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Deletion of *WT1* and *WIT1* Genes and Loss of Heterozygosity on Chromosome 11p in Wilms Tumors in Japan

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Six of 39 sporadic Wilms tumors had gross homozygous or hemizygous *WT1* and *WIT1* deletions. Two Wilms tumor-aniridia-genitourinary abnormalities-mental retardation syndrome patients had total hemizygous *WT1* and *WIT1* deletions in both constitutional and nonsporadic type tumor cells. Four of the 8 tumors with *WT1* and *WIT1* deletions showed loss of constitutional heterozygosity (LOH) for markers limited to the 11p13 region. Seven of 19 Wilms tumors with neither *WT1* nor *WIT1* deletions also had LOH on 11p; 4 in the 11p15-11p13 region, one in the 11p15 and possibly also 11p13 regions, and two solely in the 11p15 region. Thus, 15 of the 41 Wilms tumors (37%) had *WT1* and *WIT1* deletions or LOH on 11p, and only 2 of the 27 tumors whose nonneoplastic normal tissues were available for study showed LOH limited to the 11p15 region. None of the 7 non-Wilms childhood renal tumors showed *WT1* or *WIT1* deletions, or LOH on 11p. These data suggest that Japanese Wilms tumors may be characterized by a higher incidence of the gross *WT1* deletion and a lower incidence of LOH limited to the 11p15 region than the Caucasian counterparts. These molecular-genetic features may be contributing to the lower incidence of Wilms tumors in Japanese children than in Caucasian ones.

Key words: *WT1* deletion — *WIT1* deletion — Wilms tumor — Loss of constitutional heterozygosity

Wilms tumors (WTs) arise in kidneys of infants or children. Although one of the WT-associated genes, *WT1*, was recently isolated from the 11p13 region,[1,2] gross homozygous *WT1* deletion of the gene has been reported in only 10 WTs and the incidence seems quite low.[1,3–10] The gene is expressed in various tissues including fetal nephrons, gonads and hematopoietic cells, and apparently controls the urogenital system development.[1,2,11–13] The *WIT1* gene resides in the 5′ flanking region of *WT1*, and its functional role is still unknown.[14] Only large deletions encompassing both *WIT1* and *WT1* have been reported in WTs to date.[1,6–8]

In some WTs, LOH was found only in the 11p15 region, and not in the 11p13 region,[15–20] suggesting a second WT locus (*WT2*) in 11p15, where the gene for the Beckwith-Wiedemann syndrome was mapped.[18,21] The proposition that the *WT2* locus is at 11p15 was further substantiated by a micro-cell fusion study.[22]

Since the incidences of WTs markedly differ in East-Asian and Caucasian children,[23] we studied gross *WT1* and *WIT1* deletions and allelic loss on 11p in Japanese WTs, and compared the incidences of the deletions and the 11p allelic loss with those reported in the Caucasian WTs.[1,3,5,15–20] We report here that the homozygous or hemizygous *WT1* deletions appear to occur more frequently in the Japanese WTs than in the Caucasian WTs, and that the allelic loss limited to the 11p15 region may be less common in the Japanese tumors than in the Caucasian tumors.

MATERIALS AND METHODS

Tissue sample Tumors were obtained from 48 Japanese infants and children aged between one month and 10 years who were consecutively admitted to various institutions (listed in the "Acknowledgments") between August 1984 and December 1991. Normal tissues were obtained either from peripheral blood or normal renal tissue adjacent to the tumor of the same patients, and from peripheral blood of a healthy volunteer. The samples were transferred to the cytogenetic laboratory of the Saitama Cancer Center Hospital.

Of the 48 childhood renal tumors, 41 were histologically classified as WTs; these included 35 typical WTs (nephroblastic nephroblastomas)[24] (Nos. 89, 154, 165, 193, 207, 275, 312, 325, 392, 519, 521, 528, 539, 548, 571,

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575, 619, 637, 640, 667, 672, 716, 749, 771, 832, 851, 861, 871, 881, 884, 901, 902, 903, 918 and 937), 3 fetal rhabdomyomatous nephroblastomas[25] (Nos. 299, 412 and 774), 2 cystic partially differentiated nephroblastomas[26] (Nos. 74 and 384), and one nephroblastoma of a mesenchymal variant[24] (No. 791). The other 7 tumors included 3 clear cell sarcomas of the kidney[27] (Nos. 257, 593 and 608), 2 congenital mesoblastic nephromas[28] (Nos. 475 and 544), one malignant rhabdoid tumor of the kidney[28] (No. 652), and one renal cell carcinoma[29] (No. 926).

Of the 41 WTs, 39 were sporadic type and two (Nos. 165 and 299) occurred in patients with the Wilms tumor-aniridia-genitourinary abnormalities-mental retardation (WAGR) syndrome.[30] One sporadic type patient (No. 902) had bilateral WTs, and previous history of a brain tumor. All the other 40 patients had a unilateral tumor. None had external genitourinary malformations, or family history of sporadic WTs. Of the 7 non-WT patients, one (No. 926) had bilateral renal cell carcinomas. Clinical, histological and karyotypic findings of 28 of the 48 tumors were reported elsewhere.[31]

Southern blot analysis Genomic DNA was extracted from tumor and normal tissues using standard phenol/chloroform procedures. Five to 10 μg of DNA from each sample was digested with appropriate restriction enzymes, electrophoresed through 0.7 to 1.2% agarose gels, and transferred onto nylon filters (Hybond N, Amersham).

WT33 and GB16 were cDNA probes used for detection of *WT1* and *WIT1* deletions,[2, 14] respectively. The restriction fragment length polymorphism (RFLP) probes used to detect LOH included *HRAS1* (pHs-49), *INS* (pHINS6.0), *IGF2* (pHINS311), D11S12 (pADJ762), D11S323 (p5BE1.2), D11S323 (p5S1.6), D11S16 (p32-1), D11S151 (p56H2.4),[32] *WT1* (WT33),[33] D11S324 (p60H1.4), D11S325 (p8B1.25) and *CAT* (pINT800) on 11p, and D11S146 (pHBI59) and D11S147 (pHBI18P2) on 11q.[32] *BCL1* (probe B) in chromosome band 11q13 was used as a control probe.[32] The DNA probes were labeled with [^{32}P]dCTP by the random priming method.[34]

Hybridization was carried out at 42°C in 6×SSC, 10% dextran sulfate, 1% SDS, 1×Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, and 50% formamide. Filters were washed 3 times in 2×SSC at room temperature, and 3 times in 0.1×SSC and 0.1% SDS at 65°C for 30 min. Autoradiography was performed using a bioimage analyzer, FUJIX BAS 2000. The radioactivity of the *WT1*, *WIT1*, D11S16, D11S151, D11S323, D11S324, D11S325 or *CAT* fragments relative to that of the *BCL1* fragment subsequently hybridized to the same filters was compared between patients' tissues and a normal control tissue from a non-tumor-bearing individual for the detection of homozygous or hemizygous deletion of each fragment.

RESULTS

Deletion of *WT1* and *WIT1* was evaluated in WTs from 39 sporadic and 2 WAGR patients, and in other histologic types of childhood renal tumors from 7 patients. Both tumor and normal tissue DNAs were obtained from 27 patients with WT and 5 patients with other tumors to study allelic loss in tumor cells.

Deletion of *WT1* and *WIT1* in WT Of the 39 sporadic WTs, 3 (Nos. 312, 519 and 640) and 2 (Nos. 392 and 749) tumors had homozygous and hemizygous *WT1* deletion, respectively. One tumor (No. 640) showed a homozygous deletion of *WIT1*, and 4 (Nos. 312, 519, 392 and 749) tumors had hemizygous *WIT1* deletions (Fig. 1). Another sporadic tumor (No. 667) showed an altered fragment of both *WT1* and *WIT1* in addition to the respective germ line bands (Fig. 1). The two WAGR tumors (Nos. 165 and 299) had hemizygous *WT1* and *WIT1* deletion (Fig. 2). None of the other 33 WTs and 7 other histologic types of tumors showed deletion or rearrangements of *WT1* or *WIT1*. In addition to the 8 with deletions or rearrangements of *WT1* and *WIT1*, 7 of the 19 WTs with neither *WT1* nor *WIT1* deletion showed LOH on 11p (Fig. 3). None of the 5 tumors with other histologic types of tumors showed LOH on 11p or 11q (Fig. 3). The results of the study of the *WT1* and *WIT1* deletions and LOH on 11p or 11q in 32 tumors are summarized in Figs. 3 and 4.

Sporadic WTs with homozygous *WT1* deletion and homozygous or hemizygous *WIT1* deletion Of the 3 tumors with homozygous *WT1* deletion, one (No. 640) exhibited a total deletion of the gene losing all 7 *Eco*RI fragments (Fig. 1). The other 2 tumors had partial deletions; tumor 312 retained the 13 kb fragment, but had lost the other 6 fragments, while tumor 519 retained the 13 and 6.6 kb fragments and had lost the other 5 fragments (Fig. 1). The radioactivity of the retained 13 kb (No. 312) or 13 and 6.6 kb (No. 519) fragments was half that of the same fragments of the control tissue from a non-tumor-bearing individual, indicating that one allele of the *WT1* gene sequences coding exons 1 and 2 or exons 1, 2 and 3 was retained in these tumors.[35] All the 3 tumors also had homozygous (No. 640) or hemizygous (Nos. 312 and 519) deletions of *WIT1*. Nonneoplastic tissues of the 3 patients retained all the *Eco*RI fragments of *WT1* and *WIT1* as evidenced by the autoradiographic signal of these fragments, which was similar to that of the respective fragments of the control tissue.

Tumor 312 had homozygous deletion of the D11S323, D11S16, D11S151 loci, and LOH in the D11S324 locus (Fig. 4). Tumor 519 had LOH in the D11S324 and

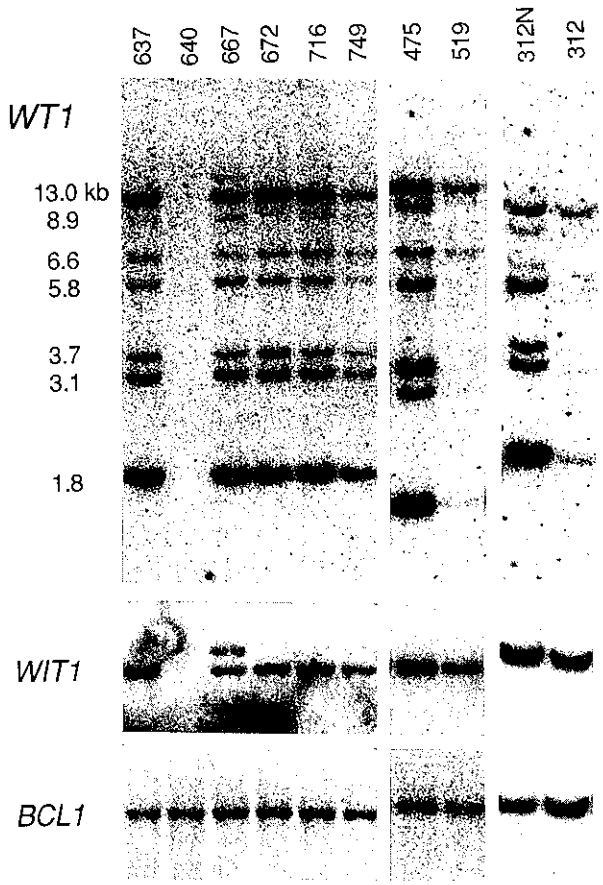

Fig. 1. Southern blots of *Eco*RI-digested DNA from 9 WTs and one normal tissue (patient 312). The same filters were successively hybridized with the WT33, GB16, and *BCL1* probes. Tumor 640 lost all the 7 *Eco*RI fragments of *WT1* and the *WIT1* fragment. Tumor 519 retained the 13.0 kb and 6.6 kb fragments and lost the other 5 *WT1* fragments, and tumor 312 retained only the 13.0 kb fragment of the *WT1* fragments. Tumor 667 showed a rearranged band in addition to the germinal bands detected by each of the *WT1* and *WIT1* probes. Tumor 749 showed hemizygous deletions of all the *Eco*RI fragments of both *WT1* and *WIT1*.

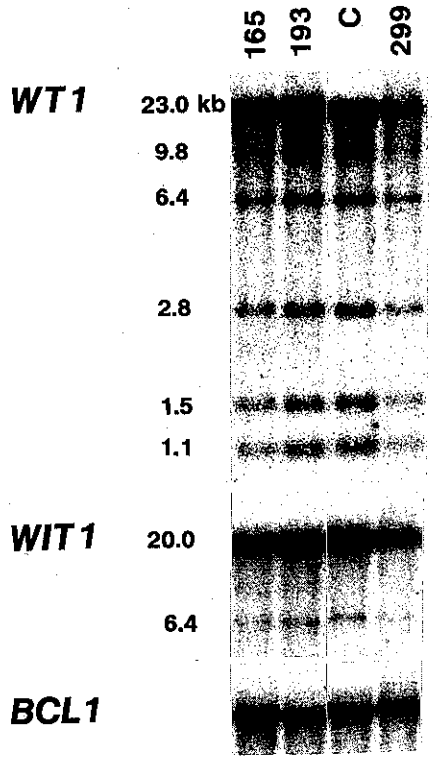

Fig. 2. Southern blots of *Bam*HI-digested DNA from 2 WAGR tumors (Nos. 165 and 299), one sporadic WT (No. 193) and one normal tissue (C). The same filters were successively hybridized with the WT33, GB16, and *BCL1* probes. Tumors 165 and 299 had the radioactivity of all the *Bam*HI fragments of *WT1* and *WIT1* decreased to half that of the normal control tissue or tumor 193.

D11S325 loci, but retained heterozygosity in the D11S16 and D11S151 loci. Tumor 640 retained heterozygosity in the D11S16, D11S324, D11S325 and *CAT* loci and had an uninformative D11S151 locus without deletion.

Sporadic WTs with hemizygous *WT1* and *WIT1* deletions

The radioactivity of the 7 *Eco*RI fragments of WT1 and the fragment of *WIT1* in 2 sporadic tumors (Nos. 392 and 749) was half that of the respective fragments of the control tissue (Fig. 1) (No. 392, data not shown in Fig. 1). The radioactivity of the *WT1* and *WIT1* fragments of nonneoplastic tissues from the 2 patients was similar to that of the control tissue.

Tumor 392 showed heterozygosity in the D11S16 and D11S151 loci, and LOH in the *CAT* locus; the radioactivity of the *CAT* fragment had reduced to half, indicating the deletion of one allele (Fig. 4). Tumor 749 showed homozygosity in the *CAT* locus with radioactivity similar to that in the control DNA, and LOH in the D11S323, D11S16, D11S151, D11S324, D11S325 loci with radioactivity half that in the control tissue, suggesting that the hemizygous deletion extended from D11S323 to D11S325.

Tumor 667 had a 20 kb *Eco*RI fragment in addition to the normal 7 *Eco*RI fragments of *WT1* (Fig. 1). The 13 and 6.6 kb *WT1* fragments exhibited radioactivity reduced to half that of the control tissue, while the other 5 fragments showed unchanged radioactivity. This tumor also had an abnormal *Eco*RI fragment of *WIT1* in addition to the normal fragment; the two fragments had almost the same radioactivity, and each fragment had half the radioactivity of that in the control tissue. The

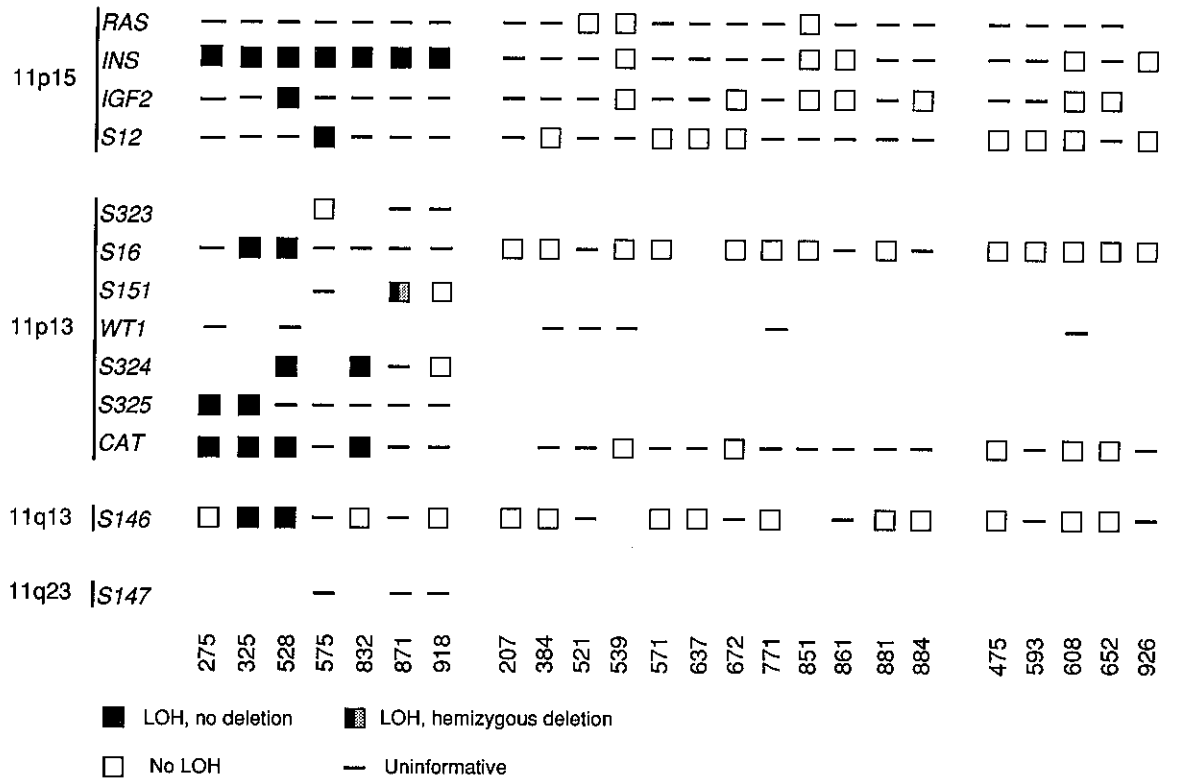

Fig. 3. LOH on 11p or 11q in 19 WTs (275–918, 207–884) and 5 non-Wilms childhood renal tumors (475–926) without *WT1* deletion.

normal tissue of patient 667 showed normal-sized *WT1* and *WIT1* bands with normal radioactivity. Since *WIT1* and exons 1 and 2 of *WT1* lie in the same 13 kb fragment,[14, 36] we assumed that a DNA fragment with an *Eco*RI site separating the 13 and 6.6 kb fragments of *WT1* was deleted from one of the 2 alleles. The D11S16, D11S324 and *CAT* loci of this tumor showed heterozygosity (Fig. 4).

WAGR tumors Both nonneoplastic and tumor tissues of the 2 WAGR patients showed the radioactivity of all the *Eco*RI or *Bam*HI fragments of *WT1* and *WIT1* decreased to half that of the normal control tissue (Fig. 2). The 2 patients had hemizygous deletion of the D11S151, D11S324 and D11S325 loci as evidenced by the radioactivity levels of these loci in both nonneoplastic and tumor tissues, which were reduced to half that of the control DNA. The 2 tumors showed heterozygosity in the *INS*, D11S12 and D11S16 loci (No. 165), or in the *INS*, *IGF2* and D11S12 loci (No. 299), and showed homozygosity in the *CAT* locus with radioactivity similar to that of the control DNA (Fig. 4). These findings indicated that the constitutional deletion extended from D11S151 to D11S325 in the 2 patients.

Sporadic WTs without *WT1* and *WIT1* deletions The remaining 33 sporadic WTs showed no *WT1* or *WIT1* alterations by Southern blot analysis. Nineteen of the 33 tumors were evaluated for the presence or absence of LOH on 11p, and the other 14 were not evaluated because no nonneoplastic normal tissue was available.

Seven of the 19 tumors showed allelic loss on 11p (Figs. 3, 5 and 6). One of the 7 (No. 871) showed LOH in the *INS* locus, and a rearranged band in addition to germinal bands in the D11S151 locus upon digestion by *Eco*RI, or *Pst*I (Fig. 7), possibly indicating deletion of a small DNA fragment in this locus.

Childhood renal tumors other than WTs The 7 tumors with histologies other than WT showed neither *WT1* nor *WIT1* deletions. Both tumor and nonneoplastic normal tissues were obtained from 5 of the 7 patients. All the 5 tumors showed heterozygosity in the 11p13 and 11p15 loci (Fig. 3). In addition, 2 tumors whose corresponding nonneoplastic tissues were not obtained, showed heterozygosity in the D11S16 locus (No. 257), or in the D11S12, D11S324, *CAT* and D11S146 loci (No. 544) (data not shown). These findings suggested absence of LOH on 11p in the 7 tumors.

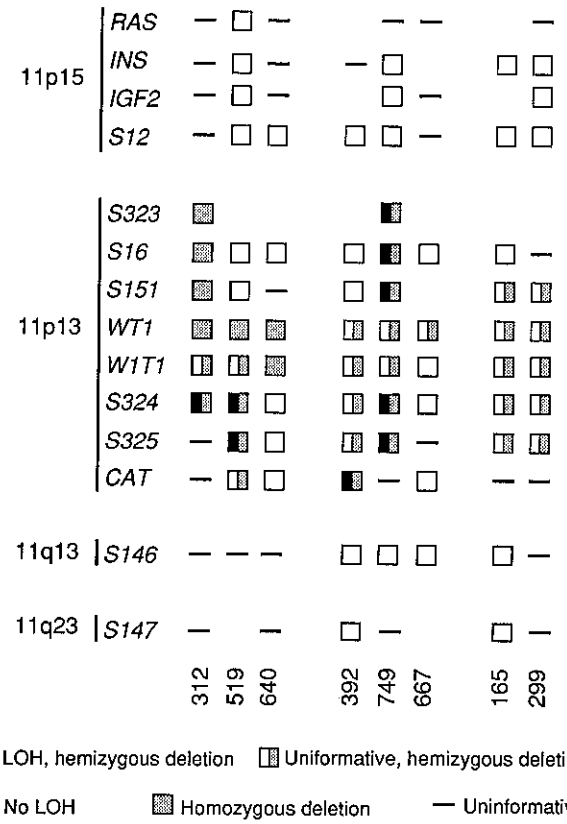

Fig. 4. LOH on 11p or 11q in 8 WTs with *WT1* deletions. Tumors 312, 519, 640, 392, 749, and 667 were from sporadic patients, and tumors 165 and 299 were from WAGR patients.

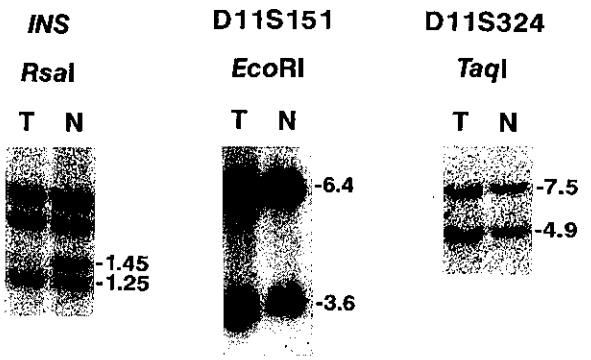

Fig. 5. Tumor 918 showed LOH in the *INS* locus, and retained heterozygosity in the D11S151 and D11S324 loci. LOH in the tumor is limited to the 11p15 region.

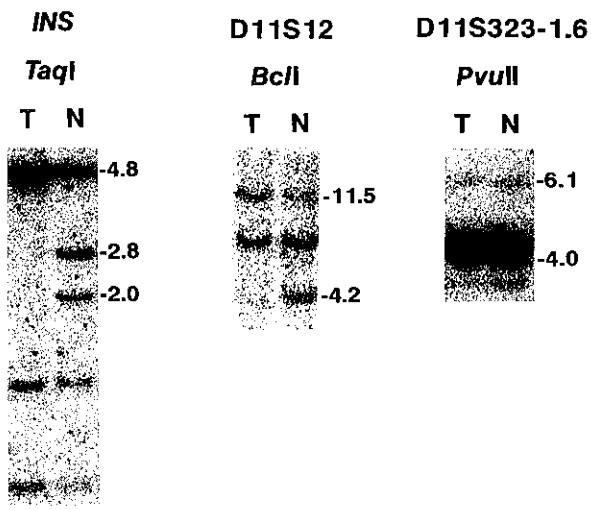

Fig. 6. Tumor 575 showed LOH in the *INS* and D11S12 loci, and retained heterozygosity in the D11S323 locus. LOH in the tumor is limited to the 11p15 region.

DISCUSSION

WT is a common pediatric solid tumor. Although the incidences of neuroblastoma, another common pediatric tumor, are almost the same in East-Asian and Caucasian children, those of WT in the 2 populations markedly differ.[23]

In the Southern blot analysis using the *WT1* probe on 39 sporadic WTs that occurred in Japanese children, we found the homozygous and hemizygous deletions of the *WT1* gene in 3 (8%) and 3 (8%) tumors, respectively. The hemizygous *WT1* deletion detected in the 3 tumors by a bioimage analyzer was confirmed by LOH of appropriate DNA markers (Nos. 392 and 749), or rearranged *WT1* and *WIT1* bands which were not shown in the normal counterpart DNA (No. 667). The previous studies on 3 series of 146 sporadic tumors which presumably arose in Caucasian children found only 4 (3%) tumors with gross homozygous deletions, and no tumors with a hemizygous deletion.[1, 3, 5] There is a significant difference in the incidence of WTs with homozygous or hemizygous *WT1* deletion between the 2 groups (chi-square test $P<0.05$). If we assume that the incidence of WT in Japanese children is one-half of that in Caucasian children, the population-based incidences of WT with the *WT1* deletion may be similar in the 2 groups.

The sizes of deletion in the 2 alleles of *WT1* were different in tumor 312 and also in tumor 519. The whole *WT1* gene was included in the large deletion of both chromosomes 11, and the difference in the sizes of deletion in the 2 alleles was not determinable in tumor 640. The *WT1* locus was intact in the nonneoplastic tissues of the 3 patients. Karyotypes of the 3 tumors were previously reported.[31] Tumor 312 had a del(11)(p13p14), tumor 640 had an inv(11)(p13q13), and tumor 519 had

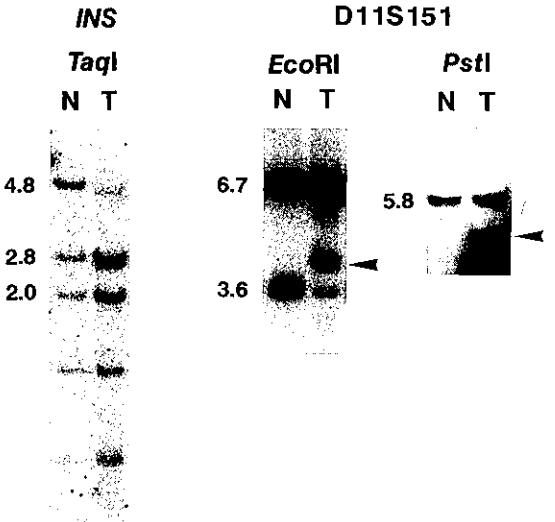

Fig. 7. Tumor 871 showed LOH in the *INS* locus; a faint 4.8 kb band represents contaminant normal tissue DNA, and a rearranged band in addition to germinal bands in the D11S151 locus with the digestion by *Eco*RI or *Pst*I. The constitutional cells showed heterozygosity by *Eco*RI digestion, and homozygosity by *Pst*I digestion. Arrowheads show rearranged bands. A faint 3.6 kb band in the tumor DNA with the *Eco*RI digestion represents contaminant normal tissue DNA. The finding suggests a small deletion in the D11S151 locus in the tumor.

no microscopic abnormality on 11p. Thus, the molecular-genetic and chromosomal findings of the 3 tumors indicate that the homozygous *WT1* deletion occurred through 2 independent somatic mutations, supporting the two-hit theory of Knudson and Strong.[37]

The chromosomal rearrangement involving 11p13 and the *WT1* deletion that occurred in both the rearranged and microscopically normal chromosomes 11 found in tumor 640 was also reported in a sporadic WT.[8]

Of the 3 tumors with a hemizygous *WT1* deletion, one (No. 392) had a del(11)(p13p14), and another (No. 749) had a normal tetraploid karyotype probably representing the karyotype of nonneoplastic cells. The other tumor (No. 667) had an abnormal karyotype with microscopically normal chromosomes 11; this finding is consistent with the molecular-genetic finding indicating a small deletion of the *WT1* gene. Two WAGR tumors had a del(11)(p12p13) (No. 165) or a t(10;11)(p13;p13) (No. 299); both had hemizygous *WT1* and *WIT1* deletions. Thus, 5 of the 8 WTs with homozygous or hemizygous *WT1* or *WIT1* deletions also showed a microscopic deletion or translocation involving the 11p13 region. These findings indicate that the microscopically detectable large chromosomal deletions play a part in the development of some WTs.

WIT1 was cloned from the 5′ flanking region of *WT1*, and its 2.5 kb transcript was altered in some WTs.[14] In the present series of WTs, the *WIT1* gene was homozygously deleted in one tumor, and hemizygously deleted in 4 sporadic and 2 WAGR tumors. The *WIT1* deletion always accompanied the *WT1* deletion. Further study is needed to clarify whether *WIT1* cooperates with *WT1* for malignant transformation of renal cells in infants and children, or whether *WIT1* was incidentally deleted because of its proximity to *WT1*.

In our series of 19 sporadic WTs having no *WT1* deletion, 7 had LOH on the short arm of chromosome 11 (Fig. 3). Two were presumed to have total deletion of a chromosome 11, and subsequent duplication of the other chromosome 11. Two showed LOH in both the *INS* (11p15) and *CAT* (11p13) loci and retained heterozygosity in the D11S146 (11q13) locus, suggesting the occurrence of somatic recombination between the *CAT* and D11S146 loci. Another tumor showed LOH in the 11p15 region and a possible small deletion in the 11p13 region, and the other 2 showed LOH in the 11p15 region and retained heterozygosity in the 11p13 region. Of the 6 sporadic tumors with *WT1* deletion, 4 retained heterozygosity in the 11p15 loci, and the other 2 had uninformative 11p15 loci; one of them retained heterozygosity in the 11p13 loci distal or proximal to *WT1* (Fig. 4). Four of the same 6 tumors showed LOH in the 11p13 region. Thus, among the 25 sporadic WTs whose corresponding normal tissues were available for LOH study, 5 (20%) had LOH in the 11p15-11p13 region, 4 (16%) had LOH limited to the 11p13 region, and only 2 (8%) showed LOH limited to the 11p15 region.

LOH in WT has been studied in various laboratories in the USA, the Netherlands, Germany and the United Kingdom.[15-20] RFLP loci on 11q were not evaluated in most tumors in these studies. Of a total of 144 sporadic tumors, 23 (16%) had LOH in the 11p15-11p13 region, 2 (1%) had LOH limited to the 11p13 region, and 18 (13%) had LOH limited to the 11p15 region. While the incidence of the LOH in the 11p15-11p13 region may be similar in Japanese and Caucasian children, the LOH limited to the 11p13 region was more common and that limited to the 11p15 region was less common in our series. Considering the lower incidence of WT in Japanese children than in Caucasian children, the difference in the population-based incidence of the LOH limited to the 11p15 region may be even more pronounced. Rare occurrences of LOH limited to the 11p15 region have just been reported in another series of Japanese WTs by Yamada and Tadokoro.[38]

We found no LOH in the 11p15 region of the tumors which developed in 2 patients with the WAGR syndrome; these patients had hemizygous *WT1* and *WIT1* deletions in the constitutional and tumor cells. Previous

studies showed LOH in the 11p15 region in 3 of the 4 WAGR tumors,[16, 39] and suggested that the two events, germinal mutations of *WT1*, and somatic mutations of *WT2*, may be necessary for the development of WT in WAGR children. Our data, however, do not support that proposal.

At least 4 mechanisms are currently thought to be responsible for the pathogenesis of WT; i.e. homozygous deletion of *WT1* as evidenced by several cases including ours,[1, 3–10] hemizygous deletion of *WT1* and homozygous mutation of *WT2* suggested in a WT studied by Haber *et al.*,[40] homozygous deletion or mutation of *WT2* suggested by micro-cell fusion studies[22] and LOH limited to the 11p15 region,[15–20] and homozygous *WT1* and *WT2* mutations suggested by LOH in the 11p15-11p13 region.[15–20] The less common occurrences of LOH limited to the 11p15 region in our and other series of Japanese WTs[38] than in Caucasian WTs suggest that homozygous *WT2* mutation unaccompanied by *WT1* mutation may be less common in Japanese WTs. Thus, the lower incidence of WT in Japanese than in Caucasian children may be related to the less common involvement of *WT2* mutation in the pathogenesis of Japanese WTs.

All tumors having *WT1* deletion or LOH on 11p were classified as typical WT or fetal rhabdomyomatous nephroblastoma. None of our 7 kidney tumors with histologies other than WT, i.e., 3 clear cell sarcomas, 2 congenital mesoblastic nephromas, one malignant rhabdoid tumor, and one renal cell carcinoma, had any genetic change on 11p. Our previous studies revealed different chromosome patterns between typical WTs and clear cell sarcomas of the kidney or congenital mesoblastic nephromas.[31] Accumulation of data on molecular-genetic and cytogenetic findings on rare non-Wilms childhood renal tumors should clarify whether these tumors develop through neoplastic processes different from those of WT.

In conclusion, the Japanese WT may be characterized by the higher incidence of *WT1* deletion and the uncommon occurrence of LOH limited to the 11p15 region. Thus, the lower incidence of WTs in Japanese children than in Caucasian children may be related to the rarer occurrence of the tumors not having *WT1* mutations, or having *WT2* mutations unaccompanied by *WT1* deletion, in the former than in the latter.

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