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Identifying molecular markers for the sensitive detection of residual atypical teratoid rhabdoid tumor cells

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Atypical teratoid rhabdoid tumor (AT/RT), a rare and highly malignant tumor entity of the central nervous system that presents in early childhood, has a poor prognosis. AT/RTs are characterized by biallelic inactivating mutations of the gene *SMARCB1* in 98% of patients; these mutations may serve as molecular markers for residual tumor cell detection in liquid biopsies. We developed a marker-specific method to detect residual AT/RT cells. Seven of 150 patient samples were selected, each with a histological and genetically ascertained diagnosis of AT/RT. Tumor tissue was either formalin fixed or fresh frozen. DNA was extracted from the patients' peripheral blood leukocytes (PBL) and cerebrospinal fluid (CSF). Multiplex ligation-dependent probe amplification, DNA sequencing, and fluorescence in situ hybridization were used to characterize the tumors' mutations. Residual tumor cell detection used mutation-specific primers and real-time PCR. The detection limit for the residual tumor cell search was 1–18%, depending on the quality of the template provided. The residual tumor cell search in PBL and CSF was negative for all seven patients. The *SMARCB1* region of chromosome 22 is prone to DNA double-strand breaks. The individual breakpoints and breakpoint-specific PCR offer the option to detect minimal residual tumor cells in CSF or blood. Even if we did not detect minimal residual tumor cells in the investigated material, proof of principle for this method was confirmed.

Keywords AT/RT, rhabdoid tumor, *SMARCB1*

Atypical teratoid rhabdoid tumor (AT/RT) is a rare and highly malignant tumor of the central nervous system, which was first described as a distinct entity in 1996 (1,2). Currently, AT/RT are World Health Organization (WHO)–classified under embryonal tumors 9508/3 and receive a WHO grade IV (3). AT/RTs account for approximately 1–2% of all tumors of the central nervous system during childhood. However, data from institutional reviews and institutional cancer registries encourage the supposition that AT/RTs constitute about 50% of all malignant brain tumors in children below the age of 1 year (4). AT/RTs have an unfavorable prognosis, and the actual number of cases has likely been underestimated in the past (5), because of the close resemblance of AT/RT to the more prevalent childhood tumor medulloblastoma (6). Rhabdoid tumors may also occur at other sites of the body, such as the kidney, the liver, and soft tissues (7). One unique feature that the majority of AT/RT and other rhabdoid tumors have in common is a biallelic alteration of the gene *SMARCB1*, a tumor suppressor gene located on chromosome 22q11.2, corresponding with Knudson's two-hit recessive model of oncogenesis (8–10). About 20% of patients carry heterozygous germline mutations of *SMARCB1*, which defines the rhabdoid tumor predisposition syndrome type 1 (RTPS1) (Online Mendelian Inheritance in Man database [OMIM] 609322) characterized by very early onset of tumor manifestation.

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SMARCB1/INI1 is a subunit of the SWI/SNF chromatin remodeling complex, an evolutionarily conserved multisubunit chromatin remodeling complex, which uses the energy of ATP hydrolysis to mobilize nucleosomes and remodel chromatin and thereby regulate transcription of target genes (11). Recently, loss of function mutations of the ATPase subunit BRG1/SMARCA4 of the SWI/SNF chromatin remodeling complex have also been implicated in the pathogenesis of AT/RT and other rhabdoid tumors (12,13). Constitutional inactivating mutations in *SMARCA4* define rhabdoid tumor predisposition syndrome type 2 (RTPS2) (OMIM 613325) (12), further underlining the importance of this complex in suppressing the generation of tumors. An inactivation or loss of the SWI/SNF complex core proteins leads to increased transcription of many genes, promoting tumorigenesis (8). These findings indeed provided the first link between chromatin remodeling complexes and tumor suppression (14). AT/RT has since obtained a special position among the different tumor entities because of its distinct pathogenesis, its association with *SMARCB1*, and its seeming independence from further accumulation of genetic alterations, as Hasselblatt et al. (15) and Kieran et al. (16) have screened AT/RT for genetic alterations in genes other than *SMARCB1* and detected none. Thus, the suggested genetic stability (17) makes the gene *SMARCB1* an ideal molecular marker, because its clones rarely escape detection or targeted treatment by change of genetic makeup (17). The most common germline mutations have been found to be nonsense and frameshift mutations that lead to a premature truncation of the protein preferentially in the region of exons 4 and 5 (18). Intragenic deletions are likewise distributed among both somatic and germline abnormalities. They may involve single exons or the complete *SMARCB1* gene and even other flanking genes. The deletions and their corresponding breakpoints, however, have not yet been examined extensively. Thus, one research objective was to identify the breakpoints of *SMARCB1* deletions, as the region is apparently prone to DNA double-strand breaks. The final objective was a proof-of-principle study of whether *SMARCB1* mutations may serve as molecular markers for the sensitive detection of residual tumor cells by means of real-time PCR of DNA from liquid biopsies, such as peripheral blood or cerebrospinal fluid (CSF). Since the risk for an early relapse of these tumors is very high, a specific and sensitive method for early detection of tumor recurrence would be highly desirable.

Materials and methods

Patient-sample selection

Seven individuals from a cohort of 150 consecutive patients were studied. The diagnosis of AT/RT was based on the histopathology and SMARCB1/INI1 staining of tumor samples, which was negative in all cases. The diagnosis was confirmed by the molecular identification of biallelic *SMARCB1* alterations. Patients with germline mutations were excluded, since their diagnostic targets were not specific for tumor cells. Tumor material was provided by the EU-RHAB neuropathology reference center (Institute of Neuropathology, University Hospital Münster), or by the Department of Pediatric Hematology and Oncology at the University Medical Center Hamburg-Eppendorf. This scientific study was covered by approval of the ethics committee and informed consent by the patients' parents.

DNA extraction

Genomic DNA from either formalin-fixed paraffin-embedded (FFPE) tumor or from fresh frozen (FF) tumor tissue, as well as from peripheral blood leukocytes (PBL) and CSF,from the patients was extracted by the QIAamp DNA mini kit for tissue and blood, according to the manufacturer's protocol (Qiagen, Hilden, Germany). Negative wild-type control DNA was extracted from PBLs of tumor-negative humans.

Breakpoint localization

Multiplex ligand-dependent probe amplification

The breakpoints of the *SMARCB1* deletions were roughly localized using multiplex ligand-dependent probe amplification (MLPA) by the SALSA MLPA kit P250 DiGeorge and SALSA MLPA kit P258 *SMARCB1* (MRC-Holland, Amsterdam, Netherlands) and further narrowed down using primer-walking PCR. All primers were designed using the National Center for Biotechnology Information (NCBI) reference sequence NT_011520.12 of chromosome 22. Primers were designed with melting temperatures (T_m) at 71°C ± 2°C. Melting temperatures were calculated using a biocalculator (Metabion International AG, Martinried, Germany). Primer-walking PCR used GoTaq Green Master Mix (Promega, Madison, WI) according to protocol; annealing temperatures were programmed at 68°C to ensure high specificity. Breakpoints were narrowed down to 200–500 bp before performing a deletion-spanning PCR. Deletion-spanning PCR was performed using DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA) as well as Invitrogen Taq DNA polymerase (Thermo Fisher Scientific). The distinct band in the agarose gel (1.2% + 1μL ethidium bromide/100 mL) was cut out and sequenced. The sequence was analyzed using the Basic Local Alignment Search Tool (NCBI Nucleotide BLAST release 2.2.28, http://blast.ncbi.nlm.nih.gov/Blast.cgi?).

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was applied on paraffin-embedded tissues using two assays with the differentially labeled bacterial artificial chromosome (BAC) clones RP11-1112A23 (labeled in Spectrum Orange) and RP11-76E8 (labeled in Spectrum Green) in assay 1 and RP11-71G19 (labeled in Spectrum Orange) and RP11-911F12 (labeled in Spectrum Green) in assay 2. A commercial probe for the centromeric region of chromosome 6 (CEP6, labeled in Spectrum Blue, Abbott Laboratories, Abbott Park, IL) served as an internal hybridization control in both assays. Labeling of probes, pretreatment of FFPE sections of tumor tissues, hybridization, washing, and evaluation were performed in accordance with recently described protocols (19). Slides were evaluated by two observers using Zeiss fluorescence microscopes (Oberkochen, Germany) equipped