Abstract

Purpose: A high-resolution genomic profiling and comprehensive targeted analysis of INI1/SMARCB1 of a large series of pediatric rhabdoid tumors was done. The aim was to identify regions of copy number change and loss of heterozygosity (LOH) that might pinpoint additional loci involved in the development or progression of rhabdoid tumors and define the spectrum of genomic alterations of INI1 in this malignancy.

Experimental Design: A multiplatform approach using Illumina single nucleotide polymorphism-based oligonucleotide arrays, multiplex ligation-dependent probe amplification, fluorescence in situ hybridization, and coding sequence analysis was used to characterize genome-wide copy number changes, LOH, and genomic alterations of INI1/SMARCB1 in a series of pediatric rhabdoid tumors.

Results: The biallelic alterations of INI1 that led to inactivation were elucidated in 50 of 51 tumors. INI1 inactivation was shown by a variety of mechanisms, including deletions, mutations, and LOH. The results from the array studies highlighted the complexity of rearrangements of chromosome 22 compared with the low frequency of alterations involving the other chromosomes.

Conclusions: The results from the genome-wide single nucleotide polymorphism array analysis suggest that INI1 is the primary tumor suppressor gene involved in the development of rhabdoid tumors with no second locus identified. In addition, we did not identify hotspots for the breakpoints in sporadic tumors with deletions of chromosome 22q11.2. By employing a multimodality approach, the wide spectrum of alterations of INI1 can be identified in the majority of patients, which increases the clinical utility of molecular diagnostic testing.

Malignant rhabdoid tumors are rare, highly aggressive neoplasms found most commonly in infants and young children. Although they may be present in any location in the body, they are predominantly found in the kidney and central nervous system (CNS). Patients may present with apparently sporadic tumors in one anatomic site or with multiple primary tumors arising in the brain, kidney, and/or soft tissues. Due to their heterogeneous histologic features, diagnosis of these lesions can often be difficult. For example, CNS atypical teratoid/rhabdoid tumor is often misclassified as medulloblastoma, primitive neuroectodermal tumor, or choroid plexus carcinoma (1).

The development of rhabdoid tumors was initially associated with alterations of chromosome 22 (2). Subsequent studies implicated the INI1/hSNF5/SMARCB1/BAF47 [MIM 601607] gene, located on chromosome band 22q11.2, as the gene responsible for the initiation of malignant rhabdoid tumors. Germ-line and somatic mutations and deletions of INI1 have been reported in renal and extrarenal rhabdoid tumors as well as atypical teratoid/rhabdoid tumors (3–5). INI1 has also been implicated in the development of epithelioid sarcoma (6), renal medullary carcinoma (7), and familial schwannomatosis (8), although it is not clear if each of these entities has a similar spectrum of mutations and deletions compared with rhabdoid tumors. In patients with malignant rhabdoid tumors, INI1 appears to function as a classic tumor suppressor gene, whereby germ-line mutations and deletions predispose to the development of these malignancies. Inactivation of both copies of the gene leads to loss of protein expression in the nucleus, which can be detected by immunohistochemistry. The
The specific function of INI1 appears to play a role in the Rb-cyclin D1 complex, which regulate transcriptional activity, resulting in the development of human rhabdoid tumors. Studies in model organisms have shown that at least one copy of INI1 is required for normal development. The SWI/SNF complex appears to regulate transcriptional activity, resulting in both repression and activation of a wide variety of target genes. INI1 plays a role in the development of human rhabdoid tumors.

Using a combination of karyotype analysis, fluorescence in situ hybridization (FISH), and direct sequence analysis, deletions and mutations of the INI1 locus have been identified in ~75% of patients. The underlying genetic basis of the remaining 25% of cases was unknown. The promoter region of the INI1 gene is not methylated in tumors, but alternative epigenetic mechanisms leading to loss of INI1 expression have not yet been explored. Copy number variations or intrinsic sequence alterations in the INI1 locus are not detected by current screening methods.

Over the last several years, oligonucleotide-based microarrays have emerged as the platform of choice for genome-wide copy number and loss of heterozygosity (LOH) analysis. Although these arrays have an intermarker distance of only a few kilobases and hence offer the potential to detect deletions and duplications at the single gene level, the limited number of SNPs within a particular locus may not be adequate to detect copy number changes or LOH at single exons.

Multiplex ligation-dependent probe amplification (MLPA) is a PCR-based assay that allows for multiple specific nucleic acid sequences to be amplified simultaneously using a single PCR primer pair. Recently, high-density probe sets for chromosome 22q11.2 have been developed to better identify and localize deletions and duplications within the DiGeorge/velocardiofacial syndrome region in 22q11.2. We showed previously that patients with constitutional and somatically acquired INI1 deletions could be detected with this high-density probe set. A comprehensive MLPA probe set specific for INI1 (SMARCB1) has now been developed, which can be used to interrogate the copy number of each of the nine exons of the gene. We hypothesized that this MLPA kit would have the sensitivity to detect whole exon deletions and duplications in tumor tissues that would be missed by standard sequence analysis and yet could be below the sensitivity of the whole genome SNP-based array.

Our previous studies suggested that the chromosome 22q11.2 breakpoints are often localized to low copy repeats (LCR) in patients with germ-line deletions of INI1. We expected, based on the increased density of the SNP arrays used herein, to more clearly refine the breakpoints in these sporadic tumors and determine if they were also localized to LCR regions in 22q11.2.

In the present study, we employed a combination of FISH, PCR-based sequence and MLPA analysis, and whole genome SNP-based array analysis with the Illumina 550K Beadchip to analyze a series of 51 primary rhabdoid tumors. The goals were to, first, achieve the most comprehensive analysis of INI1 that could be used in a clinical diagnostic setting for patient diagnosis and genetic counseling; second, detect additional recurrent alterations in the genome that could be used for identification of a second rhabdoid tumor locus; and third, determine the nature of the deletions, duplications or regions of LOH involving chromosome 22 in tumors from different anatomic locations.

**Materials and Methods**

**Case selection.** Tumor tissue was obtained from 51 patients for INI1 analysis according to the procedures approved by the Institutional Review Board at The Children’s Hospital of Philadelphia. Parental consent was obtained for genetic testing. The cases were specifically selected to include tumors for which we were previously unable to detect one or both of the inactivating deletions or mutations of INI1 and was therefore not designed to determine sensitivity or specificity of any individual assay. None of the patients had received prior chemotherapy or radiation before surgery. All of the cases were confirmed to be rhabdoid tumors by histology and/or immunohistochemistry with an antibody to INI1. DNA was extracted from tumor tissue with a Puregene kit (Gentra Systems) according to the manufacturer’s protocol and quantitated using a Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies). FISH. Touch imprints from frozen tissue, formalin-fixed tissue sections, or fixed cell pellets were analyzed by FISH. The INI1 probe was labeled by nick translation with ChromoTide Alexa Fluor 594-dUTP or UV-Vis Spectrophotometer (Nanodrop Technologies).
ChromoTide fluorescein-12-dUTP (Molecular Probes). Probes for the Ewing's sarcoma region in 22q12 were used simultaneously with the probe for INI1 as an internal control. The probes were applied to slides of the tumor cells and codenatured at 75°C on an Isotemp 125D heat block (Fisher Scientific). Slides were incubated at 37°C overnight in a moist slide moat (Boekel Scientific). They were then washed in a 0.4 X SSC solution at 73°C for 2 min followed by a 1 min wash in 2 x SSC/0.1% NP-40 and counterstained with 4',6-diamidino-2-phenylindole (Sigma). Fluorescent signals from 100 to 200 cells were evaluated at x100 with a Nikon Eclipse E800 fluorescence microscope equipped with the proper filter sets. An Applied Imaging System was used to record images of representative cells.

High-density SNP-based oligonucleotide array analysis. The high-density oligonucleotide array analysis was done using an Illumina Infinium whole-genome genotyping 550K Beadchip (Illumina). Genomic DNA (750 ng) from the tumor sample was processed using reagents and protocols supplied by Illumina in The Children's Hospital of Philadelphia Center for Applied Genomics (25).

The data were analyzed using Beadstudio software (Illumina), which allows for the visualization of several different variables relevant to the detection of copy number alterations (CNA), including the B-allele frequency (BAF) and log R ratio (LRR). The BAF represents the allelic copy ratio for the genomic SNPs. “Normal,” diploid DNA is expected to have three BAF clusters: two for the homozygous SNPs (AA and BB) with BAF close to 0 and 1, respectively, and one for the heterozygous SNPs (AB) with BAF centered at ~0.5. The LRR graph shows the log-normalized intensity ratio for each SNP in the test sample compared with a reference sample that includes DNA from 120 standard samples (from the HapMap set of 269). The presence of the expected two copies of any given SNP in a normal, diploid state would result in a LRR of ~0. Statistically significant deviations from 0 are interpreted as copy number changes. A positive LRR suggests gain in copy number and a negative LRR suggests a loss in copy number.

The BAF and LRR output data were also analyzed for CNAs using our Center for Biomedical Informatics Copy Number Analysis, Annotation and Visualization tool (CHOPPY), a set of tools based on the Circular Binary Segmentation algorithm that allows improved detection of CNAs (26). The visual output from the Beadstudio software was compared with the numerical output computed by CHOPPY. Heterozygous deletions and amplifications represented by <10 SNPs, with the exception of the INI1 locus, and copy neutral LOH events <5 kb in size were excluded from analysis. The breakpoints for the tumors with 22q11.2 heterozygous or homozygous deletions were localized with respect to the proximal and distal LCRs in 22q11.2 based on the Beadstudio and CHOPPY data. Results were compared with an in-house database of known, common copy number variations seen in 2,026 healthy controls detected with the same CHOPPY tools. All genomic positions were based on National Center for Biotechnology Information Build 36 of the human genome (hg18) from the University of California-Santa Cruz genome browser.2

The array results for chromosome 22 were then clustered using hierarchical clustering based on their copy number and LOH patterns as described previously (27). For each sample, the copy number and LOH regions were first decomposed into individual SNPs within these regions. The distance between any two samples is measured as the total number of SNPs that do not belong to the same copy number and LOH categories between the two samples. Samples with similar copy number and LOH patterns on chromosome 22 are thus clustered close together.

MLPA. MLPA was used with genomic DNA according to previously published methods based on the manufacturer's protocol using the SALSA MLPA P258 (SMARCB1) kit (MRC-Holland). This kit contains 2 probes for each of the 9 exons of INI1, probes for 9 other genes on chromosome 22, and 14 control probes from other chromosomes. The samples were processed and data were analyzed as described previously (24).

PCR sequencing mutation analysis. Oligonucleotide primers for exons 1 to 9 of the INI1 gene were designed from the intron/exon boundary sequences (GenBank accession no. AF000349-350) for PCR. PCR products for individual exons were analyzed by direct sequencing as described previously (4).

Results

A total of 51 rhabdoid tumors were analyzed using the high-density Illumina 550K SNP oligonucleotide array. The samples included 36 CNS atypical teratoid/rhabdoid tumors (34 brain and 2 spinal), 8 renal, and 7 extrarenal tumors (1 facial, 2 neck, 1 upper thigh, 2 liver, and 1 lymph node). There were 326 CNAs detected in the 51 tumors (Supplementary Table S1). Of those CNAs, only 177 were thought to be potentially pathogenic based on comparison with in-house CNA databases as well as interrogation of the genomic regions using the University of California-Santa Cruz genome browser. Ninety-seven of the 177 potentially pathogenic CNAs were localized to chromosome 22, and there were no other consistent abnormalities identified. Representative examples of the Beadstudio output for three tumors are shown in Fig. 1. Deletions or LOH involving the INI1 region were identified in 49 of 51 cases by the SNP array as shown in Fig. 2. Among the 49 tumors with alterations detected by the array, 24 (12 brain, 1 spinal, 6 renal, and 5 extrarenal) had homozygous deletions, 11 (10 brain and 1 renal) had heterozygous deletions, and 14 (10 brain, 1 spinal, 1 renal, and 2 soft tissue) had copy number neutral LOH involving chromosome 22q11.2. One brain tumor (05-188) did not have any detectable abnormality of the INI1 region by SNP array analysis aside from a small duplication that was a known normal population variant. This tumor, however, did have a homozygous deletion of one exon that was only detectable by MLPA (Table 1).

For rhabdoid tumor samples without homozygous INI1 deletions, FISH, MLPA, and sequence analysis of the nine coding exons of the INI1 gene were used to identify the second inactivating event. As shown in Table 1, combined events inactivating both copies of INI1 (deletion, mutation, or copy number neutral LOH) were detected in 50 of 51 cases (Fig. 2; Table 1). One sample (01-177) had copy number neutral LOH identified by the array but no other coding sequence mutation or exon deletion/duplication. A second sample (05-262) had a heterozygous deletion by array and FISH, although the MLPA assay was noninformative. A single-base alteration in the 3’-untranslated region (c.1220T>G) was identified in the tumor tissue from this patient. Matched normal tissue from the patient and parental blood samples were not available for comparison.

Although it was presumed to be the second inactivating event, the biological significance of this single-base change is unknown. One case (04-53) required FISH to establish both inactivating events. Direct sequencing revealed a mutation in exon 9, but neither the array nor MLPA showed a deletion at the INI1 locus (both showed small chromosome 22 deletions distal to the INI1 locus). FISH, on the other hand, revealed a deletion of 22, with loss of both INI1 and Ewing’s sarcoma signals, in 24% of cells.

Further sublocalization of the chromosome 22 deletions within 22q11.2 was done with respect to the LCRs in this

---

2 http://genome.ucsc.edu/
8 www.mrc-holland.com
region. The chromosome 22 breakpoints in cases with interstitial 22q11.2 deletions are depicted in Fig. 3. In the present series of cases, only one patient was found to have a germ-line mutation (07-06) and no patient had evidence of a germ-line deletion. These numbers are a reflection of the case selection for this study and should not be used to estimate the expected number of patients with germ-line alterations of INI1. As shown in the figure, the majority of breakpoints were located between LCR regions rather than within LCR regions or other recombination hotspots.

Fig. 1. Representative samples of chromosome 22 from Beadstudio depicting various inactivating alterations of INI1 (arrows). A, case 02-216: copy neutral LOH with contamination involving most of the long arm, with a homozygous deletion that encompasses INI1. B, case 01-146: complex duplications and deletions of chromosome 22 including a homozygous deletion in 22q11.2. C, case 07-221: exons 7 to 9 deletion of INI1 identified by MLPA associated with a three-SNP deletion.
Among the tumors with heterozygous deletions or copy number neutral LOH for 22q, mutations were identified in 18 cases by direct sequencing. Six of the CNS atypical teratoid/rhabdoid tumors had a 1143delG or 1145delC in exon 9 that appears to be the most common hotspot for mutations in tumors of the CNS (4). Furthermore, 10 tumors had single or multiple exon deletions or duplications detected by MLPA. Case 07-221 had a homozygous deletion of exons 7 to 9 that was detected by both SNP array and MLPA, and 05-174 had a duplication of exons 6 to 7 that was detected by MLPA. The exons 4 to 9 deletion identified by MLPA in 93-94 was not detected on the SNP array possibly due to contamination with normal tissue. The smaller exon deletions or duplications revealed by MLPA in the remaining cases were not observed by the array analysis.

**Discussion**

In the present study, 51 rhabdoid tumors from a variety of anatomic locations were studied using four different testing modalities to identify potential underlying genetic changes leading to the development of malignant rhabdoid tumors. Notably, despite the differences in anatomic location, half of the tumors (24 of 51) were characterized by homozygous deletions of *INI1*. This included more than one third (13 of 36) of the CNS atypical teratoid/rhabdoid tumors as well as the majority of both renal (6 of 8) and extrarenal (5 of 7) tumors.

As shown in Fig. 2, there were some differences in the patterns of structural changes of chromosome 22 in tumors from different sites made apparent by the hierarchical clustering. This series included a predominant number of CNS tumors (36 of 51) and the frequencies of CNAs versus copy number neutral LOH were fairly evenly distributed (13 tumors with homozygous deletions, 10 with heterozygous deletions, and 11 with copy number neutral LOH). Tumors with whole or large chromosome 22 deletions were exclusively from the brain. Tumors with whole arm or large regions of chromosome 22 copy number neutral LOH were mostly from brain as well. In contrast, the soft-tissue rhabdoid tumors were more likely to have smaller deletions in 22q11.22 to 22q11.23. The renal rhabdoid tumors had either copy number neutral LOH or complex CNAs. Despite these trends, due to the high prevalence of CNS tumors in these samples and the low numbers of other tumor types, it is difficult to draw conclusions about the type of inactivating events as related to anatomic location. In addition, although some CNAs involving chromosomes other than 22 were observed more than once, there were...
no other nonrandom patterns of alteration among or between the tumors from different anatomic sites.

Previous studies of rhabdoid tumors have shown that these tumors are mostly diploid, with few recurrent regions of LOH or copy number changes other than chromosome 22 (12). Our data support the fact that most of the tumors are diploid, as there was only one hyperdiploid sample (01-323) among the 51 tumors tested. However, the complex nature of some of the rearrangements of chromosome 22 was somewhat unexpected as illustrated by case 01-146 (Fig. 1B). Although most of the genome appeared balanced, chromosome 22 was disrupted by several events leading to 3 or 4 copies of the chromosome for some regions as well as heterozygous and homozygous deletions that ultimately resulted in loss of the \textit{INI1} region. In this sample, numerous genes would be subjected to dosage changes, the net effect of which is unclear.

The high-resolution SNP array data may define the approximate locations of the breakpoints leading to deletions and duplications that can yield insight into the mechanism of the inactivating events. Our previous study of patients with rhabdoid tumors and germ-line 22q11.2 deletions suggested that some of the underlying genetic changes could be related to recombination events mediated by LCRs located in chromosome 22 (23). The present cohort, on the other hand, was composed predominantly of patients with sporadic tumors, as only one patient had a germ-line mutation (07-06) and no patient had a germ-line deletion. As shown in Fig. 3, the majority of the breakpoints in the cases described here were between and not within the LCR regions. This finding is not unexpected as LCR-mediated rearrangements in the germ-line are believed to occur via nonallelic homologous recombination during meiosis (28, 29). The deletion events in the sporadic tumors are somatic events and may be the result of alternate mechanisms such as nonhomologous end joining. Thus, our analysis suggests that there may be distinct mechanisms of deletion in the setting of a germ-line deletion versus a sporadic event.

The degree of complexity for the alterations of chromosome 22 in a subset of the tumors suggests that these deletions are indeed taking place by a variety of mechanisms, which are likely to be unbalanced translocations rather than simple LCR-mediated recombinations. Cytogenetically balanced translocations involving 22q11.2 have been shown by FISH to be associated with submicroscopic deletions of \textit{INI1} as described previously (4). The partner chromosomes are variable and only limited numbers of tumors have been studied to determine whether the derivative chromosome is also deleted or if specific genes have been interrupted by the translocations. One case (00-315) had an apparently balanced t(7;22)(p15;q11.2), yet a homozygous deletion of \textit{INI1} was identified by FISH and subsequently confirmed by high-density SNP array analysis. This array also revealed a 2.5 Mb deletion in 7p15, indicating that the translocation partner chromosome was also deleted as a result of the translocation. The \textit{HOXA} gene cluster and the \textit{CREB5} genes were included in the region of loss. Whether such additional changes are ultimately related to the clinical phenotype of the patient, their response to therapy, or their clinical outcome remains to be seen.

<table>
<thead>
<tr>
<th>Table 1. INI1 inactivating events in rhabdoid tumors without homozygous INI1 deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
</tr>
<tr>
<td>----</td>
</tr>
<tr>
<td>01-090</td>
</tr>
<tr>
<td>01-175</td>
</tr>
<tr>
<td>02-07</td>
</tr>
<tr>
<td>02-203</td>
</tr>
<tr>
<td>02-237</td>
</tr>
<tr>
<td>03-1288</td>
</tr>
<tr>
<td>05-262</td>
</tr>
<tr>
<td>08-018</td>
</tr>
<tr>
<td>01-323</td>
</tr>
<tr>
<td>02-079</td>
</tr>
<tr>
<td>02-215</td>
</tr>
<tr>
<td>02-291</td>
</tr>
<tr>
<td>03-151</td>
</tr>
<tr>
<td>03-287</td>
</tr>
<tr>
<td>04-07</td>
</tr>
<tr>
<td>05-289</td>
</tr>
<tr>
<td>06-07</td>
</tr>
<tr>
<td>04-023</td>
</tr>
<tr>
<td>04-09</td>
</tr>
<tr>
<td>07-06</td>
</tr>
<tr>
<td>03-152</td>
</tr>
<tr>
<td>05-174</td>
</tr>
<tr>
<td>06-072</td>
</tr>
<tr>
<td>07-201</td>
</tr>
<tr>
<td>05-188</td>
</tr>
<tr>
<td>04-053</td>
</tr>
<tr>
<td>07-221</td>
</tr>
<tr>
<td>01-177</td>
</tr>
</tbody>
</table>

*S, sequence analysis; M, MLPA; F, FISH.*
The current study shows that, by employing a combination of SNP array analysis, MLPA, FISH, and direct sequencing, the inactivating deletions and mutations of INI1 can be identified in the vast majority of pediatric rhabdoid tumors. All 51 tumors described here had at least one detectable inactivating event, and both inactivating events were identified in 50 (98%) tumors. In the remaining case (01-177), there was loss of INI1 expression by immunohistochemistry, suggesting that the second unidentified inactivating event may have been associated with a mutation or epigenetic modification of a noncoding region of the gene.

These data strongly support the fact that INI1 is the primary gene responsible for the development of rhabdoid tumors. Although the high-density SNP array analysis did not reveal any other consistently altered regions, clinical correlative studies including outcome data are in progress to determine if some of the less prevalent changes predict differences in prognosis and response to treatment. At the present time, the SNP-based oligonucleotide array can be used to refine disease-associated CNAs and distinguish, for example, a medulloblastoma with an isochromosome 17q from a primary rhabdoid tumor with loss of 22q11.2. When indicated, molecular analysis of INI1 using MLPA and direct sequencing may then be employed. Once the tumor-associated changes are found, an analysis of germ-line DNA from the patient and parents can be analyzed to rule out an inherited or de novo germ-line mutation or deletion of INI1, so that appropriate recurrence risk assessments can be made.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

References

Genomic Analysis Using High-Density Single Nucleotide Polymorphism-Based Oligonucleotide Arrays and Multiplex Ligation-Dependent Probe Amplification Provides a Comprehensive Analysis of INI1/SMARCB1 in Malignant Rhabdoid Tumors

Eric M. Jackson, Angela J. Sievert, Xiaowu Gai, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/15/6/1923

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2009/03/13/1078-0432.CCR-08-2091.DC1

Cited articles
This article cites 28 articles, 12 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/6/1923.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/15/6/1923.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.