to more than 30% for all cases except one. In total, 52 out of 65 frozen samples provided acceptable purity for SNP array analysis (30%–99% GD2+ cells). So far, genomic analysis by UHD-SNP array has been performed for 46 frozen samples leading to clear and interpretable results. Detection of amplification events, SCAs, copy-neutral LOH (cnLOH) and even minor deletions like parts of the *ATRX* gene were possible (three examples are given in Figures 3a, 4 and 5a). The lower limit of 30% tumor cells for SNP array analysis was chosen. Enrichment of four fresh BM samples with initially very low tumor cell infiltration highly increased the tumor cell content (Figure 2, Table 2, Supplementary Figure). Genomic analyses of three samples with an original tumor cell infiltration rate of 0.05%, 0.06% and 0.08% revealed unambiguous SNP array data with different segmental and numerical aberrations including cnLOH (two examples are given in Figures 3b and 5b). In 12 samples with ≥60% infiltration rate, DMSO frozen BM samples were processed without any preceding enrichment steps.

In cases with ≥30% tumor cell infiltration for which no fresh or DMSO frozen BM samples were available, DNA was extracted from BM cytospin or smear slides. DNA was extracted from 22 cytospin slides which were stored at −20 °C. In seven cases, for which no unstained cytospin slides were available, routinely processed, i.e. formalin fixed, GD2/CD56 stained and fluorescence analyzed, cytospin slides which were stored after examination at 4 °C were used. In all 29 cases SNP array analyses were possible leading to interpretable results (two examples are given in Figure 3c and 5c). In these cases, one cytospin slide, each with 17.5 mm diameter containing $7 \times 10^5$ to $1.1 \times 10^6$ mononuclear cells (MNCs), was sufficient to perform a SNP array analysis without any additional DNA amplification step. In addition, in one case, when no further material was available, DNA was extracted from a cytospin slide after successful GD2/CD56 staining and subsequent FISH analysis.

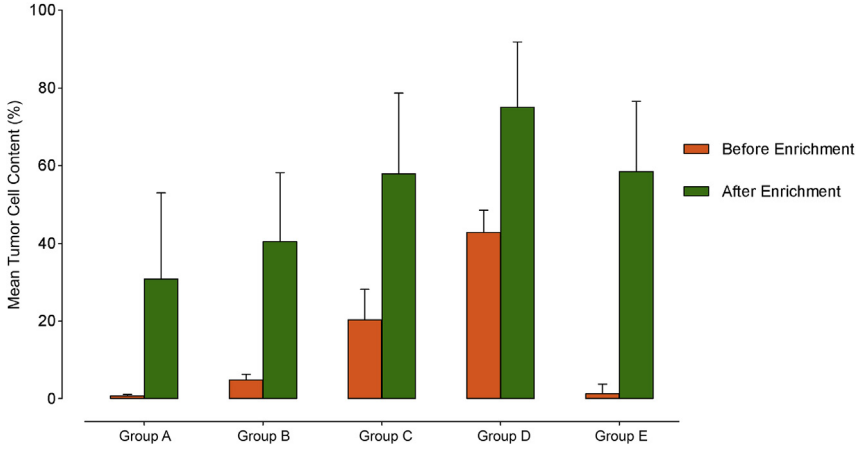


**Figure 2 – Tumor cell content before and after enrichment. The tumor cell content of archival samples before enrichment was classified into four groups: group A: 0.1%–1%, group B: 2%–9%, group C: 10%–30% and group D: 31%–50%. The fresh BM samples were listed in group E. The tumor cell content before and after enrichment in each group is represented by orange and green bars, respectively.**

**Table 1 – Enrichment information of frozen BM samples in different groups.**

| Group | Initial TCC[a] (%) | Total number | TCC[a] after enrichment | | | | | Mean of TCC[a] | | Mean of enrichment factor[b] | Samples with SNP array results |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ≥50% | ≥40% | ≥30% | Minimum | Maximum | Before enrichment | After enrichment | | |
| A | 0.1–1 | 14 | 5 | 5 | 6 | 4.8% | 70.3% | 0.75% | 31.4% | 82.5 | 5 |
| B | 2–9 | 18 | 5 | 9 | 14 | 12.3% | 86.3% | 4.8% | 40.4% | 9 | 12 |
| C | 10–30 | 24 | 14 | 19 | 23 | 15% | 95% | 20.3% | 57.5% | 3.2 | 21 |
| D | 31–50 | 9 | 8 | 9 | 9 | 47.4% | 95% | 42.8% | 75% | 1.8 | 8 |
| Total | 0.1–50 | 65 | 32 | 42 | 52 | 4.8% | 99% | 17.2% | 51.1% | 24.1 | 46 |

a TCC: Tumor Cell Content.
b Enrichment factor: fold change of tumor cell content by enrichment.

Although SNP array results of this sample looked more wavy compared to other samples, SCAs and amplification event affecting the *MYCN* gene could be detected. We were also interested to learn whether unstained conventional BM smear slides could be used for SNP array analyses. DNA was extracted from three unstained BM smear slides with a tumor cell content of 30%–80% providing sufficient amount and sufficiently intact DNA to run SNP array analyses. All three samples resulted in interpretable SNP array results with sufficient quality to identify SCAs and amplified regions (two examples are given in Figures 3d and 5d).

## 4. Discussion

In neuroblastoma, like other cancers, deaths occur mainly due to resistance of metastatic cells to cytotoxic drugs. Thus, the exact diagnosis and adequate treatment of metastatic cells are the most important issues in the clinical practice. Neuroblastoma cells can escape from the primary tumor via the blood stream and colonize the BM. (for review see e.g. Burchill, 2004). So far, it is unclear whether and to what extent the genomics of DTCs at diagnosis corresponds to that of the tumor. Moreover, even less or no information at all exists to date on the genomics of relapse DTC clones and their derivation (primary tumor versus BM infiltration). Genomic characterization of DTCs at different time points during the course of the disease would be an ideal way to unravel these questions. Interphase FISH (I-FISH) is one strategy to tackle these questions. Despite the fact that this technique is extremely sensitive, especially when combined with an immunological marker as performed for example together with GD2/CD56 in the AIPF technique (Mehes et al., 2001), it has the disadvantage of exploring only a limited number of specific genomic aberrations, usually with a need for prior knowledge of likely regions of interest. Consequently, other strategies are needed to learn about the full genomic picture of the cells. The most suited techniques for this purpose, i.e. CGH and SNP array or next generation sequencing (NGS), are only applicable when a sufficient proportion of tumor cells is available in the sample. Therefore, appropriate enrichment techniques have to be applied to obtain a sufficient purity of DTCs.

Despite the fact that a number of methods exist to isolate, enrich and characterize circulating and disseminated tumor cells (Moss et al., 1991; Sun et al., 2011), so far, only one study has performed whole genome analyses of enriched neuroblastoma DTCs. Vandewoestyne and colleagues used two different strategies to enrich rare tumor cells to be able to run array CGH experiments. DTCs from 17 BM samples were enriched by laser capture microdissection (LCM) on immuno-cytologically stained slides. Due to the limited number of tumor cells, whole genome amplification was necessary resulting in false positive and false negative array CGH results. In addition, seven samples were enriched by magnetic activated cell sorting. However, in only two out of the seven samples interpretable array CGH profiles were obtained, and these two fresh samples had more than 14% initial tumor cell infiltration. Neither frozen nor fresh samples with lower tumor cell content gave reliable results (Vandewoestyne et al., 2012).

Since many clinical BM samples have tumor cell infiltration values below the detection limit of SNP array, we tested whether fresh and/or DMSO frozen BM samples with a minimum of 0.05% (fresh) and 0.1% (frozen) initial DTC infiltration rate could be enriched sufficiently to give unambiguous SNP array results. Being in the fortunate situation to have a reliable surface marker, the ganglioside GD2, available, which is presented on the cell surface of virtually all neuroblastoma cells, GD2 antibody- and magnetic bead-based technique which is cost effective, flexible and easy to handle was successfully used. One further advantage of this system is the ability for easy monitoring of the positive and negative fractions, providing clear information on the purity of the enriched sample. With the applied magnetic bead-based technique, it was possible to enrich the original BM infiltration rate on average 24.1 times in DMSO frozen and 660 times in fresh samples. These results were achieved by optimizing the standard manufacturer's protocol by avoiding unnecessary centrifugation and manipulation steps. In addition, the amount of ferromagnetic beads and the starting volume of the cell solution were increased to obtain a high yield of DTCs. Following this protocol only one positive selection step was made to avoid losing cells by second or third rounds.

The genomic analyses were performed without any whole genome pre-amplification step to avoid any amplification-induced bias. The DTC fraction was analyzed by a UHD-SNP array technique resulting in a detailed picture of the genomic profile. Taking advantage of one of the positive aspects of the SNP array technique to visualize minor cell populations,

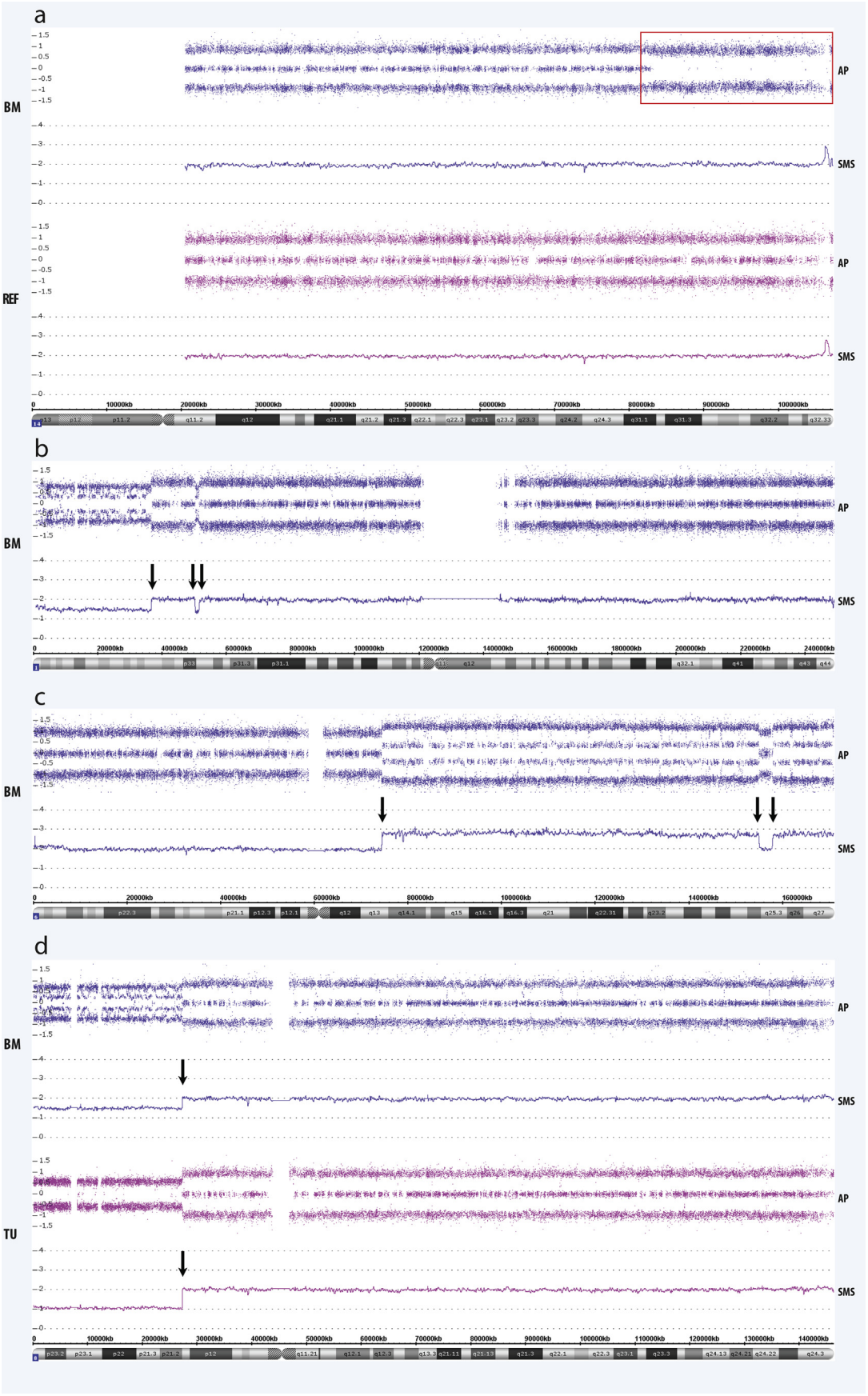


**Figure 3 – SNP array profiles of BM-derived DTCs. a. SNP array data from an enriched DMSO frozen BM sample at relapse with 5.5% tumor cell infiltration before and 85% after enrichment and the corresponding tumor free sample (REF). The red box shows a region with copy neutral**

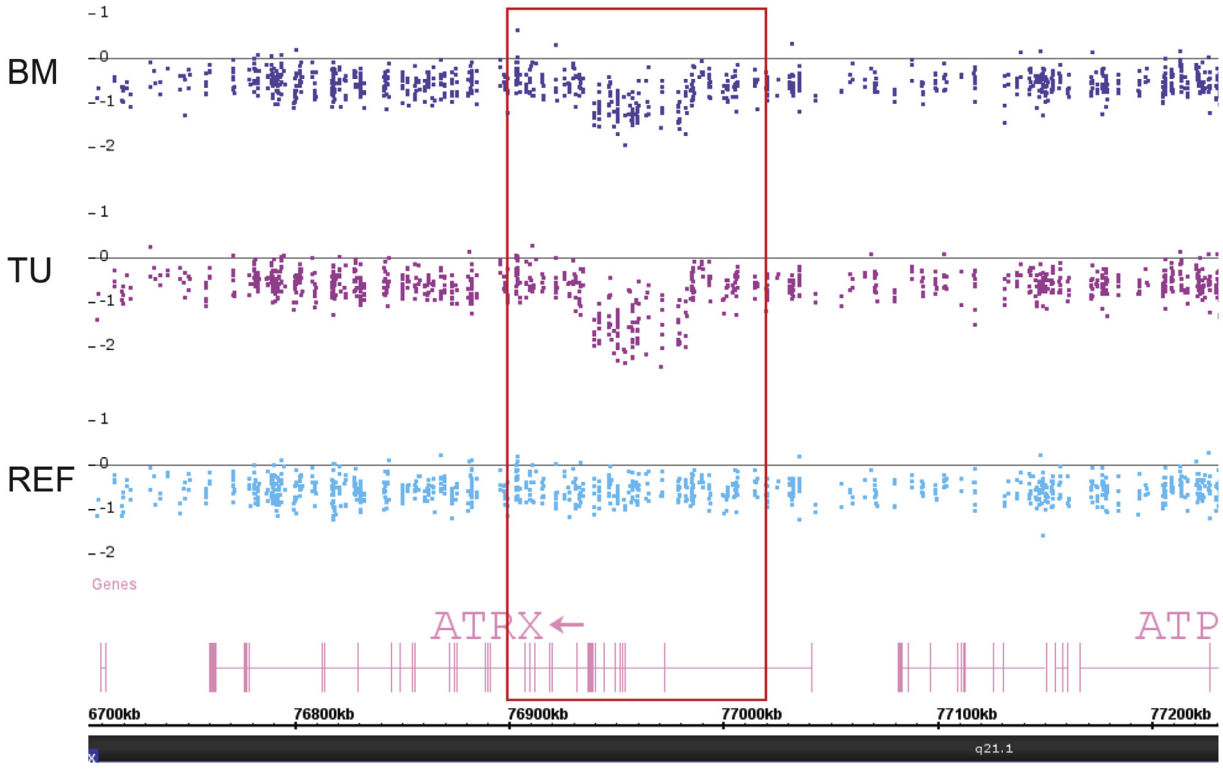


**Figure 4 – SNP array of an enriched frozen BM sample of a male patient at diagnosis with 6% tumor cell infiltration before and 40% after enrichment compared to the primary tumor (TU) and tumor free MNCs as reference (REF). The Log2 ratios show a deletion within the *ATRX* gene on Xq21.1 in the DTCs and in the tumor. The deletion concerns only parts of the *ATRX* gene and is not present in the reference sample.**

SCAs were identified even in BM samples without any enrichment technique in cases where the tumor cell content was ≥30% and in case of amplification events at even lower percentages. Thus, sufficient DNA was extracted from routine BM smears, unfixed cytospin or even fixed and GD2 stained cytospin slides to run the array experiments. Since in any amplification event there are many copies of the affected chromosomal region, the detection rate for amplification will remain high even in samples with very low tumor cell content. Detection rates for SCAs, however, will be dependent on the following factors: on the number of gained/lost segments (e.g.: only one additional 17q arm or three to four additional arms; or a loss in case of 2:1 or bigger differences like 4:2 or 5:2 situations) and furthermore on the homogeneous or heterogeneous presence of the aberration in the tumor cells. In cases with flat SNP array profiles without any segmental or numerical chromosomal aberrations, a false negative result due to low tumor cell content is most likely and should be considered. The applied UHD-SNP array technique is a robust method allowing a detailed view also of minor genomic changes such as deletions within the *ATRX* gene. Importantly, the availability of copy number probes and SNP probes in one platform allows an internal quality assessment system as these two types of information are available for every genomic locus, making detection of copy number changes very reliable. Thus, this technique provides a sensitive approach to assess numerical and structural DNA aberrations (gains, losses and amplifications) and, importantly, identifies areas with cnLOH missed by FISH, MLPA or conventional array techniques.

**LOH in chromosome 14q in the DTCs which is not detectable in the reference. In this region the smooth signal is on level 2 representing two copies while the middle track of the allele peaks is missing in this region meaning two identical copies or copy neutral LOH. b. SNP array of an enriched fresh BM sample with 0.05% tumor cell infiltration before and 68% after enrichment shows two deletions at the short arm of chromosome 1, a large deletion at the distal part (tel. – 36.4 Mb) and a small deletion at 50.3–51.6 Mb (arrows represent breakpoints). In these areas the smooth signal does not descend to 1 because of the amount of normal cells. Also the four tracks in the allele peaks are caused by the mixture of normal cells and tumor cells. c. SNP array profile of the DNA extracted from a BM cytospin slide after GD2/CD56 staining with 90% tumor cell infiltration shows two regions with 3 copies in the long arm of chromosome 6. In these regions the copy number state in smooth signal is close to level 3 (due to a mixture of 10% normal cells) and allele peaks show 4 tracks representing gains (i.e. trisomy). Breakpoints are at 74.4 Mb, 155.0 Mb and 157.9 Mb (arrows). d. SNP array profile from a BM smear slide with 50% tumor cell infiltration at diagnosis and the corresponding data from the primary tumor (TU) show a deletion of the distal part of chromosome 8p. The breakpoints at 27.3 Mb are identical in both samples (arrows). The allele peaks of the tumor disclose a loss of heterozygosity (LOH) in the deleted segment and accordingly the copy number state in smooth signal is 1. In the DTC sample the smooth signal is between 1 and 2 and the allele peaks in this region show four tracks due to a mixture of 50% normal cells and 50% tumor cells. AP: allele peaks, SMS: smooth signal.**

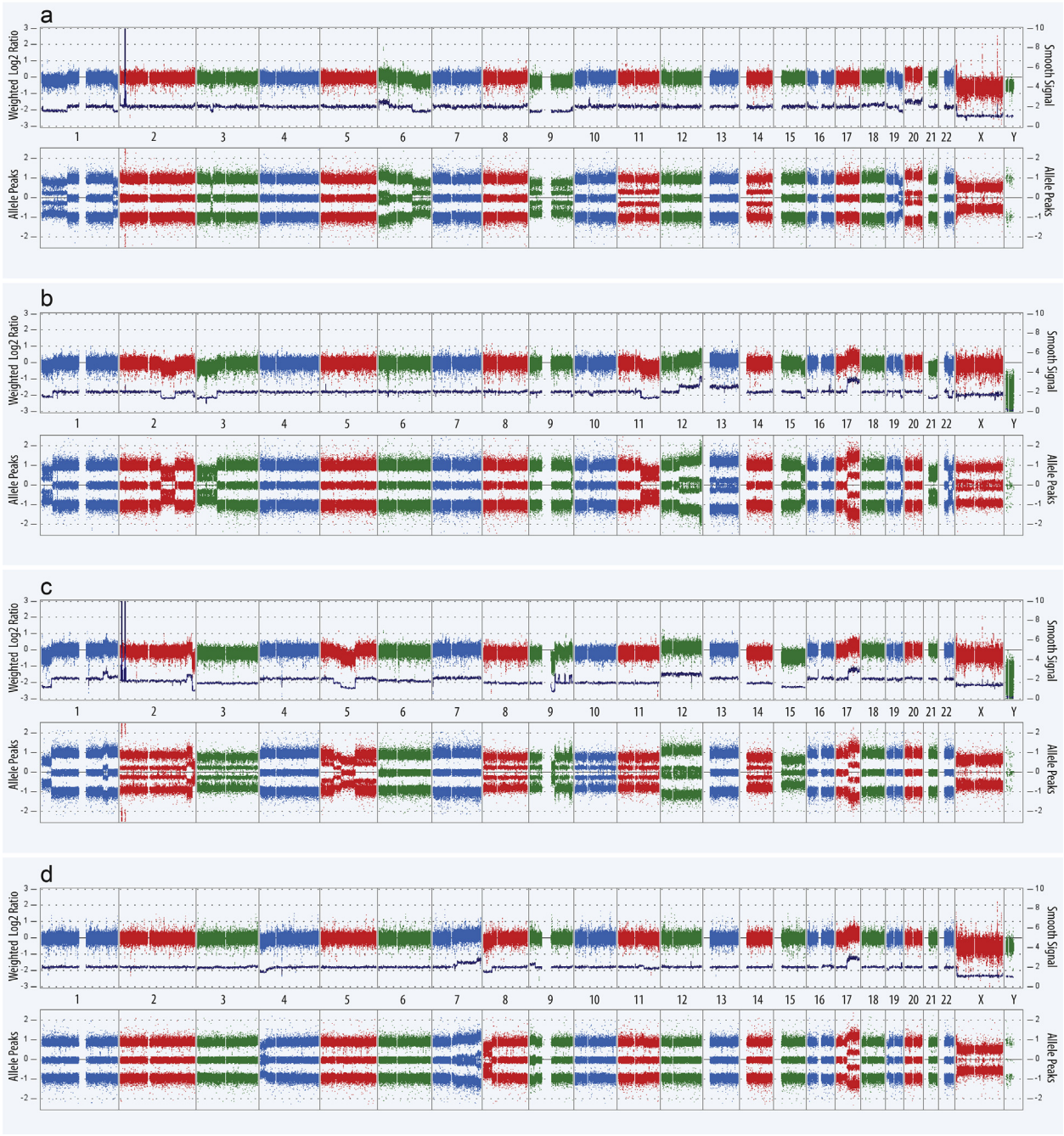


**Figure 5 – Whole genome views (WGVs) of BM-derived DTCs which were created by the ChAS software. WGVs of DTCs from a. enriched DMSO frozen BM sample with 0.69% DTC infiltration before and 60% after enrichment, b. enriched fresh BM sample with 0.05% DTC infiltration before and 68% after enrichment, c. immunostained cytospin slide with 95% DTC infiltration and d. BM smear slide with 50% DTC infiltration, show different numerical and segmental chromosomal aberrations and amplifications (short arm of chromosome 2 in a and c) in different chromosomes. The left *y*-axis in the upper box of each sample shows the weighted log 2 ratio and the right *y*-axis the copy number (smooth signal, dark blue line). The lower boxes in a, b, c and d represent the allele peaks. The numbers in the *x*-axis indicate the chromosome numbers and *X* and *Y*.**

Our study shows that enrichment of fresh and DMSO frozen BM samples from neuroblastoma patients is feasible by magnetic bead-based technique resulting in sufficient purity for whole genome analysis in the bone marrow micrometastases. In addition, in samples exceeding 30% DTCs, routinely processed BM cytospin slides and BM smears resulted in unambiguously interpretable SNP array results. All in all, BM, so far frequently overlooked by genetic studies, can serve as an ideal source to study genomic aberrations at diagnosis and especially during the course of the disease. This information will allow a better categorization of patients with disseminated disease on the basis of the genomic

**Table 2 – Enrichment information of fresh BM samples (group E).**

| Samples | TCC[a] before enrichment (%) | TCC[a] after enrichment (%) | Mean of enrichment factor[b] |
|---|---|---|---|
| 1 | 0.05 | 68.5 | 1370 |
| 2 | 0.06 | 40 | 666.7 |
| 3 | 0.08 | 47 | 587.5 |
| 4 | 5 | 78.6 | 15.7 |

a TCC: Tumor Cell Content.
b Enrichment factor: fold change of tumor cell content by enrichment.

features of DTCs which has not been possible so far. In low infiltrated BM samples the combination of a robust enrichment technique such as magnetic bead-based with a SNP array technique is an excellent and cost effective way to study the genomic profile of bone marrow micrometastases in neuroblastoma.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.10.010.

## REFERENCES

Ambros, I.M., Brunner, C., Abbasi, R., Frech, C., Ambros, P.F., 2014. Ultra-high density SNParray in neuroblastoma molecular diagnostics. Front Oncol. 4, 202.

Ambros, I.M., Zellner, A., Roald, B., Amann, G., Ladenstein, R., Printz, D., Gadner, H., Ambros, P.F., 1996. Role of ploidy, chromosome 1p, and Schwann cells in the maturation of neuroblastoma. N. Engl. J. Med. 334, 1505–1511.

Ambros, P.F., Ambros, I.M., Brodeur, G.M., Haber, M., Khan, J., Nakagawara, A., Schleiermacher, G., Speleman, F., Spitz, R., London, W.B., Cohn, S.L., Pearson, A.D., Maris, J.M., 2009. International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. Br. J. Cancer 100, 1471–1482.

Ambros, P.F., Mehes, G., Ambros, I.M., Ladenstein, R., 2003. Disseminated tumor cells in the bone marrow - chances and consequences of microscopical detection methods. Cancer Lett. 197, 29–34.

Attiyeh, E.F., London, W.B., Mosse, Y.P., Wang, Q., Winter, C., Khazi, D., McGrady, P.W., Seeger, R.C., Look, A.T., Shimada, H., Brodeur, G.M., Cohn, S.L., Matthay, K.K., Maris, J.M., 2005. Chromosome 1p and 11q deletions and outcome in neuroblastoma. N. Engl. J. Med. 353, 2243–2253.

Beiske, K., Burchill, S.A., Cheung, I.Y., Hiyama, E., Seeger, R.C., Cohn, S.L., Pearson, A.D., Matthay, K.K., 2009. Consensus criteria for sensitive detection of minimal neuroblastoma cells in bone marrow, blood and stem cell preparations by immunocytology and QRT-PCR: recommendations by the International Neuroblastoma Risk Group Task Force. Br. J. Cancer 100, 1627–1637.

Bilke, S., Chen, Q.R., Westerman, F., Schwab, M., Catchpoole, D., Khan, J., 2005. Inferring a tumor progression model for neuroblastoma from genomic data. J. Clin. Oncol. 23, 7322–7331.

Brodeur, G.M., 2003. Neuroblastoma: biological insights into a clinical enigma. Nat. Rev. Cancer 3, 203–216.

Brodeur, G.M., Pritchard, J., Berthold, F., Carlsen, N.L., Castel, V., Castelberry, R.P., De Bernardi, B., Evans, A.E., Favrot, M., Hedborg, F., et al., 1993. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J. Clin. Oncol. 11, 1466–1477.

Burchill, S.A., 2004. Micrometastases in neuroblastoma: are they clinically important? J. Clin. Pathol. 57, 14–20.

Caren, H., Kryh, H., Nethander, M., Sjoberg, R.M., Trager, C., Nilsson, S., Abrahamsson, J., Kogner, P., Martinsson, T., 2010. High-risk neuroblastoma tumors with 11q-deletion display a poor prognostic, chromosome instability phenotype with later onset. Proc. Natl. Acad. Sci. U.S.A. 107, 4323–4328.

Cheung, N.K., Zhang, J., Lu, C., Parker, M., Bahrami, A., Tickoo, S.K., Heguy, A., Pappo, A.S., Federico, S., Dalton, J., Cheung, I.Y., Ding, L., Fulton, R., Wang, J., Chen, X., Becksfort, J., Wu, J., Billups, C.A., Ellison, D., Mardis, E.R., Wilson, R.K., Downing, J.R., Dyer, M.A., 2012. Association of age at diagnosis and genetic mutations in patients with neuroblastoma. JAMA 307, 1062–1071.

Coco, S., Theissen, J., Scaruffi, P., Stigliani, S., Moretti, S., Oberthuer, A., Valdora, F., Fischer, M., Gallo, F., Hero, B., Bonassi, S., Berthold, F., Tonini, G.P., 2012. Age-dependent accumulation of genomic aberrations and deregulation of cell cycle and telomerase genes in metastatic neuroblastoma. Int. J. Cancer 131, 1591–1600.

Cohn, S.L., Pearson, A.D., London, W.B., Monclair, T., Ambros, P.F., Brodeur, G.M., Faldum, A., Hero, B., Iehara, T., Machin, D., Mosseri, V., Simon, T., Garaventa, A., Castel, V., Matthay, K.K., 2009. The international neuroblastoma risk group (INRG) classification system: an INRG Task Force report. J. Clin. Oncol. 27, 289–297.

D'Angio, G.J., Evans, A.E., Koop, C.E., 1971. Special pattern of widespread neuroblastoma with a favourable prognosis. Lancet 1, 1046–1049.

Evans, A.E., Gerson, J., Schnaufer, L., 1976. Spontaneous regression of neuroblastoma. Natl. Cancer Inst. Monogr. 44, 49–54.

George, R.E., Attiyeh, E.F., Li, S., Moreau, L.A., Neuberg, D., Li, C., Fox, E.A., Meyerson, M., Diller, L., Fortina, P., Look, A.T., Maris, J.M., 2007. Genome-wide analysis of neuroblastomas using high-density single nucleotide polymorphism arrays. PLoS One 2, e255.

Hero, B., Simon, T., Spitz, R., Ernestus, K., Gnekow, A.K., Scheel-Walter, H.G., Schwabe, D., Schilling, F.H., Benz-Bohm, G., Berthold, F., 2008. Localized infant neuroblastomas often show spontaneous regression: results of the prospective trials NB95-S and NB97. J. Clin. Oncol. 26, 1504–1510.

Ladenstein, R., Ambros, I.M., Potschger, U., Amann, G., Urban, C., Fink, F.M., Schmitt, K., Jones, R., Slociak, M., Schilling, F., Ritter, J., Berthold, F., Gadner, H., Ambros, P.F., 2001.

Prognostic significance of DNA di-tetraploidy in neuroblastoma. Med. Pediatr. Oncol. 36, 83–92.
Maris, J.M., Hogarty, M.D., Bagatell, R., Cohn, S.L., 2007. Neuroblastoma. Lancet 369, 2106–2120.
Maris, J.M., White, P.S., Beltinger, C.P., Sulman, E.P., Castleberry, R.P., Shuster, J.J., Look, A.T., Brodeur, G.M., 1995. Significance of chromosome 1p loss of heterozygosity in neuroblastoma. Cancer Res. 55, 4664–4669.
Mehes, G., Luegmayr, A., Ambros, I.M., Ladenstein, R., Ambros, P.F., 2001. Combined automatic immunological and molecular cytogenetic analysis allows exact identification and quantification of tumor cells in the bone marrow. Clin. Cancer Res. 7, 1969–1975.
Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215.
Molenaar, J.J., Koster, J., Zwijnenburg, D.A., van Sluis, P., Valentijn, L.J., van der Ploeg, I., Hamdi, M., van Nes, J., Westerman, B.A., van Arkel, J., Ebus, M.E., Haneveld, F., Lakeman, A., Schild, L., Molenaar, P., Stroeken, P., van Noesel, M.M., Ora, I., Santo, E.E., Caron, H.N., Westerhout, E.M., Versteeg, R., 2012. Sequencing of neuroblastoma identifies chromothripsis and defects in neuritogenesis genes. Nature 483, 589–593.
Moss, T.J., Reynolds, C.P., Sather, H.N., Romansky, S.G., Hammond, G.D., Seeger, R.C., 1991. Prognostic value of immunocytologic detection of bone marrow metastases in neuroblastoma. N. Engl. J. Med. 324, 219–226.
Schleiermacher, G., Michon, J., Ribeiro, A., Pierron, G., Mosseri, V., Rubie, H., Munzer, C., Benard, J., Auger, N., Combaret, V., Janoueix-Lerosey, I., Pearson, A., Tweddle, D.A., Bown, N., Gerrard, M., Wheeler, K., Noguera, R., Villamon, E., Canete, A., Castel, V., Marques, B., de Lacerda, A., Tonini, G.P., Mazzocco, K., Defferrari, R., de Bernardi, B., di Cataldo, A., van Roy, N., Brichard, B., Ladenstein, R., Ambros, I., Ambros, P., Beiske, K., Delattre, O., Couturier, J., 2011. Segmental chromosomal alterations lead to a higher risk of relapse in infants with MYCN-non-amplified localised unresectable/disseminated neuroblastoma (a SIOPEN collaborative study). Br. J. Cancer 105, 1940–1948.
Schulz, G., Cheresh, D.A., Varki, N.M., Yu, A., Staffileno, L.K., Reisfeld, R.A., 1984. Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients. Cancer Res. 44, 5914–5920.
Seeger, R.C., Reynolds, C.P., Gallego, R., Stram, D.O., Gerbing, R.B., Matthay, K.K., 2000. Quantitative tumor cell content of bone marrow and blood as a predictor of outcome in stage IV neuroblastoma: a Children's Cancer Group Study. J. Clin. Oncol. 18, 4067–4076.
Shimada, H., Ambros, I.M., Dehner, L.P., Hata, J., Joshi, V.V., Roald, B., Stram, D.O., Gerbing, R.B., Lukens, J.N., Matthay, K.K., Castleberry, R.P., 1999. The International Neuroblastoma Pathology Classification (the Shimada system). Cancer 86, 364–372.
Spitz, R., Oberthuer, A., Zapatka, M., Brors, B., Hero, B., Ernestus, K., Oestreich, J., Fischer, M., Simon, T., Berthold, F., 2006. Oligonucleotide array-based comparative genomic hybridization (aCGH) of 90 neuroblastomas reveals aberration patterns closely associated with relapse pattern and outcome. Genes Chromosomes Cancer 45, 1130–1142.
Sun, Y.F., Yang, X.R., Zhou, J., Qiu, S.J., Fan, J., Xu, Y., 2011. Circulating tumor cells: advances in detection methods, biological issues, and clinical relevance. J. Cancer Res. Clin. Oncol. 137, 1151–1173.
Vandewoestyne, M., Kumps, C., Swerts, K., Menten, B., Lammens, T., Philippe, J., De Preter, K., Laureys, G., Van Roy, N., Speleman, F., Deforce, D., 2012. Isolation of disseminated neuroblastoma cells from bone marrow aspirates for pretreatment risk assessment by array comparative genomic hybridization. Int. J. Cancer 130, 1098–1108.
Zhe, X., Cher, M.L., Bonfil, R.D., 2011. Circulating tumor cells: finding the needle in the haystack. Am. J. Cancer Res. 1, 740–751.