Combined cytogenetic and array-based comparative genomic hybridization analyses of Wilms tumors: amplification and overexpression of the multidrug resistance associated protein 1 gene (MRP1) in a metachronous tumor

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Abstract

Tumor samples from a variety of Wilms tumors (WT) obtained from three patients were analyzed by cytogenetic and array-based comparative genomic hybridization (CGH) methods. The tumors represented different stages of tumorigenesis and included a unilateral primary WT and contralateral nephrogenic rest (case 1), a primary WT and a contralateral metachronous WT (case 2), and a recurrent WT with lung metastases (case 3). All six specimens exhibited abnormal karyotypes characteristic of different WT levels of progression. Array-based CGH examinations of 57 genes that are commonly amplified in various cancers revealed a 2.6-fold genomic amplification of the multidrug resistance-associated protein 1 (MRP1) gene in the metachronous WT, but no amplification in the primary tumor. This sole amplification event in our series was also confirmed by Southern blot analysis. Furthermore, quantitative reverse transcriptase polymerase chain reaction showed a sixfold overexpression of the MRP1 gene in this metachronous WT relative to the primary tumor. Our findings suggest that for most of the genes examined in this series genomic amplification does not play a role in WT pathogenesis. Isolated amplification and overexpression of the MRP1 gene in the metachronous WT, however, suggest that this gene may be an important factor in the development and progression of metachronous tumors. © 2003 Elsevier Science Inc. All rights reserved.

1. Introduction

Wilms tumor (WT) is an embryonal tumor of the kidney that affects about 1 in 10,000 children, usually within the first 3 years of life [1]. Wilms tumors usually present as sporadic and unilateral lesions. Bilateral involvement of the kidneys occurs more frequently in familial cases and is usually synchronous. Metachronous occurrence of WT is relatively rare [2]. The tumor is characterized by the combination of blastemic, stromal, and epithelial cell types. Abnormal persistence of pluripotential embryonal residua, known as nephrogenic rests (NR), can be found in the proximity of many WT lesions, particularly those of the metachronous form [3,4]. Therapy and prognosis are generally dependent upon the clinical staging and tumor presentation. Treatment of anaplastic or metachronous WT is especially challenging. Diffuse anaplasia, in particular, confers an adverse prognosis, and in metachronous WT the contralateral disease develops following treatment for the primary tumor, which frequently involves nephrectomy [2], thereby significantly lowering the cure rate for these patients. This emphasizes the importance of identifying patients who are at high risk for development of anaplastic or metachronous WT and for progression of the disease.

In the last decade, genetic markers have emerged as a powerful tool in diagnosing and monitoring many cancers, including several types of solid tumors. Mutations in the WT1 gene at 11p13, the only WT-associated gene that has
been cloned thus far, were found in about 10% of sporadic examined cases [5]. Additionally, loss of heterozygosity (LOH) at 11p15 has been detected in about half of the non-familial WT tumors. This region contains a second putative WT gene, designated WT2 [6]. Identification of other WT-associated genetic alterations (e.g., LOH 1p [7], LOH 7p [8], and LOH 16q [9]), as well as nonrandom chromosome numerical changes and epigenetic changes, supports the existence of other genes important in WT formation and progression.

The gene-dosage effect through amplification was found to play an important role in solid-tumor formation and is used as a prognostic marker for tumor aggressiveness, like MYCN in neuroblastoma [10]. Array-based comparative genomic hybridization (CGH) is a newly developed method that allows simultaneous screening of 57 different genes reported to undergo genomic amplification in various cancers. In our study, we applied advanced methods such as spectral karyotyping (SKY) and array-based CGH to analyze cytogenetic changes and to detect gene amplification events that may play a role in WT development in six WT samples (1 NR and 5 WT samples). We now report the amplification and overexpression of the multidrug resistance associated protein 1 (MRP1) gene at 16p13.1 in a metachronous WT, to our knowledge the first such report.

2. Materials and methods

2.1. Patients

The patients were treated for WT at Dana Children’s Hospital, Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel. Tumor samples were sent for routine cytogenetic analysis at the Genetic Institute. None of the patients had a family history of renal neoplasia. The main clinical features of the three reported patients were as follows. Case 1 was a 2-year-old girl with hemihypertrophy who underwent a biopsy in both kidneys at the same time. Pathological examination revealed bilateral NR, but WT was determined only in the left side. Case 2 was a 5-year-old girl with a primary WT in the right kidney who received chemotherapy for 6 months and was found to have a metachronous WT in the left kidney 3 months later. Pathologic examination revealed NR and WT lesions in both kidneys. Case 3 was a 9-year-old boy with recurrent extrarenal WT in the left side of the pelvis. The primary WT that had been detected 3 years earlier was not available for chromosomal and array-based analyses. Chemotherapy and subsequent autologous bone marrow transplantation failed to prevent the development of local recurrence and lung metastases.

The treatments of all three reported patients were carried out according to the National Wilms Tumor Study Group-5 protocols and included vincristine and actinomycin D.

Chromosomal analysis in peripheral blood leukocytes showed karyotypes of normal constitution for all patients studied.

2.2. Chromosome studies

Chromosome analysis was performed on primary short-term cultures derived from five renal biopsies and the lung metastasis. The short-term cultures set-up, the chromosome preparations and the Giemsa-banding (G-banding) staining were performed according to standard cytogenetic techniques as previously described [11]. Spectral karyotyping analysis was performed according to the manufacturer’s instructions (Applied Spectral Imaging [ASI], Migdal Ha’Emek, Israel) in cases 2 and 3 to refine G-banding findings [12]. Karyotypes were established according to the International System for Human Cytogenetic Nomenclature (ISCN 1995) [13].

2.3. DNA and RNA extraction

High-molecular weight genomic DNA was extracted from frozen tissues stored at −70°C using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). Total RNA was isolated using Tri reagent (Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions.

2.4. Array-based CGH analysis

Detection of gene amplifications was performed by array-based CGH using the AmpliOnc I microarray and the GenoSensor reader system (Vysis, Downers Grove, IL, USA) according to the manufacturer’s instructions. Briefly, 1 μg tumor DNA (test) and control DNA (reference) from a normal male (supplied by the manufacturer) were labeled with Alexa Fluor 488 (green fluorophore) and Alexa Fluor 594 (red fluorophore) (Molecular Probes, Leiden, The Netherlands), respectively, using nick translation enzymes (Vysis). For each sample, 500 ng tumor DNA was mixed with the control DNA at a 1:1 ratio, denatured, and allowed to prehybridize (Vysis) at 37°C for 1 hour. Following prehybridization, the DNA mixture was cohybridized to an AmpliOnc I microarray (Vysis) containing 59 different probes (P1, PAC, or BAC clones), corresponding to 57 different genes. Following hybridization, the free probe was washed away and the target spots were counterstained with a DAPI IV-mounting solution (Vysis). Hybridization signals images in green, red, and blue were then analyzed by the GenoSensor reader system based on 4’,6-diamidino-2-phenylindole (DAPI) staining that identified target spots and their location on the grid. The green-to-red ([G/R], test/reference) ratio at each spot was automatically calculated by the custom software after subtracting the background for each color, and Pearson’s coefficient of correlation was determined. Target spots that were not segmented correctly or that had a Pearson’s coefficient less than 0.8 were excluded. The level of amplification was averaged between three replicate spots for each gene, and the final DNA copy number was then determined by normalizing the signal ratio for each genomic sequence to the two-copy reference DNA. The list of the 59 gene targets on the AmpliOnc I microarray can be found at the manufacturer’s Web site (http://www.vysis.com), including genes, cytogenetic loci, and location on the grid.
2.5. Southern hybridization analysis

Five micrometers of genomic DNA from the primary and metachronous WT samples (case 2) and a control kidney (normal kidney tissue displaying a normal karyotype; data not shown) was digested with EcoRI and BamHI restriction enzymes (New England Biolabs, Beverly, MA, USA), electrophoresed, blotted, and hybridized with $^{32}$P-labeled MRP1 and GAPDH probes using standard protocols [14]. The 742-bp MRP1 probe (bases 2069–2810; GenBank identifier [GI]: 563909) and 304-bp GAPDH probe (bases 79–383; GI: 182976) were generated from the corresponding coding region using reverse transcriptase polymerase chain reaction (RT-PCR). The primer sequences were as follows: for MRP1, 5'-CAC CGT GAG GAA TGC CAC AT-3' (forward) and 5'-CTT CCC TGG ACC GCT GAC GC-3' (reverse); for GAPDH, 5'-GGA GTC AAC GGA TTT GGT AG-3' (forward) and 5'-GCC TTC TCC ATG GTG GTG AG-3' (reverse). The GAPDH gene was used as a control for the amount of loaded DNA. Relative MRP1 intensities of different specimens were scanned and analyzed by a Kodak EDAS 290 system (Eastman Kodak, Rochester, NY, USA). The relative change in MRP1 DNA copy number was then calculated after normalization to GAPDH.

2.6. Quantitative RT-PCR assay for MRP1 expression

The RT-PCR analysis for the MRP1 gene was performed on the primary and metachronous WT samples (case 2) and the normal kidney control. Complementary DNA (cDNA) was synthesized from 1 μg total RNA using 100 pmol oligo dT$_{12-18}$ (Roche Diagnostics, Mannheim, Germany), 500 μM each DNTP (Pharmacia, Uppsala, Sweden), and 100 U RNasin (Promega, Madison, WI, USA), and 100 U Superscript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD, USA) in a total volume of 10 μL. The reaction was incubated at 42°C for 1 hour and terminated by heating at 90°C for 2 minutes.

A multiplex PCR of MRP1 and GAPDH (reference control gene) was performed using forward primers that were end-labeled with hexachlorofluorescein. The primers for MRP1 were 5'-TGG GCA GAC CTC TTC TAC TC-3' (forward) and 5'-AGG ATG ATG AGG GCA CAC AC-3' (reverse) generating a 203-bp PCR product (bases 393–576; GenBank identifier [GI]: 1221829). The sequence of GAPDH forward (H11032) and reverse (H11034) generating a 203-bp PCR product (bases 393–576; GenBank identifier [GI]: 563909) and 304-bp MRP1 probe (bases 2069–2810; GenBank identifier [GI]: 1221829) were generated from the corresponding coding region using reverse transcriptase polymerase chain reaction (RT-PCR). The primer sequences were as follows: for MRP1, 5'-CAC CGT GAG GAA TGC CAC AT-3' (forward) and 5'-CTT CCC TGG ACC GCT GAC GC-3' (reverse); for GAPDH, 5'-GGA GTC AAC GGA TTT GGT AG-3' (forward) and 5'-GCC TTC TCC ATG GTG GTG AG-3' (reverse). The GAPDH gene was used as a control for the amount of loaded DNA. Relative MRP1 intensities of different specimens were scanned and analyzed by a Kodak EDAS 290 system (Eastman Kodak, Rochester, NY, USA). The relative change in MRP1 DNA copy number was then calculated after normalization to GAPDH.

3. Results

3.1. Cytogenetic analysis

Table 1 lists the patients’ clinical data, tumor characterization, cytogenetic findings based on G-banding and SKY analyses, and the DNA amplification results. Karyotypes of all the tumors (i.e., four WT, one NR, and one lung metastasis) were first established by conventional G-banding staining. The SKY analysis was then added to refine the results and define marker chromosomes as identified by G-banding (cases 2 and 3; Fig. 1A, 1C, and 1D). All the tumors showed cytogenetic aberrations (Table 1). The WT and the NR biopsies in case 1 revealed a pseudodiploid karyotype, the primary tumor karyotype in case 2 displayed hyperdiploidy, and the metachronous WT (of case 2) and both tissues (renal and lung) of case 3 displayed hypodiploid karyotypes. Most tumors displayed a monochonal karyotype, except the primary tumor in case 2, which showed three different clones.

Characterization of the cytogenetic alterations revealed that chromosomes 1 was involved in four different tumors. Trisomy 1q was detected in the primary and metachronous WT of case 2 (Fig. 1A and 1B) and in the WT and lung metastasis of case 3 (Fig. 1C and 1D), resulting in monosomy 1p in case 3. An i(7)(q10) resulting in monosomy 7p and trisomy 7q was observed as the sole cytogenetic finding in the samples of the NR and unilateral WT of case 1. In this case, the percentage of the abnormal cells was correlated with the severity of the pathological diagnosis: 34% (11/32) in the left kidney (NR and WT) and 3% (1/36) in the left kidney (NR). Monosomy 7p was also found in the primary
tumor of case 2 due to a translocation between chromosomes 7q and 1q (Fig. 1A).

We detected cytogenetic involvement of chromosome 11p only in the recurrent tumors (kidney and lung) of case 3 (Fig. 1C and 1D). In the kidney biopsy, the short arm of chromosome 11 (including bands p13 and p15) was deleted. In the lung metastasis, however, an additional part of chromosome 11q was also missing. Monosomy 22 was detected in three biopsies (two renal and one lung) taken from case 2 (metachronous WT) and case 3 (WT and lung metastasis) (Fig. 1B, 1C, and 1D).

Other nonrandom whole-chromosome aberrations (including trisomies 6, 8, 12, 13, and 18) and partial monosomies (4q, 17p, and 22q) were detected in the WT biopsies of cases 2 and 3 (Fig. 1). Chromosome 16, however, did not seem to be involved in any of the chromosomal abnormalities detected by G-banding or spectral karyotype staining.

3.2. Array-based CGH analysis

To determine the variations of the G/R ratios of the genes, the mean ratio for measuring deviations was first determined in normal control kidney DNA (46,XY karyotype). The mean ratio of G/R fluorescence for control DNA for all 59 target genes was 1.015, with a standard deviation (SD) of 0.119. A value of the mean +2 SD was set as the cutoff level for the normal gene copy number and the normal range was, therefore, defined as 0.77–1.26. A significant increase in gene copy number (amplification) was considered as a G/R fluorescence ratio above 2, corresponding to four copies.

The results of the array-based CGH are illustrated in Fig. 2A. A single significant amplification of 2.6-fold (the average of two independent experiments) of the MRP1 gene was detected in the metachronous WT of case 2. The MRP1 amplification was restricted to the metachronous tumor and was not present in the primary tumor. No other significant gene amplification events were detected in either of the remaining tumors, including the multidrug resistance 1 (MDR1) gene, which has a similar function as MRP1.

3.3. Southern blot analysis

Because no cytogenetic alterations involving chromosome 16 were detected in the metachronous WT specimen, Southern blotting was used to confirm amplification results. Southern blot analysis (Fig. 2B) showed a 3.2-fold increase of MRP1 gene dosage in the metachronous WT sample (case 2) that corresponded well with the finding of a 2.6-fold amplification of MRP1 that had been revealed by array-based CGH. No increase of MRP1 gene dosage was detected in the DNA from the primary WT of case 2 and the control kidney.

3.4. Quantitative RT-PCR

Quantitative RT-PCR was performed to determine whether MRP1 amplification in the metachronous WT of case 2 was associated with MRP1 overexpression. The MRP1 mRNA expression level was calculated relative to GAPDH expression in the metachronous WT and was, on the average, sixfold (6.1 ± 0.32) higher than its expression in the primary tumor (Fig. 2C). In the normal kidney tissue, the relative expression level was considerably lower than in the WT samples: 0.04, 0.7, and 4.4 for the control kidney, primary WT, and metachronous WT, respectively. The increase in MRP1 gene expression (RMRP1) relative to the normal kidney was 17.5-fold in the primary tumor and 110-fold in the metachronous tumor.

4. Discussion

Wilms tumor is characterized by complex and diverse genetic alterations, but most of the genes involved in WT
tumorigenesis are unknown, as reviewed by Dome and Coppes [16]. In this study, we examined one NR and five WT specimens to identify chromosomal changes and gene amplification events associated with WT. Such changes can eventually provide clues to the clinical progression of the disease and contribute important information for the patient’s therapeutic management. The WT specimens we examined were highly distinctive from one another; they included unilat-
eral primary or recurrent tumor, with or without NR, and metachronous and metastatic WT. Their histologic classification was also heterogeneous and included favorable histology as well as focal and diffuse anaplasia.

Our cytogenetic results showed nonrandom chromosomal changes similar to those previously reviewed by other authors [1,16,17]. The possibility of comparing the karyotype of two different tumors from the same patient was an advantage because it allowed us to determine the timing of the chromosomal changes and to confirm tumor origin.

The most common genetic alteration in our series was structural abnormalities of chromosome 1 (4/5 WT) generating trisomy 1q or monosomy 1p. The high incidence of chromosome 1 abnormalities is well documented in WT as well as in other cancers, including solid tumors and leukemia [18]. This finding supports an important role for genes in this region in tumor progression [11,17,19].

Loss of heterozygosity at 7p or 11p is regarded mainly as an initiation event in WT development [20,21]. In our study, monosomy 7p was found in two of the three patients. The finding of monosomy 7p as the sole cytogenetic abnormality in the WT and the contralateral NR of case 1 is in agreement with previous reports, supporting a role for a putative tumor suppressor gene at 7p in tumor initiation [8,20], which would point to a possible diagnostic value. This result is also in agreement with reported findings of identical cytogenetic alterations in the NR and the WT tissues, supporting the assumption that NR represent precursor lesions to nephroblastoma [3,4].

Involvement of chromosome 11p is the most frequent genetic abnormality studied in WT [17]. This region contains the WT1 gene locus and is imprinted in Beckwith–Wiedemann syndrome [22]. We detected alterations of chromosome 11p only in the recurrent WT and the lung metastasis of case 3.

Fig. 2. *MRP1* amplification and overexpression in the metachronous WT. (A) Array-based CGH analysis. A representative image of a hybridized microarray showing a 2.6-fold amplification of the *MRP1* gene (green signals in yellow box) in the metachronous WT of case 2 relative to reference control. (B) Dosage analysis of *MRP1* by Southern blotting. The DNA from a control normal kidney (lane 1), the metachronous WT (lane 2), and the primary WT of case 2 (lane 3) was digested with EcoRI (left panel) and BamHI (right panel) and hybridized to *MRP1* (top) and *GAPDH* (control probe; bottom). When normalized to the kidney control, the metachronous WT showed a 3.2-fold increased dosage of *MRP1*. (C) Quantitative RT-PCR analysis of *MRP1* gene. Representative gel scans (GeneScan, ABI). Band sizes (in base pairs) are shown in the upper boxes; peak areas are shown in the lower boxes; top the metachronous WT; bottom the primary kidney tumor of case 2.
Both tumors displayed monosomy 11p in addition to isochromosome 1q and monosomy 22q. The remaining alterations detected in this case, such as i(13q) and partial monosomy 17 in the WT, and partial deletion of 11q in the metastasis, were probably secondary changes that developed through clonal evolution.

Clinical data suggest a possible prognostic significance for LOH 11q and LOH 22q because of their association with tumor recurrence and adverse outcome [23]. Our results further support this possibility with the finding of monosomy 22q in the metachronous WT and the recurrent WT of cases 2 and 3, respectively, and monosomies 11q and 22q in the lung metastasis of case 3 (Figs. 1B, 1C, and 1D). Finally, nonrandom cytogenetic changes, in particular, trisomies 6, 8, 12, 13, and 18 and partial monosomies 4q, 11q, 17q, and 22q, were also implicated in WT, and may be mainly associated with WT progression [11,24,25]. In our small series, nonrandom trisomies were detected mostly in the primary tumor of case 2; partial deletions occurred in the metachronous WT of the same patient.

The CGH analysis of WT provided extensive data on changes in the DNA copy number. The use of array-based CGH allowed rapid screening for alterations in the copy number of specific genes that were reported to be involved in tumorigenesis. Of the six analyzed tissue samples, only the metachronous tumor (case 2) exhibited a single significant amplification (2.6-fold) of the MRP1 gene, a finding further confirmed at the DNA level by Southern blot analysis (Fig. 2A and 2B). The MRP1 gene belongs to the superfamily of the ATP binding cassette (ABC) transporters. The gene confers multidrug resistance to some anticancer agents (e.g., doxorubicin, daunorubicin, vincristine, actinomycin D, and colchicine) by functioning as an efflux pump to decrease intracellular drug concentrations in cancer cells; see review by Ramachandran and Melnick [26]. Amplification and/or overexpression of one of these multidrug resistance genes may be an obstacle for the success of cancer therapy and is, therefore, associated with tumor progression, thereby adversely affecting patient outcome.

In WT, overexpression of MDR1 gene has been reported [27], but MRP1 expression was not detected in two primary cell cultures from WT [28]. MRP1 is ubiquitously expressed in many tissues and is overexpressed in various multidrug-resistant cell lines [26,29]. Recently, Efferth et al. [30] described MRP1 expression in 32 nephroblastomas, but MRP1 amplification in clinical specimens has not yet been described. Simultaneous amplification and overexpression of a gene associated with multidrug resistance in WT is documented here for the first time, to our knowledge. We also present novel data on the possible contribution of MRP1 to WT progression. In our case 2, the expression studies using quantitative RT-PCR showed a high expression of MRP1 (six-fold) in the metachronous WT compared with its primary counterpart tumor. Furthermore, when MRP1 overexpression in the tumors was normalized to its expression in a normal kidney, we found that MRP1 expression in both the primary and the metachronous WT were significantly higher (17- and 110-fold, respectively).

The metachronous tumor in our case 2 was diagnosed only 3 months after the cessation of chemotherapy for the primary WT. In a retrospective study, Paulino et al. [31] found that the median time interval to development of a metachronous tumor was considerably longer in children who received chemotherapy compared with children who did not (24 vs. 9 months). It is therefore possible that the rapid development of the metachronous tumor in this patient was due, at least in part, to the multidrug resistance phenotype acquired by the WT cells via MRP1 amplification and overexpression.

The extent and nature of gene amplification events vary widely among different tumors. MRP1 amplification was the sole amplification event in the WTs examined in our study. Due to the small sample size, however, we cannot determine the exact extent of gene amplification involvement in the complex genetic changes in WT. The absence of detectable amplification does not necessarily mean lack of function, and some of the oncogenes that we had tested in this investigation may still play a role in WT development, probably via other genetic mechanisms.

In conclusion, cytogenetic analysis is an important auxiliary tool in the diagnosis of WT. Our focus has also been on the detection of amplified genes that could serve as potential genetic markers to correlate with patient prognosis and assist in therapeutic management. By combining cytogenetic techniques with array-based CGH, we were able to maximize the identification of structural abnormalities and amplification events involved in the initiation and progression of WT in our patients.

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