Impact of Disseminated Neuroblastoma Cells on the Identification of the Relapse-Seeding Clone

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Abstract

Purpose: Tumor relapse is the most frequent cause of death in stage 4 neuroblastomas. Since genomic information on the relapse precursor cells could guide targeted therapy, our aim was to find the most appropriate tissue for identifying relapse-seeding clones.

Experimental design: We analyzed 10 geographically and temporally separated samples of a single patient by SNP array and validated the data in 154 stage 4 patients.

Results: In the case study, aberrations unique to certain tissues and time points were evident besides concordant aberrations shared by all samples. Diagnostic bone marrow–derived disseminated tumor cells (DTCs) as well as the metastatic tumor and DTCs at relapse displayed a 1q deletion, not detected in any of the seven primary tumor samples. In the validation cohort, the frequency of 1q deletion was 17.8%, 10%, and 27.5% in the diagnostic DTCs, diagnostic tumors, and DTCs at relapse, respectively. This aberration was significantly associated with 19q and ATRX deletions. We observed a significant increased likelihood of an adverse event in the presence of 19q deletion in the diagnostic DTCs.

Conclusions: Different frequencies of 1q and 19q deletions in the primary tumors as compared with DTCs, their relatively high frequency at relapse, and their effect on event-free survival (19q deletion) indicate the relevance of analyzing diagnostic DTCs. Our data support the hypothesis of a branched clonal evolution and a parallel progression of primary and metastatic tumor cells. Therefore, searching for biomarkers to identify the relapse-seeding clone should involve diagnostic DTCs alongside the tumor tissue. Clin Cancer Res; 23(15); 4224–32. ©2017 AACR.

Introduction

The clinical behavior of neuroblastomas is very heterogeneous ranging from spontaneous regression or maturation to highly malignant and metastatic disease (1–4). It is generally assumed that differences in the genetic background are, at least to a large degree, responsible for this clinical heterogeneity. Overall, low-risk neuroblastosoms mostly have whole chromosomal gains without structural aberrations. High-risk neuroblastomas, for example, mostly stage 4, on the contrary, present with different structural aberrations in the form of gains or losses of large chromosomal segments, referred to as segmental chromosomal aberrations (SCAs), MYCN amplification (MNA), and/or mutations as well as copy-number alterations affecting certain genes or parts thereof (5–13). Whether genetic markers might help to identify the relapse-seeding clone and to categorize stage 4 patients into different prognostic subgroups is still a matter of debate.

Intratumor heterogeneity (ITH) in solid tumors can be responsible for treatment failure and drug resistance (14, 15). Despite the huge degree of intertumor genetic heterogeneity, studies on ITH in neuroblastoma have, thus far, mainly focused on MNA (16–21). As a single biopsy likely fails to capture the genetic complexity of a heterogeneous tumor, the relapse-seeding subclone may be overlooked (14).

Bone marrow (BM) is the most common site for disseminated tumor cells (DTCs) in neuroblastoma, and its infiltration at diagnosis and relapse is a hallmark of the majority of stage 4 disease. Depending on the sensitivity of the detection technique, BM infiltration at diagnosis can be found in more than 90% of stage 4 patients (22). In previous studies, we showed that a full genomic and transcriptomic characterization of DTCs in neuroblastoma patients is feasible (23, 24). In the case of adult cancers, DTCs are generally considered to be metastasis precursor cells.
Translational Relevance

Because relapse is the major cause of death in patients with neuroblastoma, early detection and characterization of the relapse-seeding clone would help to develop or choose an appropriate treatment. However, due to intratumor heterogeneity, single or even multiple tumor biopsies may fail to identify the relapse-seeding clone. In this study, we therefore focused on the genomic evolution of neuroblastoma tumors and bone marrow–derived disseminated tumor cells (DTCs) by analyzing geographically and temporally separated samples of 155 patients with stage 4 neuroblastoma. Our data support a branched clonal evolution and a parallel progression of primary and metastatic tumor cells. We provide evidence that the analysis of DTCs at diagnosis besides the tumor biopsies may improve the probability for detecting the relapse-seeding clone. This highlights the unmet need to study genetic changes in diagnostic DTCs alongside tumor tissue to better characterize and predict possible relapses.

and may develop into a metastatic relapse after a period of dormancy (25–27). In this article, we use the term DTCs for any BM-derived tumor cells regardless of the rate of BM infiltration.

In order to better understand tumor progression and how tumor ontogeny proceeds to metastasis and relapse, we analyzed different BM-derived DTCs and tumor samples of a single patient with neuroblastoma taken at diagnosis and at relapse, using high-density SNP array. For a more global conclusion on the clinical impact of genomic aberrations of DTCs, we compared the results of this patient with the genomic results obtained from samples at different time points and sites of 154 patients with stage 4 neuroblastoma.

Materials and Methods

Clinical report of the case study

A 4-year-old boy with a history of constipation, frequent infections, right knee pain, and a mass in the left middle abdomen was admitted to the St. Anna Children’s Hospital, Vienna, in 1993. CT scan detected a mass in the left retroperitoneum. Laboratory tests showed an increase in urinary catecholamines (vanillylmandelic acid: 1.8 folds, homovanillic acid: 1.5 folds, and dopamine: 3 folds) and neuron-specific enolase in the blood. Lactic dehydrogenase was in the normal range. At this time, the patient had a BM involvement with 6% GD2-positive cells. He underwent surgery with diagnosis of stage 4 neuroblastoma. After resection of the para-aortic tumor, pathologic examination reported a 4.5 × 4.5 × 9.4-cm nodular neuroblastoma tumor. After 4 months of chemotherapy, abdominal CT and metaiodobenzylguanidine scans showed no evidence of a remaining tumor. The patient received high-dose chemotherapy followed by an autologous peripheral stem cell transplantation and radiotherapy of the primary tumor site. The patient was in complete remission till 28 months after diagnosis, when he experienced a relapse with a tumor in the right frontoparietal region of the skull and BM involvement. Palliative chemo- and radiotherapy for the metastatic site resulted in an improvement in clinical and imaging signs. He experienced generalized pain 8.5 months after the relapse, and an MRI showed different metastatic lesions in the paravertebral region of the thorax. Palliative radiotherapy was started for paravertebral site but had to be stopped due to the patient's poor general condition. He remained on analgesic therapy and passed away 9.5 months after the relapse.

Patients and samples

For the case study, we analyzed 10 different samples of a patient with stage 4 neuroblastoma including seven samples from different regions of the primary tumor and BM-derived DTCs at diagnosis as well as a metastatic tumor biopsy and BM-derived DTCs at the time of relapse (Supplementary Table S1). A tumor-free peripheral blood sample was used as reference.

For the validation cohort, we analyzed 122 BM-derived DTC samples (73 samples at diagnosis, 40 samples at relapse, and nine samples during therapy or with unknown time point) and 81 primary tumors (50 tumors at diagnosis and 31 tumors after induction chemotherapy) of 154 patients with stage 4 neuroblastoma. Only patients with positive BM were entered into the study. For 43 patients, more than one sample at different time points and/or different sites was analyzed (Supplementary Table S2). In cases where more than one primary tumor sample per patient was available, we combined the information of the different samples and counted shared aberrations as one per primary tumor.

Immunofluorescence detection

BM samples were analyzed by GD2 immunofluorescence staining for quantification of the tumor cell load using an automatic detection device, RC Detect/MetaCyte (MetaSystems GmbH; ref. [28]).

Sample preparation

Tumor and BM samples collected for the case study had been stored in the gas phase of liquid nitrogen. BM samples were enriched by applying a density-gradient centrifugation (Lymphoprep, AXIS-SHIELD PoC AS) followed by a magnetic bead–based enrichment technique using FITC-labeled anti-GD2 antibody (14.18 delta CH2 clone) and anti-FITC microbeads (Miltenyi Biotec) as already described in detail (23). The BM at diagnosis had a 6% DTC infiltration rate before and a 50% rate after enrichment. The tumor cell content of the BM sample at relapse was unknown; however, 45% DTC infiltration was obtained after enrichment. Cryosection slides were prepared from seven different regions of the primary tumor and from the tumor biopsy at relapse. After hematoxylin and eosin staining, the tumor cell–rich regions were selected for DNA extraction. DNA was extracted using the high salt extraction method (29).

For the validation cohort, DNA was extracted from 122 BM-derived DTC samples. In case of BM samples with ≤50% tumor cell infiltration, tumor cells were enriched using the magnetic bead–based technique. In cases where no fresh or DMSO-frozen BM was available, DNA was extracted from immunostained or unstained cytopsin slides for samples with ≥30% tumor cell infiltration rate. Furthermore, tumor DNA was extracted from fresh or snap-frozen tumor tissues and also from the tumor cell–free mononuclear cell fractions as reference.

Genomic analysis

Genomic profiles of tumor cells were generated using Cytocan HD Arrays (Affymetrix Inc.), and the data were
analyzed using the ChAS software (Affymetrix Inc.; refs. 23, 30). CytoScan HD has a genome-wide coverage with more than 2.6 million copy number and SNP markers. This array has more than 99% sensitivity, which can reliably detect copy-number changes even as small as 25 to 50 kb size with a high specificity. Because it includes 750,000 SNP probes, it is possible to detect regions with copy-neutral LOH and low level of mosaicisms. Subclonal frequencies were calculated by evaluating the smooth signal level and the allele difference track pattern and by considering the tumor cell content of the samples as described before (23, 30, 31).

I-FISH
Interphase fluorescent in situ hybridization (I-FISH) experiments were performed on tumor touch imprints (TTIs) and BM cytospin slides for the detection of 1q deletion in the diagnostic DTCs and seven primary tumor samples of the case study. The cells were fixed in 4% formaldehyde at 4°C for 15 minutes and, after rinsing in PBS, were digested in 0.05% pepsin in 1 N HCl for 1 to 2 minutes. The slides were dehydrated in ethanol series: 70%, 95%, and 100%, 3 minutes each. Two probes (digoxin-labeled RP11-951L14 and biotin-labeled RP11-312G23, kindly provided by Dr. Mariano Rochci, Bari, Italy) were added. Denaturation was performed on a heating plate at 78°C for 8 minutes, and hybridization was carried out at 37°C overnight in a humid chamber. The slides were washed with formamide solution for 15 minutes at 42°C and with 2 × standard saline citrate (SSC) for 7 minutes twice and incubated with mouse anti-biotin and sheep anti-digoxin antibodies at 37°C for 30 minutes in a humid chamber. After washing in 4 × SSC/0.1 Tween 20 at 42°C for 2 minutes, the slides were incubated with goat anti-mouse Cy3 and rabbit anti-sheep FITC in 37°C for 30 minutes. Slides were covered with mounting medium plus DAPI after washing in Tween 20 and PBS.

Statistical analysis
To investigate the association between different aberrations and sample types, a generalized linear model was fitted using the logistic link function. The modelling of correlated data (i.e., samples from the same patient at different time points) was performed using the generalized estimating equation approach developed by Liang and Zeger (32). The Fisher exact test was used to determine the association between different chromosomal aberrations. Kaplan–Meier curves were generated for event-free survival (EFS) and overall survival (OS). Curves were compared using a log-rank test. For measuring EFS, “time to event” was defined as the time from diagnosis to the time of the first relapse, progression, or death.

Ethics statement
Ethical approval was obtained from the St. Anna Children’s Hospital ethics committee, and the patients’ parents gave their informed consent.

Data access
Affymetrix CytoScan HD array data of this study have been submitted to NCBI Gene Expression Omnibus under accession number GSE84291.

Results
To study the clonal expansion during tumor development, we reconstructed the phylogenetic tree for the samples of a stage 4 patient by using chromosomal aberrations as markers of clonal origin (Fig. 1). For this purpose, we assumed that identical breakpoints occurred at the same time point during clonal evolution and that the number of aberrations increased with time. A schematic visualization of the way how the temporal sequence of different events was deduced from the appearance of different chromosomal aberrations is provided in Supplementary Fig. S1. In total, we found 91 breakpoints and three whole chromosome copy-number changes in the 10 different samples of the patient (Supplementary Fig. S2; Supplementary Table S3). Eighteen breakpoints were homogeneously present in all cells of all samples, representing the genomic profile of the common ancestor clone. We detected 37, five, and 21 breakpoints exclusively in the primary tumor, diagnostic DTCs, and relapse samples, respectively (Fig. 2). The primary tumor harbored genetically distinct subpopulations indicating extensive ITH. Except for a terminal deletion at 5p, which was found in 50% of cells of the relapse tumor, all other aberrations detected in the relapse samples were shared between tumor and DTCs.

A terminal deletion affecting the long arm of chromosome 1 (231,262kb-pter) was homogeneously present in the metastatic tumor and DTCs at relapse as well as in approximately 30% of the diagnostic DTCs. However, this aberration was undetectable in any of the seven analyzed primary tumor samples (Supplementary Fig. S3). Furthermore, chromosome Y loss (LOY) and a gain in the AGBL4 gene were homogeneously present in the relapse samples and in the DTCs at diagnosis but were found in only about 35% of the primary tumor cells. The analyzed relapse samples showed a number of focal copy-number changes affecting single genes or parts thereof (e.g., PTPRD and ATRX intragenic deletions, or affecting two to three adjacent genes) (Supplementary Table S4). Deletions affecting ZMYM5, ARID2, ATRX, and RBM53 were present in all samples. Deletions in PTPRD, RBM53 (with different breakpoints from the deletion shared between all samples), and GALT13 and gains in MIR1246, LOC375295, NRP2, PARD3B, DNAdam1, SIRT1, HERCa, and CADPS2 were present only in the relapse samples. GALT13 was deleted in the DTCs at diagnosis with different breakpoints. AGBL4 was deleted in the relapse samples, DTCs at diagnosis, and a subclone of the primary tumor.

The applied SNP array technique is able to detect copy-number changes in samples with even less than 10% tumor cell content. In addition, in order to search for tumor cell clusters with 1q deletion, we applied I-FISH with probes flanking the breakpoint. The I-FISH pattern indicating a deletion of the terminal region of 1q (one single spot and one pair of signals) was found in none of the seven TTIs. However, this hybridization pattern indicating 1q deletion was found in the diagnostic DTCs (Supplementary Fig. S4).

To determine the general frequency of 1q terminal deletion, we analyzed the SNP array data of 203 BM-derived DTCs and primary tumor samples of 154 patients with stage 4 neuroblastoma (Supplementary Table S2).

In the validation cohort, 17.5% of patients had at least one sample with 1q terminal deletion (Supplementary Table SSA). In addition to the 1q terminal deletions resulting in partial monosomies in 22 samples, we detected a deletion in the trisomic q arm that resulted in a 1q imbalance with loss of the distal region in 11 samples. The frequency of this aberration was 17.8%, 10%, and 27.5% in the DTCs at diagnosis, diagnostic tumors, and DTCs at relapse, respectively (Fig. 3A). The
breakpoints of all 33 samples clustered within a 14,551 kb region (226,934–241,485 kb). The smallest region of overlap (SRO) in samples with 1q deletion spanned the terminal 7,766 kb of the q arm (Fig. 4). This region consists of 93 genes including 80 protein-coding genes (Supplementary Table S6). By analyzing the R2 database (R2: Genomics Analysis and Visualization Platform, http://r2.amc.nl), we observed that among these genes, low expression of RGS7, AKT3, C1orf101, COX20, and CNST significantly decreased OS and/or EFS in patients with neuroblastoma in many of datasets (refs. 9, 33–36; Supplementary Table S7). The same result was observed for the FMN2 gene which was deleted in all samples but two.

For 10 patients (including the case study) with 1q deletions found in at least one of their samples, more than one analyzed sample from different time points and/or different sites was available. We observed spatial and/or temporal heterogeneity of 1q deletion between the different samples in six of these patients. In addition to the discrepancy observed in the studied case, we found 1q deletions only in the DTCs at relapse but not in the corresponding primary tumors. In one case, a 1q deletion was present in the second relapse but neither in the primary tumor.

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primary tumor nor in the first relapse sample (Supplementary Table S5A). We also investigated the association between 1q deletion and other frequent aberrations in the DTCs at diagnosis and at relapse (Supplementary Fig. S5). Because we found 1q deletions only in patients ≥18 months and mainly in samples without MNA, we performed the statistical evaluation in patients ≥18 months without MNA. The 1q deletion was significantly associated with 19q and ATRX deletions at diagnosis (Fig. 3B). However, a significant correlation did not exist for DTCs at relapse. Both aberrations were also detected in all samples of the case study.

Terminal deletions of 19q were found in 18.2% of patients. The frequency of this aberration was 15.1% in the DTCs at diagnosis, 27.5% in the DTC samples at relapse (Fig. 3A). We observed spatial and/or temporal heterogeneity of the 19q deletion in five of nine patients with more than one analyzed sample (Supplementary Table S5B). The SRO for samples with chromosome 19 deletions spanned from 57,633 kb to the terminal of the q arm (Supplementary Fig. S6). This region consists of 70 genes (43 of them are ZNF family genes) including 62 protein-coding genes (Supplementary Table S6). According to the R2, downregulation of ZNF264 and AURKC decreases OS and/or EFS of patients with neuroblastoma (Supplementary Table S7).

Intragenic deletions within the ATRX gene were observed in 13.6% of patients. We found this aberration exclusively in the samples without MNA of patients ≥18 months. The frequency of this deletion was about 12% in the diagnostic DTCs and primary tumors and 15% in the DTCs at relapse (Fig. 3A). In only one of four patients with more than one analyzed samples, we observed temporal heterogeneity of the ATRX deletion. In this case, the deletion was detected in the primary tumor but not in the DTCs at diagnosis (Supplementary Table S5C).

The three aberrations of interest were found exclusively (for ATRX deletions) or mainly (for 1q and 19q deletions) in the samples without MNA of patients ≥18 months. Therefore, in order to eliminate these confounding variables, the survival analysis was restricted to patients ≥18 months without MNA. Within this patient cohort, only 19q deletion found in diagnostic DTCs was considered to be a significant marker for EFS. Five-year EFS was 13% and 12% and 39% and 11% for nine patients with and 33 patients without 19q deletions, respectively (P = 0.022). Similarly, a nonsignificant effect of 19q deletions on OS was observed (5-year OS: 26% ± 16% vs. 35% ± 12%, P = 0.186).
Although 1q and ATRX deletions in the diagnostic DTCs did not reach statistical significance, we observed an effect by these aberrations on EFS. Five-year EFS was 23% ± 14% versus 38% ± 11% for 11 patients with 1q deletions compared with 31 patients without this aberration ($P = 0.589$), and 19% ± 16% versus 37% ± 11% for eight patients with ATRX deletions compared with 34 patients without this aberration ($P = 0.247$; Fig. 5).

Due to a low number of diagnostic tumors and tumors after induction therapy, we combined these two groups for survival analyses. Interestingly, 1q and ATRX deletions in the primary tumors showed no effect on OS and EFS rates. Although 19q deletions in the primary tumors decreased OS and EFS of patients, this effect was stronger when found in the diagnostic DTCs (Supplementary Fig. S7). To have a general overview of the effect of these aberrations on the survival of stage 4 patients regardless of age and the status of MYCN, we also performed the survival analysis for the whole population, without age and MNA adjustment. 1q, 19q, and ATRX deletions in the diagnostic DTCs had comparable effects on EFS and OS compared with the presence of these aberrations in the diagnostic DTCs after eliminating age and MNA (Supplementary Fig. S8).

**Discussion**

By comparing the genomic profile of DTCs and tumor samples at different time points, we were able to identify the evolutionary relationship of different clones, as well as the presumed order of chromosomal aberration events during tumor evolution in a neuroblastoma patient. Genomic data showed that the primary and metastatic tumor samples and DTCs at diagnosis and at relapse had a common ancestor. Despite this common origin, a parallel progression model of primary tumor and metastatic samples explains our data best. Presumably, tumor cells left the primary tumor site early and disseminated into the BM where they accumulated additional genomic aberrations independent of the primary tumor. At the primary tumor site, a branched clonal evolution of the cancer genome with stepwise accumulation of somatic aberrations resulted in extensive ITH.

Temporal heterogeneity concerning copy-number changes and gene mutations was reported in neuroblastoma (37–39). In line with the studies performed on matched primary and relapse neuroblastomas, despite a decrease in subclonal copy-number changes in the relapse samples compared with the primary tumors (39), a number of de novo aberrations were found in the relapse samples (37, 39). The question arises whether the accumulation of these new aberrations in the relapse clone was a result of selective pressure exerted by drug exposure or not. However, data on the genomic evolution of neuroblastomas do not necessarily support the view of drug-mediated genomic evolution as sole cause for acquiring genomic aberrations. Schleiermacher and colleagues showed that accumulation of chromosomal aberrations occurs not only in patients...
receiving chemotherapy but also in patients who had not received chemotherapy (40). In addition, similar to previous studies (37, 39), we identified some unique aberrations in the primary tumor that were no longer present in the relapse samples. These aberrations may tell us about their minor impact concerning driving the tumor cell progression and chemoresistance. These data support the importance of performing biopsies for genomic analyses of relapsed neuroblastomas.

Most strikingly, our data indicate that the metastatic tumor and DTCs at relapse developed from a subclone that was already present in the BM at diagnosis but was either not detected or did not exist in the primary tumor. The aberration unique to this clone was a terminal deletion of 1q. This aberration, originally described by Fieuw and colleagues (41), was present in 30% of diagnostic DTCs and in all cells of the relapse samples but was not detected in any of the primary tumor subclones of the case study. Two additional aberrations (LOV and a gain within the AGBL4 gene) present in the relapse samples were detected in the diagnostic DTCs, but these aberrations were only subclonal in the primary tumor and the detectability differed geographically within the tumor. Nevertheless, we cannot rule out the possibility that the assumed relapse-seeding clone existed either in a region of the primary tumor that was not sampled or in a very low number of tumor cells and was therefore not detected by the applied techniques in the sampled regions.

Our data indicate that for two reasons the identification of the genomic profile of the relapse clone may be missed when analyzing only the primary tumor: first, sampling error due to ITH and, second, independent evolution of the relapse clone(s) after diagnosis. However, we showed that the relapse-seeding clone may be present in the metastatic tissue (BM) at diagnosis. Therefore, we hypothesize that by sampling and analyzing the BM at diagnosis in addition to the primary tumor, the chance for detecting relapse-specific aberrations at an early time point increases.

To verify which tissue (tumor vs. DTCs) harbors the 1q terminal deletion at a higher frequency, we searched in a large group of stage 4 patients. Fieuw and colleagues found 1q deletions in 7.8% of stage 4 primary tumors (41). In our cohort, the frequency of 1q deletions was 10% in tumors at diagnosis. In addition to the significantly higher frequency of this aberration in the relapse samples, the frequency of 1q deletion in the diagnostic DTCs was nearly double that of the primary tumors. In cases with spatial and/or temporal heterogeneity of 1q deletion, this aberration was present in the DTCs at diagnosis and/or relapse but was not found in the primary tumors. The presence of this aberration in the DTCs at diagnosis did not significantly increase the likelihood of an adverse event; therefore, further studies are needed to investigate the possible role of 1q deletion as a prognostic marker and to ravel the role of the genes in this region in tumor progression. There are some interesting genes in the SRO of this aberration. RGS7 (regulator of G-protein signaling 7) is a protein coding gene and the most proximal gene within the SRO. The regulators of G-protein signaling pathway play an important role in signaling transduction, cellular activities, and carcinogenesis (42, 43). This candidate tumor-suppressor gene found to be methylated in renal cell carcinoma and associated with prognosis (44). Another interesting gene in this region is AKT3. The protein coded by this gene is a member of the serine/threonine protein kinase family. AKT kinases play a role as regulators of metabolism, translation, proliferation, survival, and angiogenesis (45). The AKT signaling pathway is important in tumor development, disease aggressiveness, and drug resistance (46). AKT3 plays an important role in brain development, and various mutations in this gene are linked to neurological disorders (45). Although AKT3 upregulation or increased copy number of its chromosomal region was reported in different cancers (46), recent data indicate that AKT3 inhibits cancer cell migration. It has been shown that knockdown of AKT3 increases the metastatic potential of tumor cells (47). Another gene is FMN2, a member of the formin homology protein family. Increased FMN2 protein level inhibits degradation of p21 and promotes cell-cycle arrest (48, 49). Based on the R2 database, lower expression of these three genes decreases the survival of neuroblastoma patients.

19q terminal deletion, which was significantly associated with 1q deletion, could represent another potential marker for relapse-seeding clones since this aberration had a higher frequency in the relapse samples and significantly decreased the EFS of patients when found in the DTCs at diagnosis. Similar to our findings, this aberration was reported by Cobrinik and colleagues in non–MYCN-amplified tumors (50).

Deletion within the ATRX gene was also significantly associated with 1q deletion. Somatic alterations of the ATRX gene were reported to occur in 10% to 22% of stage 4 neuroblastoma tumors. Seventy percent of these alterations, which are exclusively present in patients ≥18 months with non-MNA tumors, were found in the form of deletions within this gene (6, 9–12). This aberration is proposed to define a subgroup of high-risk neuroblastomas resulting in elongation of telomeres by alternative lengthening of telomeres, ALT (10, 12). In line with these reports, we found intragenic ATRX deletions in 13.6% of stage 4 patients, exclusively older than 18 months without MNA. There was neither a considerable difference in the frequency between tumor and DTC samples nor between the different time points of sample acquisition, which suggests a driver role for this aberration.

Significant decreased EFS was observed only for patients with 19q deletion in the diagnostic DTCs. Although statistical significance was not achieved for decreased EFS of patients with 1q or ATRX deletions in the diagnostic DTCs and also not for decreased OS for patients with 19q deletion, we have to bear in mind that the survival analyses were performed for a prognostically highly unfavorable patient group (stage 4, ≥18 month). Larger studies are warranted to investigate and validate the independent prognostic importance of these aberrations.

In summary, we provide evidence for a branched clonal evolution with early dissemination and parallel evolution of the primary tumor and DTCs in a patient with neuroblastoma. The relapse-associated aberration found in a single, well-characterized patient with neuroblastoma was verified in a large number of tumor and DTC samples. Our results indicate that the presumed relapse-seeding clone can be found with a different frequency in DTCs as compared with tumor biopsies. This study demonstrates the unmet need to study genetic changes in diagnostic DTCs alongside tumor tissues to better characterize and predict possible relapses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Authors' Contributions

Conception and design: M.R. Abbasi, I.M. Ambros, P.F. Ambros
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.R. Abbasi, F. Rifatbegovic, C. Brunner, U. Pötschger, I.M. Ambros, P.F. Ambros
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