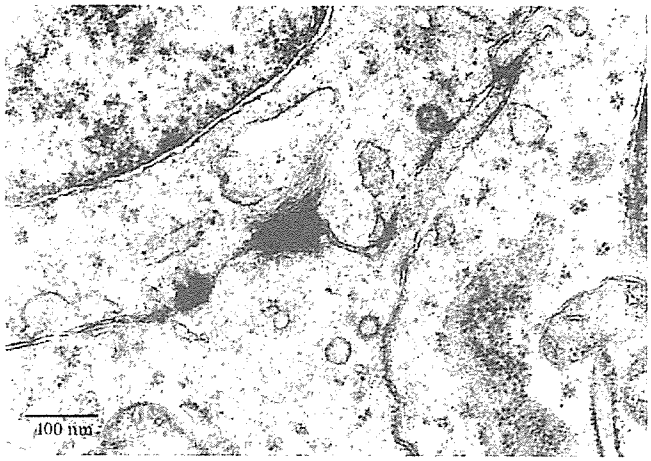

Figure 4 Electron microscopy. The tumor cells exhibit intercellular desmosomes with distinct tonofilaments.

differentiation were seen in the cytoplasm. No specific neurosecretory vesicle was found in the cytoplasm.

Gene analysis

Total RNA was extracted from frozen tumor tissue using an RNeasy Kit (Qiagen, Hilden, Germany) and was reverse transcribed to cDNA using a First-Strand cDNA synthesis Kit (Amersham, Piscataway, NJ, USA). PCR was performed using the following primers: EWS/WT1, ESBP1 (CGAC TAGTTATGATCAGAGCAGT)[2] and 10BA (TGCTGCCTGG GACACTGAAC); EWS-FLI1, 22.3 (TCCTACAGCCAAGCT CCAAGTC)[3] and 11.3 (ACTCCCCGTTGGTCCCCTCC); and EWS/ERG, ESBP1 and EU15 (CATGTACGGGAGGTCT GAGGGGT).[4] The amplified products were run on a 2% agarose gel. The products were then purified using MicroSpin S-400 Columns (Amersham) and cloned into pGEM-T vectors via the pGEM-T Vector System (Promega, Madison, WI, USA). Sequencing of the subcloned PCR products was performed using a DyEnamic ET Terminator Cycle Sequencing Kit (Amersham) with M13-40 and reverse primers.

The aforementioned histological diagnosis was also confirmed by RT-PCR of snap-frozen tumor tissues taken from the first recurrent tumor and the second recurrent masses, including both the solid cellular portion and the desmoplastic portion (Fig. 5). Each tissue specimen exhibited the same *EWS-WT1* chimeric fusion (data not shown). The chimeric transcript was finally confirmed by cloning and sequencing of the PCR product, resulting in the identification of an in-frame fusion of *EWS* exon 9 and *WT1* exon 8 (Fig. 6). Neither *EWS-FLI1* nor *EWS-ERG* chimeric transcripts associated with the Ewing family of tumors were detected (data not shown).

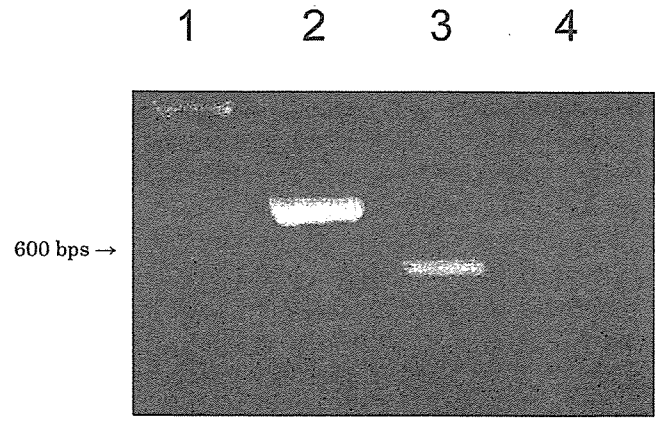

Figure 5 Molecular analysis. Reverse transcriptase–polymerase chain reaction of the first recurrent tumor tissue shows an in-frame fusion of *EWS* and *WT1* in lane 2, which contains a longer band than that of the positive desmoplastic small cell tumor (DSCT) control. A smaller product, corresponding to the fusion of *EWS* exon 7 to *WT1* exon 8, is also visible. Lane 1, 100 bp ladder marker; lane 2, present case; lane 3, positive DSCT control; lane 4, *NCR-NB3* negative control neuroblastoma cell.

	EWS exon 9	WT1 exon 8
Present case	cgaggtggcttcaataagcctggtg	gtgagaaaccataccagtgtgactt

Figure 6 Sequence of *EWS-WT1* junction. The fusion point of the longer product is shown. A vertical line indicates the nucleotide position of the junction.

DISCUSSION

Several epithelial neoplasms, many of which are malignant in adults, were initially considered in the differential diagnosis of the slow-growing tumor and repeated recurrences seen in the present case. Several kinds of malignant small cell tumors of soft tissues including rhabdomyosarcoma (especially alveolar type), extraskeletal Ewing sarcoma family of tumors (ESFT),[5] neuroblastoma metastases, and non-Hodgkin lymphoma (in addition to DSCT) must be ruled out in pediatric patients. The immunohistochemical reactivity of desmin or vimentin was not necessary to confirm a diagnosis of rhabdomyosarcoma because myogenin, which is a myogenic regulatory protein, was not reactive. The absence of CD99, CD45, NSE, S100, and GFAP reactivity enabled ESFT, non-Hodgkin lymphoma, and neuronal tumors to be ruled out. Electron microscopy revealed an epithelioid fine structure with distinct desmosomes, similar to the findings for a previously reported extra-abdominal DSCT.[6] No evidence of intracytoplasmic neurosecretory vesicles, often seen in neuroblastomas, or aggregates of thick myofilaments with electron-dense zones, indicating rhabdomyosarcoma, was found.

Another possibility in the differential diagnosis of small round cell tumors in children is DSCT. Recently, intra-

abdominal DSCT was recognized as a small cell tumor that usually occurs in the intra-abdominal region of children or young adults. DSCT is characterized by small cell proliferation, prominent stromal desmoplasia, and intra-abdominal serosal involvement. A distinct molecular property of this tumor is the presence of an in-frame fusion of the *EWS* gene and the *WT1* gene.[7–11]

Intra-abdominal DSCT has been described in the retroperitoneum, pancreatic region, gastric wall, and the pelvic, mesenteric, and omental regions.[7,9,12] Light microcopy has revealed a divergent differentiation pattern (small cell proliferation in an intermingled pattern) because the majority of cases exhibit a trabecular, basalioid, or glandular architecture associated with abundant interstitial fibrous proliferation, and, in some cases, solid cellular areas.[7] The small cells are typically positive for cytokeratin, EMA, vimentin, desmin, or NSE immunohistochemical staining.[7] Immunoreactivity for S100, Leu7, or LeuM1 has been observed in some cases.[6,13–20]

Cytogenetic investigation of intra-abdominal DSCT has revealed a consistent chromosomal translocation: t(11;22)(p13;q12). This translocation causes an in-frame fusion between the *EWS* gene and the *WT1* gene, producing a chimeric transcript. In most cases of intra-abdominal DSCT, the first seven exons of *EWS* are fused to the last three exons of *WT1*. *EWS-ERG* or *EWS-FLI1* fusion transcripts have also been described in some DSCT cases.[10–12,21]

The soft-tissue tumor in the present case was similarly characterized by divergent immunoreactivity pattern of EMA, cytokeratin, desmin, and vimentin in the solid and trabecular areas of the tumor. Electron microscopy showed the tumor cells to be arrayed with distinct desmosome-type intercellular junctions[6] and with intracytoplasmic intermediate fibrils in a few cells. These characteristics were morphologically and immunohistochemically consistent with a diagnosis of DSCT, despite the extra-abdominal location of the tumor. Molecular analysis of the first and second recurrent tumors showed the same in-frame fusion transcript, even though the tumor tissues had different morphological phenotypes.

Extra-abdominal DSCT is extremely rare, although ovarian or paratesticular involvement has been described.[13–15] DSCT of the soft tissue and bone of the hand, intracranial DSCT, and pleural cavity DSCT have also been reported.[16–19] To the best of our knowledge, no extra-abdominal DSCT confirmed by RT-PCR to exhibit an *EWS-WT1* chimeric fusion have been reported in Japanese studies. The morphological, immunohistochemical, ultrastructural, and molecular features of soft-tissue DSCT are similar to those of intra-abdominal DSCT. Gerald *et al.* reviewed and summarized 109 cases of DSCT, of which 103 were located in the abdominal cavity, four were in the thoracic region, one was in the cranial fossa, and one was in the hand.[20] Swanson *et al.* described 12 cases of polyphenotypic small cell tumors in children; two of these tumors arose from bone, six arose from soft tissue or the axial skeleton, two arose from the CNS, and two arose from the retroperitoneum.[1] All of the tumors exhibited primitive round cell features or neuroectodermal characteristics with or without myogenic, epithelial, or combined differentiation.

The present patient exhibited an uncommon form of the *EWS-WT1* fusion transcript: a fusion of *EWS* exon 9 and *WT1* exon 8, instead of the more common fusion of *EWS* exon 7 and *WT1* exon 8 seen in intra-abdominal DSCT.[8,22] Interestingly, two cases of DSCT of soft tissue and bone and one case of DSCT of the kidney exhibited the same type of fusion transcript.[6,19,23] This molecular aberration might be a variant of the in-frame *EWS-WT1* fusion transcript, because the *EWS-WT1* fusion gene has been reported to exhibit molecular heterogeneity, such as the fusion of *EWS* exon 10 and *WT1* exon 8[24] or the fusion of *EWS* exon 7 and *WT1* exon 9.[12] In contrast, intra-abdominal DSCT with a fusion of *EWS* exon 9 and *WT1* exon 8 has not been previously described. Furthermore, in spite of the cytological heterogeneity of the tumor phenotypes in the present case, the same in-frame *EWS-WT1* fusion was noted in the solid area and the desmoplastic epithelioid region. The genetic findings in the present case suggest that fusion gene heterogeneity may be related to the tissue-specific phenotypes of DSCT, although further investigation of other cases of extra-abdominal DSCT is needed to confirm this speculation.

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Association of 11q Loss, Trisomy 12, and Possible 16q Loss with Loss of Imprinting of Insulin-Like Growth Factor–II in Wilms Tumor

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We evaluated the *WT1* and *IGF2* status and performed chromosome and/or comparative genomic hybridization analysis in 43 tumor samples from patients with Wilms tumor. On this basis, we classified them into 4 groups: *WT1* abnormality, loss of heterozygosity (LOH) of *IGF2*, loss of imprinting (LOI) of *IGF2*, and retention of imprinting (ROI) of *IGF2*, which were seen in 12%, 30%, 16%, and 42% of the tumors, respectively. Patients in the LOI group were older than those in other groups ($P < 0.01$), and tumors in the *WT1* group had fewer cytogenetic changes than did those in the other groups ($P < 0.01$). It was found that 11q− and +12 were more frequent in the LOI group than in the *WT1*+LOH+ROI group ($P < 0.01$ and $P < 0.01$). There was no difference in the incidence of 16q− between the LOI group and the other groups; however, when we excluded 16 tumors with LOH on 11p15, 16q− tended to be more frequent in the LOI group than in the *WT1*+ROI group ($P = 0.06$). The association of 11q− or +12 with LOI of *IGF2* found in the present study suggests that many tumors with no *WT1* abnormalities need overexpression of *IGF2* together with biallelic inactivation of the tumor-suppressor gene on 11q and/or overexpression of growth-promoting genes on chromosome 12. The 11q gene may code for one of the proteins that constitute a CTCF insulator complex, and its mutation, deletion, or haploinsufficiency may cause insulator abnormalities that might lead to LOI of *IGF2*.

INTRODUCTION

Wilms tumor is the most common kidney tumor in childhood. A tumor-suppressor gene, *WT1*, was isolated in the 11p13 chromosomal region, but deletion or mutation has been found in only 15%–20% of Wilms tumors (Huff, 1998; Nakadate et al., 2001). Loss of imprinting (LOI) of insulin-like growth factor–II (*IGF2*), a paternally expressed gene at 11p15.5, has been reported to occur in 40%–70% of tumors (Ogawa et al., 1993; Rainier et al., 1993), and it was associated with a pathological subtype that occurs in a later stage of renal development (Ravenel et al., 2001). Several studies found the type of loss of heterozygosity (LOH) on 11p that is always caused by loss of the maternal chromosome in 30%–40% of tumors investigated (Schroeder et al., 1987; Grundy et al., 1994; Nakadate et al., 2001). LOI or LOH of *IGF2* may cause overexpression of a gene that gives tumor cells a growth advantage or modifies their differentiation stage (Sakatani et al., 2005), and *IGF2* is the primary candidate for being the *WT2* gene. Cytogenetic, comparative genomic hybridization (CGH), and LOH analyses of Wilms tumors showed gain or loss of specific chromosomes or chromosomal regions, indicating that *WT1*-wild-type tumors had more genomic alterations than *WT1*-mutant-type tumors (Nakadate et al., 1999; Hing et al., 2001; Ruteshouser et al., 2005). Furthermore, association of the long arm loss of chromosome 16 (16q−) with LOI of *IGF2* in Wilms tumor was recently reported (Mummert et al., 2005). However, 16q− was found in only a small portion of the tumors with LOI investigated, and no other cytogenetic abnormalities are known to be associated with LOI in the tumors. These studies indicate that Wilms tumor is a genetically heterogeneous disease, and further

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studies are needed to clarify the genetic/epigenetic and cytogenetic background of the tumor.

We evaluated the *WT1* and *IGF2* status and performed chromosome and/or CGH analysis of 43 Wilms tumors, on the basis of which we classified them into 4 genetic/epigenetic groups: *WT1* abnormality, LOH of *IGF2*, LOI of *IGF2*, and retention of imprinting (ROI) of *IGF2*. We analyzed the relationship between cytogenetic and genetic/epigenetic changes and found an association of LOI of *IGF2* with 11q– and +12 and possibly also with 16q–.

MATERIALS AND METHODS

Patient Samples

Tumor samples were available from 68 Japanese infants or children ranging in age from 2 months to 8 years who underwent surgery or biopsy between August 1984 and February 2003. These samples were selected on the basis of tissue availability and were not gathered consecutively. Of the 68 patients, 21 were registered in the Japan Wilms Tumor Group Study (JWITS). Samples of normal tissue were obtained from either the peripheral blood or normal renal tissue adjacent to the tumor from the same patients. Informed consent was obtained from the parents, and the study design was approved by the ethics committee of Saitama Cancer Center. The tumors were staged according to the National Wilms' Tumor Study group (NWTS) staging system, and most patients were treated according to NWTS protocols (d'Angio et al., 1989). None of the 68 patients had a family history of Wilms tumor. One patient (275) had Drash syndrome, and another patient (953) had bilateral tumors; the remaining patients had sporadic and unilateral tumors (Table 1).

Histological Examination

In all tumors, the diagnosis of Wilms tumor was made with routine hematoxylin- and eosin-stained pathology slides by local pathologists from each institution according to the classification proposed by the Japanese Pathological Society and/or the NWTS pathology panel (Beckwith et al., 1978; Japanese Pathological Society, 1988). Twenty-one cases that were registered at the JWITS were also reviewed by the pathology panel.

Cytogenetic, Fluorescence In Situ Hybridization, and CGH Studies

Chromosomes from tumor cells were studied by methods reported previously (Nakadate et al., 1999), and karyotypes were described according to the International System of Human Cytogenetic Nomenclature (ISCN, 1995). Fluorescence in situ hybridization (FISH) using Vysis probes [CEP 3 (chromosome 3 centromere), CEP 12 (chromosome 12 centromere), CBFB (16q22), and MLL (11q23); Downers Grove, IL) were carried out as described previously (Watanabe et al., 2002). CEP 12 was used to detect trisomy 12 and CEP 3 was used as a control because chromosome and CGH analyses detected 2 copies of chromosome 3 in almost all Wilms tumors, and the CBFB and MLL probes were used to detect 16q– and 11q–, respectively. Karyotypes of 11 of the 43 tumors described in Table 1 were reported previously (Nakadate et al., 1999).

CGH analysis was performed as described previously (Kumon et al., 2000). A chromosomal region was considered overrepresented or underrepresented if the average ratio profile was above 1.25 or below 0.75, respectively.

Analyses of WT1 Abnormalities and Allelic Loss on 11p and 11q

DNA preparation and digestion and Southern blot analysis using a *WT1* cDNA probe (WT33; Call et al., 1990), PCR–single-strand conformation polymorphism (SSCP) and subsequent direct-sequencing analysis, and allelic loss analysis on 11p and 11q were performed as described previously (Nakadate et al., 2001). Whether there was allelic loss on 11p and 11q was determined by PCR using microsatellite markers of D11S922, *TH*, *IGF2*, D11S932, *PAX6*, D11S903, D11S4100, *NCAM*, D11S1885, D11S29, and D11S1364 and using the restriction fragment length polymorphism (RFLP) sites of *WT1* (Tadokoro et al., 1993). The primer sequences used for PCR were obtained from the Genome Database (http://www.gdb.org). The results of the allelic loss analysis on 11p and 11q for 21 of the 43 tumors described in Tables 1 and 2 were reported previously (Nakadate et al., 2001).

The results of the study of promoter hypermethylation of *WT1* were reported previously (Satoh et al., 2003).

Analysis of *IGF2* Allelic Expression and Loss

The *Apa*I/*Ava*II polymorphism site in exon 9 was used to evaluate allelic expression of *IGF2*. PCR with genomic DNA from normal tissue and identification of heterozygous specimens after *Ava*II and *Hinf*I digestion were performed as described previously (Watanabe et al., 2002). RT-PCR products from the tumor RNA also were

TABLE 1. Clinical, Genetic, Karyotypic and CGH findings in 43 Wilms Tumors

Patients number	Age/Sex	Stage of disease	*WT1* Abnormality	Karyotype	CGH	CEP 12/CEP 3	CBFB
Tumors with *WT1* abnormalities and LOH or ROI of *IGF2* (n = 5)							
275*	1 y 0 m/F	I	Mutation in exon 8	48,XX,+3,+6	ND		
832*	9 m/F	II	Mutation in exon 2	45,XX,del(3)(p12p14),−7	ND		
949*	1 y 3 m/F	II	Promoter methylation	44,X,-X,dic r(1;11)(p3?q3?;q25?p1?),inv(9)(p11q12)c	ND		
2375	1 y 9 m/M	IV	Homozygous deletion	46,XY	N		
M289	5 y 4 m/F	II	Mutation in exon 7	ND	enh(18),dim(11p13–11q12, 19,22)		
Tumors with LOH of *IGF2* and no *WT1* abnormalities (n = 13)							
325*	1 y 6 m/M	I	None	47,XY,+8,del(14)(q22)	ND		
528*	4 y 1 m/F	II	None	56,XX,+5,+7,+7,+9,+10,+12,+13,+18,+19,+22	enh(1q,4p,7,8,9,10,12,13,18)	3/2	2
575	3 y 11 m/M	II	None	46,XY	enh(1q)		
871	1 y 4 m/F	I	None	NM	N	2/2	2
918*	4 y 6 m/M	III	None	45,X,-Y	ND		
1075	2 y 4 m//M	IV	None	NM	N		
1390	4 y 0 m/M	I	None	NM	enh(Yq)		
1570*	11 m/F	II	None	51,XX,+7,+8,+10,+12,+13,?del(16)(q22)	enh(7,12,13),dim(16q22–qter)	3/2	1
1658*	2 y 8 m/M	III	None	46,XY,der(16)t(1;16)(q21;q12)	ND		
1752	1 y 0 m/F	III	None	46,XX	N		
2488	3 m/F	I	None	46,XX	N		
M134	10 m/F	II	None	ND	enh(6q),dim(7p)		
M204	3 y 9 m/F	IV	None	ND	enh(8,9,20),dim(Y)		
Tumors with LOI of *IGF2* and no *WT1* abnormalities (n = 7)							
548	3 y 1 m/M	II	None	NM	enh(6,8,9,12)		
1206*	3 y 10 m/F	II	None	50,XX,+12,inc/11q- detected by FISH (MLL)	ND	3/2	2
1207	4 y 4 m/F	III	None	76–87 complex changes	enh(12),dim(9,10p,11q,16q,18p)		
1435*	6 y 1 m/F	III	None	53,XX,+12,inc	ND	3/2	2
1535*	3 y 8 m/F	II	None	46,XX,dup(1)(q21q25),der(11)t(1;11)(q21;q22),del(16)(q22)	enh(1q,4p15–pter),dim(11q13–qter,16q)		
M269	4 y 6 m/F	IV	None	ND	enh(7q,14q21–qter),dim(7p,X)		
M291	8 y 0 m/F	I	None	ND	enh(1q,6,9p,12,13,18q),dim(1p,11q,19)		

(Continued)

TABLE 1. Clinical, Genetic, Karyotypic and CGH findings in 43 Wilms Tumors (Continued)

Patients number	Age/Sex	Stage of disease	*WT1* Abnormality	Karyotype	CGH	CEP 12/CEP 3	CBFB
Tumors with ROI of *IGF2* and no *WT1* abnormalities (n = 18)							
884	2 m/M	III	None	46,XY	N	2/2	2
953	1 y 1 m/F	V	None	47,XX,add(2)(p25), del(7)(q11q22),+8	ND		
1371	5 m/F	IV	None	NM	N		
1420	6 m/F	Unknown	None	46,XX	N		
1879	7 m/M	I	None	46,XY	N		
2011	2 y 7 m/M	II	None	55,XY,+2,+6,+7,+8, +10,+del(12)(q23) +del(12)(q23),+13,+14	enh(1q,2,6,7q21–qter,8,10, 12pter–q23,13,15), dim(1p,18p)		
2385	1 y 4 m/F	IV	None	46,XX	N		
2677	4 y 4 m/F	II	None	46,XX	enh(2)		
2749	5 y 2 m/M	II	None	46,XY	dim(22)		
M126	2 y 5 m/F	III	None	ND	enh(2p14–pter,3q,6,7,8, 12,13,17)		
M175	1 y 9 m/F	I	None	ND	N		
M188	1 y 0 m/M	I	None	ND	N		
M196	1 y 5 m/F	III	None	ND	N		
M232	1 y 2 m/F	II	None	ND	N		
M233	5 y 3 m/F	IV	None	ND	enh(6,8)		
M238	6 m/F	I	None	ND	enh(7,8,10,12,13,17,18)		
M258	4 m/M	I	None	ND	N		
M290	2 y 1 m/M	II	None	ND	enh(1q,6,7,9,12),dim(18p,Y)		

*Karyotypes of these tumors were reported previously (Nakadate et al., 1999).
Abbreviations: NM, no mitotic cells; ND, not done; N, normal; 3/2, 3 copies of CEP 12 and 2 copies of CEP 3 detected by FISH; 2, 2 copies of CBFB detected by FISH.

TABLE 2. Allelic Status of 11p and 11q and *IGF2* Imprinting Status in *WT1*, LOH of *IGF2*, and LOI of *IGF2* Wilms Tumor Groups

	p15					p13		p11	q21–22	11q23					
	S922	*IGF2*	*IGF2-LOI*	*TH01*	*S932*	*PAX6*	*WT1*	*S903*	*S4100*	*NCAM*	*S1855*	*S29*	*S1364*	*WT1* abnormality[a]	11q-detected by CGH/ cytogenetics
Tumors with *WT1* abnormalities and LOH, LOI, or ROI of *IGF2* ($n = 5$)															
275	—	●	—	●	●	—	●	—	—	○	—	○	—	Mutation in exon 8	Not detected
832	—	—	—	●	●	●	●	—	○	○	—	—	○	Mutation in exon 2	Not detected
949	—	—	—	●	—	—	●	●	●	—	—	—	●	Promoter methylation	Not detected
M289	—	○	□	○	○	○	●	●	—	—	○	○	○	Mutation in exon 7	Not detected
2375	○	○	□	○	○	—	▲	—	○	○	—	—	○	Homozygous deletion	Not detected
Tumors with LOH of *IGF* and no *WT1* abnormalities ($n = 13$)															
325	●	—	—	—	—	—	●	—	○	○	—	○	○	None	Not detected
528	—	●	—	●	●	●	—	—	●	—	●	—	●	None	Not detected
575	●	●	—	●	●	—	○	—	○	○	—	—	○	None	Not detected
871	●	—	—	●	—	●	—	—	—	○	○	○	○	None	Not detected
918	●	—	—	—	—	○	○	○	—	—	○	○	○	None	Not detected
1075	—	—	—	●	●	—	○	○	○	○	○	—	○	None	Not detected
1390	—	—	—	●	—	—	●	○	○	○	○	—	—	None	Not detected
1570	—	●	—	—	●	●	—	—	—	●	●	●	—	None	Not detected
1658	—	—	—	●	—	—	—	—	—	—	○	○	—	None	Not detected
1752	●	—	—	●	●	—	●	●	—	—	—	○	○	None	Not detected
2488	●	—	—	—	●	●	●	—	○	—	○	○	○	None	Not detected
M134	—	●	—	—	●	●	●	●	○	○	○	○	—	None	Not detected
M204	—	—	—	—	●	●	●	—	—	●	—	—	○	None	Not detected
Tumors with LOI of *IGF2* and no *WT1* abnormalities ($n = 7$)															
548	—	○	■	○	○	—	○	—	○	—	—	○	○	None	Not detected
1206	—	○	■	○	○	○	○	○	○	—	—	●	●	None	Detected
1207	—	○	■	○	—	—	—	○	—	—	—	—	●	None	Detected
1435	—	○	■	—	—	○	○	—	○	○	—	○	○	None	Not detected
1535	○	○	■	○	—	—	○	—	—	●	●	●	—	None	Detected
M269	○	○	■	○	○	○	○	○	○	—	—	○	○	None	Not detected
M291	○	—	■	○	—	○	○	—	●	—	●	●	●	None	Detected

[a]Details of *WT1* abnormality are described in the text.
● Loss of heterozygosity; ○ Retention of heterozygosity; — Not informative; ■ Loss of *IGF2* imprinting; □ Retention of *IGF2* imprinting; ▲ Homozygous *WT1* deletion.

digested with *Ava*II and *Hinf*I, and allelic expression of *IGF2* was determined.

Statistical Analysis

The significance of differences in various clinical and cytogenetic aspects of the disease among the 4 genetic/epigenetic groups of tumors was determined by the chi-square or Fisher's exact tests. Differences in the mean age of the patients and in the average number of chromosome changes between any 2 of the 4 groups were examined with Welch's t test.

RESULTS

Allelic Loss on 11p and 11q

Allelic loss on 11p and 11q was analyzed in Wilms tumor samples from 68 patients. Informative 11p15 loci were found in normal tissue from 64 of the patients; the 11p15 loci in the tissue from the other 4 patients were uninformative. Of the 64 informative tumors, 16 showed LOH. Of the 48 tumors without LOH, 27 were informative for the *Apa*I/*Ava*II polymorphism site of the *IGF2* gene. Thus, 43 tumor samples were the subject of the present study.

Three tumors (949, 528, and 1570) showed LOH for the entire chromosome 11; 1 tumor (M204) showed LOH on 11p15–11q23, retaining heterozygosity in the more distal 11q locus; 3 tumors (575, 918, and 1075) showed LOH limited to the 11p15 region; and 9 (275, 832, 325, 871, 1390, 1658, 1752, 2488, and M134) showed LOH limited to the 11p15–11p13 region (Table 2). Of the 27 tumors without LOH on 11p15, 1 (M289) showed

LOH limited to 11p13–11p11, and 4 (C1206, C1207, C1535, and M291) showed LOH on 11q (Table 2).

WT1 Abnormalities

Of the 9 tumors with LOH limited to the 11p15–11p13 region, 2 showed a *WT1* mutation; one (275) had a missense mutation in exon 8 (G to A conversion in nucleotide 1064; Haber et al., 1991), and the other (832) had a nonsense mutation (C to T conversion in nucleotide 550) in exon 2 (Table 2). Another tumor (C949) was found to have *WT1* promoter methylation, which was examined in 21 of the 43 tumors, of which only 1 showed the methylation (Satoh et al., 2003). This tumor had a ring chromosome containing chromosomes 1 and 11. Because the incidence of promoter methylation was quite low, and no other tumors showed a ring chromosome containing chromosome 11 and LOH for the entire chromosome 11, the other 22 tumors whose *WT1* promoter methylation status was not examined were assumed to be unmethylated.

Of 27 tumors without LOH on 11p15, 1 (M289) with LOH limited to the 11p13–11p11 region had a missense mutation in exon 7 (G to T conversion in nucleotide 895), and another (C2375) with retention of heterozygosity (ROH) for the entire chromosome 11 had homozygous deletion of the 6.6-kb fragment of *WT1*, detected by Southern blotting with a *WT1* cDNA probe and *Eco*RI digestion (Call et al., 1990; Table 2).

LOI of *IGF2*

Of the 27 tumors with ROH in 11p15 and the informative *Apa*I/*Ava*II polymorphism site of *IGF2*, 7 showed LOI of *IGF2* (Tables 1 and 2, Fig. 1). Of the 20 ROI tumors, 2 (M289 and C2375) showed *WT1* abnormalities as described before.

Four Groups of Tumors Classified by *WT1* and *IGF2* Status

We classified 43 Wilms tumors into 4 groups on the basis of major genetic abnormalities: *WT1* abnormality, LOH of *IGF2*, LOI of *IGF2*, and tumors without *WT1* or *IGF2* abnormalities. Three tumors with a *WT1* abnormality and LOH on 11p15–11p13 were included in the *WT1* group because *WT1* abnormalities are believed to have a stronger impact on tumorigenicity than LOH of *IGF2*. Thus, of the 43 tumors, 5 were classified into the *WT1* group, 13 into the LOH group, 7 into the LOI group, and 18 into the ROI group (Table 1).

CGH patterns and/or karyotypes were available for all 43 tumors (Table 1). Four tumors (528, 1206, 1435, and 1570) with a hyperdiploid karyotype (≥50 chromosomes) and trisomy 12 with or without other changes were also studied by FISH using the CEP 3, CEP 12, and CBFB probes. All 4 tumors were shown to have trisomy 12, and 1 was shown to have 16q–. One tumor (1206) was shown to have 11q– using FISH with the *MLL* probe.

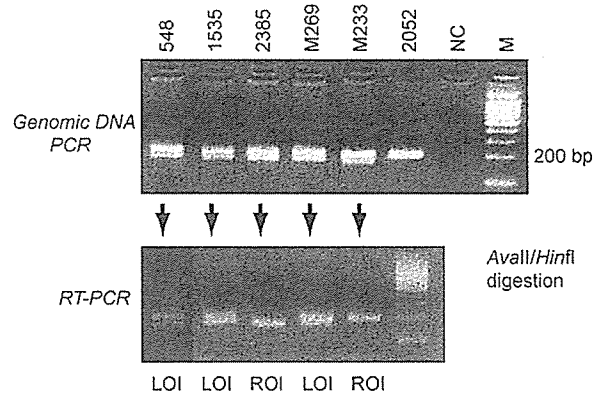

Figure 1. Electrophoretic patterns of products of genomic DNA PCR or reverse-transcription PCR after *Ava*II and *Hin*fI digestion. Normal tissue from samples 548, 1535, 2385, M269, and M233 was observed to have heterozygous *IGF2* alleles, and normal tissue from sample 2052 was observed to have homozygous *IGF2* alleles, in upper lanes; loss of imprinting was found in tumor tissue from samples 548, 1535, and M269 and retention of imprinting in tumor tissue from samples 2385 and M233, in lower lanes [NC, negative control (H_2O); M, size marker].

Clinical Characteristics of Patients in Each Tumor Group

The mean age of the patients was higher in the LOI group than in the *WT1* ($P = 0.03$), the LOH ($P = 0.01$), the ROI ($P < 0.01$), or the *WT1* + LOH + ROI ($P < 0.01$) groups (Table 3). There were no differences in stage distribution among the 4 groups. The tumors of 42 patients were classified as having a favorable histology, and the tumor of 1 patient (1390) was classified as having unfavorable histology (the diffuse anaplasia type). Of the 43 patients, 41 were alive with no evidence of disease at the last follow-up (November 30, 2004). Two patients had died: the patient who had the diffuse anaplasia–type tumor died of the disease, and the patient in the *WT1* group who had Drash syndrome (275) died of renal failure.

Association of Chromosome Abnormalities with *IGF2* and *WT1* Status

Ten chromosome/CGH abnormalities were seen in 4 or more tumors (Table 3). Loss limited to 11q was more frequent in the LOI group than in the *WT1* ($P = 0.08$), the LOH ($P < 0.01$), the ROI ($P < 0.01$), or the *WT1* + LOH + ROI ($P < 0.01$)

test. Thus, the present study and that of Yuan et al. (2005) lead to the same conclusion: chromosomal loss limited to 11q is associated with LOI of *IGF2* in Wilms tumor.

It has been hypothesized that 11q harbors a tumor-suppressor gene involved in the development of Wilms tumor (Radice et al., 1995; Nakadate et al., 2001). The association between 11q− and LOI of *IGF2* found in the present study suggests that Wilms tumors with overexpression of *IGF2* require deletion/mutation of the putative 11q gene in order to develop to full-blown tumors. As we have shown (Tables 1 and 2, Fig. 2), the present CGH and cytogenetic study detected physical loss of 11q DNA, rather than mitotic recombination, in the 4 tumors with LOI and 11q LOH. The gene on 11q may code for one of the proteins that constitute a CTCF insulator complex, and mutation, deletion, or haploinsufficiency of the gene may cause insulator abnormalities that might lead to LOI of *IGF2* (Ohlsson et al., 2001).

The present study also found an association between trisomy 12 and LOI of *IGF2*. We previously proposed that hyperdiploid tumors (≥50 chromosomes) make up a unique subgroup of Wilms tumors characterized by the absence of *WT1* abnormalities and nonrandom gains of chromosomes, usually including trisomy 12 (Nakadate et al., 1999). The present study added another characteristic, namely, the tendency to show LOI of *IGF2*, to the list of characteristics of hyperdiploid tumors. *CCND2* and *CDK4*, which are growth-promoting genes on chromosome 12, are overexpressed in Wilms tumors (Faussillon et al., 2005), and it is speculated that tumors with LOI of *IGF2* also need trisomy 12 in order to proliferate in an accelerated manner.

Ravenel et al. (2001) reported that patients who had Wilms tumors with LOI of *IGF2* were older than those who had tumors with normal imprinting and that the tumors with LOI were more likely to be of a pathological subtype associated with a later stage of renal development. The present study confirmed that patients with tumors with LOI were older than those who had tumors of other subtypes. Chromosome changes were most frequent in the LOI group and least frequent in the *WT1* group (Table 3). We suggest from the findings described above that tumors with LOI need far more genetic events to develop into full-blown tumors than do those with certain genetic types of tumors; it will take time to accumulate the genetic and epigenetic events that might explain why patients with LOI of *IGF2* are older.

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