Identification of Novel Regions of Deletion in Familial Wilms' Tumor by Comparative Genomic Hybridization

Rachel A. Altura, Marcus Valentine, Hao Li, James M. Boyett, Patricia Shearer, Paul Grundy, David N. Shapiro, and A. Thomas Look

ABSTRACT

Wilms' tumor, an embryonic renal neoplasm diagnosed primarily in young children, can occur in either a noninheritable (sporadic) or a familial form, with the latter presenting earlier and more often at bilateral sites. Although familial Wilms' tumor is thought to develop through inherited and acquired mutational inactivation of the two alleles of predisposing tumor suppressor genes, only a small percentage of cases can be accounted for by mutations affecting the WT1 gene or linkage to the Beckwith-Weidemann syndrome of the 7p53 region on the short arm of chromosome 11. To find chromosomal regions that might contain genes important in the development of this disease, we used comparative genomic hybridization to analyze tumor specimens from familial cases for chromosomal regions that were consistently lost. Although inherited lesions of tumor suppressors are most often inactivating point mutations, accompanying somatic lesions in the malignant clones are often chromosomal deletions; therefore, consensus regions of loss in familial tumors are likely to harbor genes linked to familial predisposition. There were extensive genomic aberrations among the eight familial cases studied, with an average of 6.5 changes/tumor (range, 0-22). The most consistent findings with likely biological relevance were deletions of chromosomes 4 (consensus, 4q12-qter), 9 (consensus, 9p21-pter), 20p, and 3 (consensus, 3q13-q21). These regions have not been previously implicated in Wilms' tumor and may harbor novel genes that could aid attempts to understand the familial predisposition as well as the development and progression of these tumors.

INTRODUCTION

Wilms' tumor affects approximately 1 of every 10,000 children worldwide, making it the most common renal neoplasm of childhood (1, 2). Like retinoblastoma, it can occur in either a sporadic (nonhereditary) or familial form. Although the latter is estimated to comprise only 1.5% of all Wilms' tumors, the percentage of individuals carrying a germline mutation in a gene predisposing to this tumor is probably much higher (3, 4). The molecular pathogenesis of familial Wilms' tumor remains unclear, but it is thought to follow a "two-hit" model, in which an initial mutation affects a tumor suppressor locus in the germline, but does not become transforming until a second (somatic) mutation inactivates the wild-type allele in a target progenitor cell (5). In contrast, sporadic Wilms' tumors may develop through mechanisms involving two sequential somatic mutations that affect tumor suppressor loci (5).

Recent findings have implicated more than one tumor susceptibility locus in the genesis of Wilms' tumor. Overall, 10-15% of patients have mutations in the WT1 gene on chromosome 11, band p13 (6-8). Some of these mutations occur somatically, whereas others are inher-

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3 The abbreviations used are: LOH, loss of heterozygosity; CGH, comparative genomic hybridization; FISH, fluorescence in situ hybridization; DAPI, 4',6-diamidino-2-phenylindole.
standard procedures (19). None of the patients from whom the tumors were obtained had constitutional cytogenetic abnormalities. None had Beckwith-Weidemann syndrome, Denys-Drash syndrome, or WAGR (Wilms’ tumor, aniridia, genitourinary malformations, and mental retardation). One patient had Simpson-Golabi-Behmel syndrome (Table 1).

CGH. CGH was performed with indirectly labeled fluorochrome-conjugated DNA as described previously (18, 20). Briefly, tumor DNA was labeled by nick translation with biotin 14-dATP; normal DNA (isolated from human male lymphocytes) was labeled with digoxigenin 11-dUTP. The fragments used for hybridizations had an average size ranging between 600 and 1200 bp. Three hundred ng of labeled tumor and normal (reference) DNA were combined with 15 mg of human Cot1 DNA and precipitated with ethanol. The precipitate was then resuspended in 10 ¡l of hybridization buffer (50% formamide, 10% dextran, and 2× SSC), denatured at 70°C for 6 min, and hybridized to normal metaphase spreads. Before hybridization, metaphase preparations were denatured at 70°C from 1 to 3 min in a 70% formamide solution and then dehydrated in a series of 70, 80, and 95% ethanol solutions. Hybridizations were performed at 37°C for 72 h. Slides were then washed, and bound DNA fragments were detected with avidin-FITC (green fluorescence) and antidigoxigenin rhodamine (red) according to standard immunostaining protocols. Controls included normal DNA. contamination of the tumor DNA sample with nontumor DNA, and antidigoxigenin rhodamine (red) according to standard immunostaining protocols. Chromosomes were counterstained with DAPI (blue) in an antifade solution (120 ng/ml).

Digital Image Analysis. A Zeiss fluorescence microscope and CCD camera were used to collect images from metaphase spreads. Three color images representing the tumor DNA (green), reference DNA (red), and DAPI counterstain (blue) were acquired from an average of 20 metaphase spreads/ hybridization. Only those metaphases showing uniform hybridization and consistency of color change between chromosome homologues were analyzed. Green:red fluorescence intensity ratios were calculated with IP-Labs software using the smart capture package (Vysis) as described previously (20). In brief, the chromosomes were identified according to the DAPI-banding pattern. Green and red fluorescence intensities were then scored along the vertical medial axis of each chromosome and were normalized so that the average green:red ratio for the whole metaphase was 1. From 10 to 15 observations/ chromosome number were combined to obtain a mean ratio and its SD. In contrast to most authors reporting CGH results (18, 20), we did not use threshold values to select chromosomal regions that were underrepresented or overrepresented. Rather, any change in fluorescence intensity was considered potentially important, subject to statistical confirmation (see below). The centromeric regions were excluded from analysis. Controls included normal-normal DNA hybridizations as well as CGH experiments on tumors with known cytogenetic abnormalities.

RESULTS

Chromosomal Regions Involved in DNA Sequence Gains and Losses.

Seven of the eight tumors that were analyzed with CGH showed evidence of DNA sequence gains, losses, or both. One case showed no DNA imbalance. The mean number of changes per tumor was 6.5 (range, 0–22), consisting of 2.25 increases (range, 0–8) and 4.25 decreases (range, 0–14). Composite results of CGH screening are presented in Table 2 and Fig. 1. Extra copies of whole chromosomes, including chromosomes 12 (63% of cases), 6 (38% of cases), and 8 (25% of cases), were the most common form of DNA sequence gain. One tumor had multiple copies of chromosomes 2, and 7–10, and 12. Duplicated regions included 1q (one case), 4p (one case), and 20q (one case). We did not find amplification of single chromosomal bands in any case.

DNA sequence losses occurred most often (=3/8 tumors analyzed) in chromosomes 3, 4, 9, 16, and 20. In four of eight cases, chromosome 4 was either lost entirely or had a discrete deletion on 4q (consensus, 4q21-ter). Two such cases involved siblings in the same family. Regions within the short arm of chromosome 9 were also lost in 50% of the cases (consensus, 9p21-ter). 16q was missing in two cases, whereas in another the whole chromosome was lost. Two cases lacked the short arm of chromosome 20 (with duplication of the long arm in 1), whereas in another case the entire chromosome was missing. Additional notable losses included a discrete region on 3q (consensus, 3q12–21) as well as the entire chromosome 3 in two other cases. Less common areas with DNA sequence loss included chromosomes 1p, 6p, 9q, 11p, 11q, 14q, 17p, 17q, and 19q. All of these changes were seen in one case, with the exception of 11p and 17p deletions, each seen in two cases. Whole losses of various other chromosomes were apparent in several tumors (Fig. 1 and Table 2).

Statistical Analysis. Because of the potential variability of CGH experiments (e.g., due to fluctuations in normal metaphase preparations, sources of normal DNA, contamination of the tumor DNA sample with nontumor DNA, and visual identification of chromosomes), we assessed our fluorescence intensity findings with a mixed effects statistical model (21). The chromosomal segments themselves were considered fixed effects, and each chromosome was analyzed separately. Simultaneous 99% confidence intervals were calculated to adjust for multiple estimations. Confidence intervals containing a green:red ratio of 1 were considered evidence of neither a deletion nor an amplification of a chromosomal region. Confidence intervals entirely below 1.0 identified deleted regions and entirely greater than 1.0 identified regions of chromosomal gain.

FISH. Two-color FISH analysis was used to verify results at a 4q locus believed to lie within the consensus region of loss determined by CGH. Interphase nuclei from case 4 were hybridized to the P1 clone RMC04P007 from the 4q22–4q24 locus along with the α-satellite probe from chromosome 4 as described previously (19). Similar hybridization assays were performed to investigate the possibility of loss of the INK4A (also p16, MTS1, or CDKN2) gene, which maps to the 9p21 locus, in tumors with a deletion on the p arm of chromosome 9. Interphase nuclei from tumor cases 5 and 7 were hybridized to an INK4A probe (also containing INK4B DNA); a P1 probe containing the IL3BP gene (22), located on the q arm of chromosome 9, served as the probe control. Deletion was defined as the presence of a lower number of single-copy probes compared to centromeric or locus-specific control probes.
### DISCUSSION

We undertook this analysis to identify genomic regions that might harbor tumor suppressor genes linked to familial Wilms' tumor susceptibility based on the fact that tumor cells frequently contain somatically acquired deletions of chromosomal regions containing tumor suppressor genes inactivated by an inherited mutation of the allele retained by the tumor. Several tumors had shared regions of chromosomal loss including regions that have not been described in previous studies of sporadic or familial Wilms' tumors, notably 4q21-qter and 9p21-pter, both deleted in 50% of the cases, and 3q12-21 and 20p, each lost in 38%.

Deleted only infrequently in sporadic cases of Wilms' tumor (23), the 4q21-qter region was the only common area of deletion in the tumors of two siblings in the same family. This observation, along with the high level of 4q loss overall in familial cases, indicates that this region may harbor a gene important in predisposition to familial Wilms' tumor. Of the tumor suppressor genes cloned to date (24), none resides on chromosome 4. However, in a recent report on the transfer of human chromosomes into cultured cells initially exposed to ionizing radiation, Verhaegh et al. (25) reported that chromosome 4 was the only chromosome associated with the inhibition of DNA synthesis, a critical checkpoint in the cells' response to injury (25). When this checkpoint is breached, as in retinoblastoma with loss of pRb function, the cell can proceed, uninhibited, through the cell cycle and constitutively replicate its DNA. Verhaegh et al. (25) narrowed the region responsible for inhibition of DNA replication to 4q25–34.

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#### Table 2 DNA sequence gains and losses, by chromosome, in eight cases of familial Wilms' tumors

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*Extra copies of a chromosome.

*Loss of one or more copies of a chromosome.

#### Fig. 1. Summary of the chromosomal gains and losses in familial Wilms' tumor. Gains (right side) and losses (left side) of the chromosomes for each of the eight tumors studied. Chromosomes 6, 8, and 12 represent the most frequent sites of chromosomal gain and chromosomes 3, 4, 9, 16, and 20 the most frequent sites of loss.
which is within the consensus region of loss in our familial Wilms’ tumor cases and therefore may contain a novel tumor suppressor gene.

9p deletion, seen in 50% of our familial Wilms’ tumor cases, has been characterized previously in many tumor types, but not Wilms’ tumor, as a major contributor to malignant transformation. Examples include familial melanoma (with genetic linkage to a locus on 9p), leukemia, glioma, squamous cell carcinoma, and others (26–29). Tumor development in these diseases is thought to involve loss of one or both of two closely linked genes (INK4A and INK4B) on chromosome band 9p21 (30–33). Given that familial Wilms’ tumor patients do not have an increased incidence of melanoma or other malignancies and that our FISH results demonstrate two copies of INK4A/INK4B per cell, it is unlikely that mutation of these genes is responsible for predisposition to familial Wilms’ tumor; nonetheless, other genes in this general area could play a role in tumor development, as indicated by reports of putative tumor suppressors outside the INK4A region, but still within the 9p21 band (34).

Although most of the remaining individual regions of chromosome loss detected using CGH analysis are mentioned only rarely in the published literature, an exception is the 3q12–21 consensus region. In a LOH study of osteosarcomas (35), 3q was the most common region of loss (75% of cases) with a consensus region, 3q21–qter, that was compatible with our CGH finding. Other notable similarities were LOH at 4q35 in 20% of the osteosarcomas compared to a 4q21–qter consensus region in 50% of our cases and LOH at 20p12 in 30% of the osteosarcomas, an area of deletion in 38% of familial Wilms’ tumors. This overlapping pattern of chromosomal loss between osteosarcoma and familial Wilms’ tumors suggests that genes within these shared regions might play an important role in the development of embryonal neoplasms.

The search for a familial Wilms’ tumor predisposition gene initially focused on the 11p13 and 11p15 genomic regions because of tumour-specific LOH at these loci. However, these regions have been excluded by genetic linkage studies (15, 16). LOH at 16q, seen in 20% of the sporadic Wilms’ tumor cases and subsequently shown to be a poor prognostic indicator in patients with this disease, led to additional linkage studies that ruled out the 16q region as a potential site for the familial predisposition gene (17). Negative linkage results were obtained for the 1q21–31 locus associated with an inherited pattern of parathyroid adenomas, tumors of the jaw, parathyroid carcinoma, and Wilms’ tumor (hereditary hyperparathyroidism-jaw tumor or HPT-JT syndrome; Ref. 36). Thus, our CGH findings provide several new regions of genetic loss that should be useful in guiding future genetic linkage studies of Wilms’ tumor families.

Classical cytogenetic studies have shown that Wilms’ tumors commonly carry extra copies of several chromosomes (37–40). In one such study, 52% of 31 Wilms’ tumor cases were hyperdiploid, with chromosome 12 trisomy the most common cytogenetic abnormality observed (37). Nonrandom additions of chromosomes 6, 8, and 18 were also noted in this and other studies as well (37–40). Similarly, DNA sequence gains in the form of extra copies of whole chromosomes were the most common findings in our CGH analysis. Extra copies of chromosome 12 occurred in 63% of the cases, with +6 (38%) and +8 (25%) also frequently found. 16q loss was noted in 38% of our cases compared with 20% of the sporadic cases studied for LOH (14). Unlike the LOH data, which showed 1p loss in a relatively high percentage of sporadic tumors (12%; Ref. 14), we did not observe loss affecting 1p in our familial study. A recent study completed in our laboratory on the CGH of 22 sporadic Wilms’ tumors also showed some similarities with the familial cases. Specifically, the sporadic tumors also had frequent gains of chromosomes 6 and 12 as well as loss affecting 16q. Losses of 4q, 9p, and 20p, however, were not observed in the sporadic tumors. The parallels between the cytogenetic and CGH findings and the comparisons between the sporadic and familial cases reinforce the validity of novel regions of nonrandom chromosomal loss as reported here.

* D. N. Shapiro, personal communication.
Areas of significant chromosomal gain or loss can be difficult to quantify. Before accepting calculated green:red fluorescence intensity ratios, we evaluated sets of experimental data using a mixed effects model (21) based on underlying assumptions of random variability. Possible sources of random variability include the normal lymphocyte metaphase preparations, contamination of the tumor DNA sample by normal cells, and visual examination of the chromosomes for numerical identification. The sensitivity of the statistical model was demonstrated in several cases in which a predicted deletion was later confirmed by the FISH assay. We therefore suggest that future CGH investigations would benefit from the addition of formal statistical analysis to objectively identify overrepresented and underrepresented regions.

Our CGH results highlight several chromosomal regions (3q, 4q, 9p, and 20p) that may contain a gene or genes conferring predisposition to familial Wilms’ tumor. Further analysis of tumor specimens with specific probes from these areas, as well as genetic linkage studies in families with Wilms’ tumor, should help to better define the precise loci involved.

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