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A high incidence of *WT1* abnormality in bilateral Wilms tumours in Japan, and the penetrance rates in children with *WT1* germline mutation

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Background: Bilateral Wilms tumours (BWTs) occur by germline mutation of various predisposing genes; one of which is *WT1* whose abnormality was reported in 17–38% of BWTs in Caucasians, whereas no such studies have been conducted in East-Asians. Carriers with *WT1* mutations are increasing because of improved survival.

Methods: Statuses of *WT1* and *IGF2* were examined in 45 BWTs from 31 patients with *WT1* sequencing and SNP array-based genomic analyses. The penetrance rates were estimated in *WT1*-mutant familial Wilms tumours collected from the present and previous studies.

Results: We detected *WT1* abnormalities in 25 (81%) of 31 patients and two families, which were included in the penetrance rate analysis of familial Wilms tumour. Of 35 BWTs from the 25 patients, 31 had small homozygous *WT1* mutations and uniparental disomy of *IGF2*, while 4 had large 11p13 deletions with the retention of 11p heterozygosity. The penetrance rate was 100% if children inherited small *WT1* mutations from their fathers, and 67% if inherited the mutations from their mothers, or inherited or had *de novo* 11p13 deletions irrespective of parental origin ($P = 0.057$).

Conclusions: The high incidence of *WT1* abnormalities in Japanese BWTs sharply contrasts with the lower incidence in Caucasian counterparts, and the penetrance rates should be clarified for genetic counselling of survivors with *WT1* mutations.

Wilms tumour (WT; OMIM 194070) arises from the developmental kidney (Rivera and Haber, 2005). Wilms tumour and retinoblastoma are typical embryonal tumours. The *WT1* gene was altered in <25% of sporadic WTs (Haruta *et al*, 2012), whereas the *RB1* gene was shown to be altered in >90% of hereditary and non-hereditary retinoblastoma (Leiderman *et al*, 2007), indicating genetic heterogeneity and homogeneity of WT and retinoblastoma, respectively. Bilateral WT is thought to be hereditary, and the germinal mutation of *WT1* located in 11p13 and alterations of 11p15 were reported in 17–38% and 55%, respectively, of bilateral WTs in the series reported from USA, UK and Australia (Huff, 1998; Scott *et al*, 2012; Hu *et al*, 2013). Carriers with *WT1* mutations are now increasing because multidisciplinary therapies have improved the survival rates of patients with bilateral WTs and those with a unilateral WT (UWT) with a *WT1* germline mutation (Royer-Pokora *et al*, 2008; Hu *et al*, 2013). The penetrance rates of *WT1*-mutant familial WT (FWT) are needed for genetic counselling of WT survivors. However, investigators have never examined

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the incidence of *WT1* and 11p15 abnormalities in bilateral WTs of East Asian children, and have not yet tried to estimate the penetrance rates of *WT1*-mutant FWT.

WT1 is a multifunctional protein that acts as a transcriptional activator or repressor, is predominantly expressed in the embryonic kidney, and plays a pivotal role in its development (Huff, 2011). Insulin-like growth factor II (*IGF2*; OMIM 147470) is an imprinted gene expressed by the paternal allele, and encodes a foetal polypeptide growth factor (Foulstone *et al*, 2005). In contrast, *WT1* is biallelically expressed in normal foetal tissues and WTs (Little *et al*, 1992). The loss of heterozygosity (LOH) and loss of imprinting (LOI) of *IGF2* have been reported in 30–40% and 30–70% of sporadic WTs, respectively, and these alterations cause the overexpression of *IGF2*, which is involved in Wilms tumorigenesis (Schroeder *et al*, 1987; Ravenel *et al*, 2001; Haruta *et al*, 2008).

Both *WT1* and *IGF2* genes are located on the short arm of chromosome 11 (11p) and uniparental disomy (UPD) on 11p, involving either the region limited to 11p15 or that including both 11p15 and 11p13, is regularly accompanied by maternal allele loss and paternal allele duplication (Schroeder *et al*, 1987). We previously reported that small homozygous *WT1* mutations and paternal UPD (pUPD) of 11p occurred in one-third of unilateral and bilateral WTs with various *WT1* abnormalities (Haruta *et al*, 2008). Based on these genetic findings of human WT, Hu *et al* (2011) showed that the combined occurrence of the upregulation of *Igf2* and ablation of *Wt1* resulted in WT in transgenic *Wt1-Igf2* mice; however, the upregulation of *Igf2* or ablation of *Wt1* by themselves did not lead to malignant tumours .

The inheritance of *WT1* mutations have been poorly studied in FWTs, and only 13 hereditary WT families with *WT1* abnormalities have been described in the literature (Yunis and Ramsay, 1980; Kousseff and Agatucci, 1981; Nakagome *et al*, 1984; Lavedan *et al*, 1989; Pelletier *et al*, 1991; Kaplinsky *et al*, 1996; Jeanpierre *et al*, 1998; Pritchard-Jones *et al*, 2000; Shibata *et al*, 2002; Zirn *et al*, 2005; Regev *et al*, 2008; Fencl *et al*, 2012; Melchionda *et al*, 2013). In addition, the parental origins of *de novo* small *WT1* mutations and large 11p13 deletions encompassing *WT1* were reported previously in two and eight individuals, respectively (Huff *et al*, 1990; Nordenskjold *et al*, 1994). The aim of the present study was to determine the incidence rates of *WT1* and *IGF2* abnormalities in bilateral WTs in Japanese children, and was to compare the results with those reported in bilateral WTs of Caucasian children. In addition, we summarised the present and previous findings on the penetrance rate for children who inherited various types of *WT1* abnormalities from their fathers or mothers, or had *de novo WT1 (DNWT1)* abnormalities that occurred in the paternal or maternal germ cell, and tried to clarify whether parental inheritance and *WT1* abnormality types may affect the penetrance rate of hereditary WT.

MATERIALS AND METHODS

Patients and samples. Forty-five tumour samples were available from 31 Japanese infants or children with bilateral WT, ranging in age between 2 and 26 months, who underwent surgery or biopsy between August 1996 and 2011 (Table 1); 11 of the 45 tumours and 7 of the 31 patients were described in a previous series of patients with *WT1*-mutant WT (Shibata *et al*, 2002; Haruta *et al*, 2008). In one of the seven patients, data on the 11p15 status was added and shown as Bilateral Wilms tumour 23 (BWT23) (Table 1; Shibata *et al*, 2002). In addition, five patients, including one with UWT of a *DNWT1* mutation (UWTG1), one with familial and UWTG2, one with Wilms tumour–aniridia–genitourinary malformation-mental retardation (WAGR) syndrome-associated UWTG8 and two with sporadic and UWTS1 and 5 were incorporated into our previous study for a comparison of the data with those of *WT1*-mutant bilateral WTs (Table 2). Normal tissue samples were obtained from either peripheral blood (PB) or normal renal tissue adjacent to the tumour from the same patients. Tumours were staged according to the National Wilms Tumor Study Group (NWTS) staging system and most patients were treated according to the NWTS protocols (D'Angio *et al*, 1989; Oue *et al*, 2009). Malformations found in patients with bilateral WT are listed in Table 1. None of the patients in the present study showed hemihypertrophy or malformations associated with Beckwith–Wiedemann syndrome (BWS; OMIM#130650). One (BWT9) died of the disease, another (BWT27) with premature chromatid separation (PCS) syndrome died of infection (Matsuura *et al*, 2006) and 29 were alive at the last follow-up.

This study was approved by the Ethics Committee at Saitama Cancer Center, and written informed consent was obtained from parents for samples from the Japan Wilms Tumor Study Group (JWiTS; Oue *et al*, 2009). Since written informed consent was not obtained in a subset of patients collected before 2001, identifying information was removed prior to their analysis, in accordance with the Ethical Guidelines for Clinical Research enacted by the Japanese Government. The Ethics Committee approved the waiver of written informed consent for the latter samples.

Histological examination. The diagnosis of WT was made in all 45 tumours, with routine haematoxylin and eosin-stained pathology slides by pathologists at each institution or the JWiTS pathology panel according to the classification proposed by the Japanese Society of Pathology (The committee on histological classification of childhood tumors, 2008). In addition, a pathological review of 29 tissue specimens was performed by the JWiTS pathology panel.

Analysis of *WT1* and allelic loss on 11p and 11q. Copy number and LOH analysis using single-nucleotide polymorphisms (SNP) arrays, Affymetrix Mapping 50K-Xba and 250K-Nsp arrays (Affymetrix, Santa Clara, CA, USA) was conducted as described previously (Haruta *et al*, 2008). Copy numbers and LOH were calculated using CNAG and AsCNAR programmes with paired or anonymous references as controls (Nannya *et al*, 2005; Yamamoto *et al*, 2007). Gross *WT1* deletions were analysed by Southern blotting using a *WT1* cDNA probe and *BCL1* in chromosome band 11q13, or by SNP arrays or the multiplex ligation-dependent probe amplification (MLPA) method (Salsa MLPA kit, MRC-Holland, Amsterdam, the Netherlands). To detect small *WT1* mutations, defined as missense, nonsense, frame-shift or splice-site mutations, all coding exons including flanking intronic sequences of *WT1* were amplified from genomic DNA by PCR, and PCR products were directly sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

COBRA of the CTCF6 site at *H19*-DMR or MS-MLPA of the IC1 (*H19*-DMR) and IC2 (KvDMR) regions. We determined the methylation status of 11p15 region in tumour and PB samples by combined bisulfite restriction assay (COBRA; Watanabe *et al*, 2006) and/or methylation specific (MS)-MLPA (Salsa MS-MLPA kit, ME030BWS/SRS) assay. Combined bisulfite restriction assay of CTCF6 at *H19*-differentially methylated region (*H19*-DMR) showed that the mean methylation percentage ± 2 s.d. of five normal kidney and two PB samples was $53.6 \pm 5.6\%$, and we defined more than the mean percentage $+2$ s.d. as the hypermethylated state. Methylation specific-MLPA analysis was used to detect the methylation status of the IC1 (*H19*-DMR) and IC2 (KvDMR) regions. The methylation statuses were defined according to the manufacturer's instructions.

Statistical analysis. Differences in the incidence of clinical and genetic characteristics between any two genetic subtypes of

Table 1. Genetic and epigenetic alterations in *IGF2* (11p15) and *WT1* (11p13) loci in 31 patients with bilateral Wilms tumours

Tumour	Age/sex	11p15 SNP	*H19*-DMR	*IGF2* Status	*WT1* allele 1	*WT1* allele 2	Other CGH changes	Anomaly/reference/heredity
BWT1R	1 year 1 month/F	UPD	Hypermethyl. (C)	pUPD	ex 1, 370C>T/G124X	The same as allele 1, UPD11pt-11cen	del(7)(q11.1q21.11)	None
BWT2L	1 year 2 months/F	ROH	Normal Methyl. (C)	ROI	Deletion of 21.8 Mb	Deletion spanning exons 4–9	None	WAGR syndrome/No. 1[a]
BWT2R	—	ROH	Normal methyl. (C)	ROI	Deletion of 21.8 Mb	ex 7, 927-956del30ins5/T309fs379X	None	WAGR syndrome/No. 16[a]
BWT3R	1 year 2 months/M	UPD	Hypermethyl. (C, M)	pUPD	Deletion in ex 1∼5	The same as allele 1, UPD11pt-11cen	UPD3pter-3p14.2,7p-q+	None/No. 2[a]
BWT4L	12 months/M	UPD	Hypermethyl. (C)	pUPD[b]	ex 9, 1168C>T/R390X	The same as allele 1, UPD (MLPA)	NE	None
BWT4R	—	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 9, 1168C>T/R390X	The same as allele 1, UPD, 11pt-p13	7p-q+	None
BWT5L	9 months/M	UPD	Hypermethyl. (C, M)	pUPD	ex 9, 1168C>T/R390X	The same as allele 1, UPD 11pter-p12	None	Hypospadia/cryptorchidism/No. 26[a]
BWT6R	12 months/M	UPD	Hypermethyl. (C, M)	pUPD	IVS1+1G>A	The same as allele 1, UPD (MLPA)	NE	None
BWT7R	7 months/F	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 1, 268delA/S90Afs128X	The same as allele 1, UPD11pt-11cen	None	None
BWT8R	2 months/M	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 3, 646delC/L216Cfs2X	The same as allele 1, UPD11pt-11cen	1q+	None
BWT9L	1 year 1 month/M	ROH	Normal Methyl. (C)	ROI	ex 1, 172delC/P58Rfs32X	Deletion of 1.6 Mb	None	Urogenital anomaly/No. 19[a]
BWT9R	—	UPD	Hypermethyl. (C, M)	pUPD	ex 1, 172delC/P58Rfs32X	The same as allele 1, UPD11pt-11cen	UPD17q21.33-qter	Urogenital anomaly/No. 31[a]
BWT10L	1 year 2 months/F	UPD	Hypermethyl. (C)	pUPD[b]	ex 9, 1186G>A/D396N	The same as allele 1, UPD11pt-11p12	None	Drash syndrome/No. 32[a]
BWT10R	—	UPD	Hypermethyl. (C)	pUPD[b]	ex 9, 1186G>A/D396N	The same as allele 1, UPD11pt-11cen	UPD3pter-p21.33	Drash syndrome/No. 33[a]
BWT11L	12 months/F	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 7, 938C>A/S313X	The same as allele 1, UPD11pt-11cen	None	None
BWT11R	—	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 7, 938C>A/S313X	The same as allele 1, UPD (MLPA)	NE	None
BWT12R	11 months/F	UPD	Hypermethyl. (M)	pUPD	ex 6, 818C>G/Y271X	The same as allele 1, UPD11pt-11cen	None	None
BWT13L	12 months/M	UPD	Hypermethyl. (M)	pUPD	IVS2-1G>A	The same as allele 1, UPD11pt-11cen	UPD3pt-3p21	None
BWT13R	—	UPD	Hypermethyl. (M)	pUPD	IVS2-1G>A	The same as allele 1, UPD11pt-11p13	None	None
BWT14L	11 months/F	UPD	Hypermethyl. (M)	pUPD[b]	ex 9, 1180C>T/R394W	The same as allele 1, UPD11pt-11cen	None	Drash syndrome
BWT15L	9 months/M	UPD	Hypermethyl. (C, M)	pUPD	ex 8, deletion	The same as allele 1, UPD11pt-11cen	None	None
BWT15R	—	UPD	Hypermethyl. (C, M)	pUPD	ex 8, deletion	The same as allele 1 (UPD)/11pt-p13	None	None
BWT16L	12 months/M	UPD	Hypermethyl. (C)	pUPD	ex 8, 1084C>T/R362X	The same as allele 1, UPD11pt-11cen	None	None
BWT17	12 months/M	UPD	Hypermethyl. (C)	pUPD	ex 1, 97_98ins5/Q32Rfs59X	The same as allele 1, UPD11pt-11cen	7p-q+	None
BWT18L	11 months/F	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 4, 714G>A/W238X	The same as allele 1, UPD11pt-11cen	None	None
BWT18R	—	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 4, 714G>A/W238X	The same as allele 1, UPD11pt-11p12	UPD3pt-3p21	None
BWT19R	1 year 2 months/F	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 4, 682delC/Q228Kfs2X	The same as allele 1, UPD11pt-11cen	None	None
BWT20L	1 year 1 month/F	UPD	Hypermethyl. (C, M)	pUPD	ex 9, 1168C>T/R390X	The same as allele 1, UPD11pt-11p13	None	None
BWT20R	—	UPD	Hypermethyl. (C, M)	pUPD	ex 9, 1168C>T/R390X	The same as allele 1, UPD11pt-11cen	None	None
BWT21R	10 months/F	UPD	N. D.	UPD	ex 9, 1180C>T/R394W	The same as allele 1, UPD (MLPA)	NE	Drash syndrome
BWT22R	2 years 2 months/M	ROH	Normal Methyl. (C)	ROI[b]	Deletion of 3.2 Mb	IVS6+1-IVS6+3del3	3p-,4q-,7p-	Hypospadia/cryptorchidism
BWT23L	7 months/F	UPD	Hypermethyl. (C, M)	pUPD	ex 9, 1168C>T/R390X[c]	The same as allele 1, UPD11pt-11p12	None	OD, FGS/No. 5[d]//FWT1
BWT23R	—	UPD	Hypermethyl. (C, M)	pUPD	ex 9, 1168C>T/R390X[c]	The same as allele 1, UPD11pt-11cen	None	OD, FGS/No. 5[d]/FWT1
BWT24R	1 year 8 months/F	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 1, 144C>A/Y48X[c]	The same as allele 1, UPD11pt-11cen	7p-,7p-q+, 9q-	*de novo* mutation/No. 36[a,e]/DNWT9-2
BMT25L	1 year 7 months/F	UPD	Hypermethyl. (C, M)	pUPD[b]	ex 9, 1168C>T/R390X[c]	The same as allele 1, UPD11pt-11cen	1q+	None/FWT2-2

Table 1. (Continued)

Tumour	Age/sex	11p15 SNP	*H19*-DMR	*IGF2* Status	*WT1* allele 1	*WT1* allele 2	Other CGH changes	Anomaly/reference/heredity
BWT26L	7 months/M	ROH	Normal Methyl. (C)	ROI	Wild type	Wild type	UPD1q	None
BWT26R	—	ROH	Normal Methyl. (C)	ROI	Wild type	Wild type	UPD1q	None
BWT27R	10 months/M	UPD	Hypermethyl. (C, M)	pUPD	Wild type	Wild type	+2,UPD11,+12,+17,+18	PCS syndrome
BWT28L	1 year 1 month/M	ROH	Hypermethyl. (C)	LOI	Wild type	Wild type	Normal	None
BWT28R	—	ROH	Hypermethyl. (C)	LOI	Wild type	Wild type	+6,+12	None
BWT29	9 months/F	ROH	Hypermethyl. (C, M)	LOI[b]	Wild type	Wild type	1q+,4p+,+7,7q-,UPD8, UPD9,+10,+12,15q+,+20, UPD22	None
BWT30L	1 year 5 months/F	UPD	Hypermethyl. (C, M)	pUPD[b]	Wild type	Wild type	+8,+10,UPD11,+12	Coarctation of the aorta
BWT30R		ROH	Normal Methyl. (C)	ROI[b]	Wild type	Wild type	UPD2,+5,+7,+8, -10,+12,+18	Coarctation of the aorta
BWT31L	1 year 2 months/F	UPD	Hypermethyl. (C)	pUPD	Wild type	Wild type	1q+,+3,+6,+8, +9,UPD11pt-11cen, +12,+13,16q	None
BWT31R		ROH	Normal Methyl. (C)	ROI	Wild type	Wild type	Normal	None

Abbreviations: BWT = bilateral Wilms tumour; C = methylation analysis of CTCF6 by COBRA; CGH = comparative genomic hybridisation; COBRA = combined bisulfite restriction assay; DMR = differentially methylated region; ex = exon; F = female; FGS = focal glomerular sclerosis; FWT1 = familial WT1; LOI = loss of imprinting; M = male; M, methylation analysis of H19-DMR by MS-MLPA; methyl. = methylation; MLPA = multiplex ligation-dependent probe amplification; ; NE = not examined; PB = peripheral blood; PCS = premature chromatid separation; pUPD = paternal *IGF2* UPD; ROH = retention of heterozygosity; ROI = retention of *IGF2* imprinting; SNP = single-nucleotide polymorphism; UPD = uniparental disomy; WAGR = Wilms tumour – aniridia – genitourinary malformation-mental retardation. See Table 3 for FWT1.

[a]Case numbers reported by Haruta *et al*, 2008.

[b]Above pUPD, LOI or ROI indicates that normal methylation pattern at *H19*-DMR was identified in PB by COBRA.

[c]Germline origin of the mutation was confirmed.

[d]Case number reported by Shibata *et al*, 2002.

[e]Stage was corrected from I to V in the present Table 1.

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Table 2. Genetic and epigenetic alterations in *IGF2* (11p15) and *WT1* (11p13) loci in 10 syndromic or familial and 20 sporadic patients with unilateral Wilms tumours

Tumour	Age/sex	Stage	*IGF2*	*WT1* allele 1	*WT1* allele 2	Malformations/heredity	References
Patients with syndromic or familial unilateral Wilms tumours ($n=10$)							
UWTG1	1 year 6 months/F	I	UPD[a]	ex 1, 144C>A/Y48X[b]	The same as allele 1, UPD11pt-11cen	None/*de novo* mutation (DNWT9-1)	The present study
UWTG2	1 year 4 months/M	I	pUPD (C)	ex 9, 1168C>T/R390X[b]	The same as allele 1, UPD11pt-11cen	Hypospadia/familial WT (FWT2-1)	The present study
UWTG3	12 months/F	I	pUPD (C)	ex 8, 1604G>A/C355Y	The same as allele 1, UPD11pt-11cen)	Drash syndrome	No. 29 (Haruta *et al*, 2008)
UWTG4	1 year 9 months/F	III	pUPD (C)	ex 3, 665_666delinsT/S222fs299X	The same as allele 1, UPD11pt-11cen	Hermaphroditism	No. 35 (Haruta *et al*, 2008)
UWTG5	9 months/F	II	pUPD (C)	ex 2, 549C>T/Q184X[b]	The same as allele 1, UPD11pt-11cen	None/None	No. 25 (Haruta *et al*, 2008)
UWTG6	1 year 7 months/F	III	ROH/ROI (C)	Deletion of 2.4 Mb	ex 10, 1297–1298insT/R433fsNo-stop	WAGR syndrome	No. 20 (Haruta *et al*, 2008)
UWTG7	5 months/F	I	ROH/LOI (C)	Deletion of 6.5 Mb	ex 9, 1180C>G/ R394G	WAGR syndrome	No. 14 (Haruta *et al*, 2008)
UWTG8	1 year 3 months/M	I	ROH/ROI (C)	Deletion spanning exons 1–10	ex 4, 721_730del10/M309fs313X	WAGR syndrome	The present study
UWTG9	1 year 3 months/M	I	ROH/ROI (C)	Deletion of 4.4 Mb	ex 7, 938-939ins8/S313fs380X	WAGR syndrome	No. 17 (Haruta *et al*, 2008)
UWTG10	1 year 3 months/F	III	ROH/ROI (C)	Deletion of 14.4 Mb	ex 7,938C>A/S307X	WAGR syndrome	No. 18 (Haruta *et al*, 2008)
Patients with sporadic and unilateral Wilms tumours ($n=20$)							
UWTS1	1 year 5 months/F	I	pUPD (C)	ex 1, 231_232insC/W78fs53X	The same as allele 1, UPD11pt-11cen	None/None	The present study
UWTS2	1 year 4 months/F	IV	pUPD (C)	ex 8, 1068_1069ins23/F357fs380X	The same as allele 1, UPD11pt-11cen	None/None	No. 34 (Haruta *et al*, 2008)
UWTS3	9 months/F	I	pUPD (C)	ex 1, 204_206delinsCC/P68fs162X	The same as allele 1, UPD11pt-11cen	None/None	No. 27 (Haruta *et al*, 2008)
UWTS4	9 months/M	I	pUPD (C)	ex 7, 1021C>T/Q341X	The same as allele 1, UPD11pt-11cen	None/None	No. 28 (Haruta *et al*, 2008)
UWTS5	1 year 3 months/M	II	pUPD (C)	ex 8, 1084C>T/R362X[c]	The same as allele 1, UPD11pt-11cen	None/None	The present study
UWTS6	4 years 8 months/F	III	pUPD 11p15 (C)	Deletion of 3.9 Mb	Deletion spanning exons 3–7	None/None	No. 13 (Haruta *et al*, 2008)
UWTS7	3 years 5 months/M	III	ROH/ROI (C)	Deletion of 3.3 Mb	Deletion spanning exons 4–10	None/None	No. 12 (Haruta *et al*, 2008)
UWTS8	3 years 1 month/M	III	ROH/ROI (C)	Deletion of 1.3 Mb	Deletion spanning exons 1–10	None/None	No. 11 (Haruta *et al*, 2008)
UWTS9	1 year 11 months/M	II	ROH/ROI (C)	Deletion of 13.1 Mb[c]	ex 2, 538_539insTC/K177fs299X	None/None	No. 21 (Haruta *et al*, 2008)
UWTS10	1 year 3 months/F	II	Monosomy 11 (C)	Loss of chromosome 11	Promoter methylation	None/None	No. 3 (Haruta *et al*, 2008)
UWTS11	1 year 11 months/M	III	ROH/ROI (C)	Deletion of 1.8 Mb	Deletion spanning exons 1–5	None/None	No. 7 (Haruta *et al*, 2008)
UWTS12	2 years 5 months/M	III	ROH/ROI (C)	Deletion of 3.1 Mb	Deletion spanning exons 6–10	None/None	No. 9 (Haruta *et al*, 2008)
UWTS13	2 years 3 months/M	I	ROH/ROI (C)	Deletion of 9.1 Mb	Deletion spanning exons 2–5	None/None	No. 8 (Haruta *et al*, 2008)
UWTS14	5 years 7 months/F	II	ROH/ROI (C, P)	Deletion of 21.4 Mb	ex 7, 895G>T/D299Y	None/None	No. 24 (Haruta *et al*, 2008)
UWTS15	2 years 7 months/M	I	ROH/ROI (C)	Deletion of 3.5 Mb[c]	ex 10, 1291_1297delins8/L401fsNo-stop	None/None	No. 23 (Haruta *et al*, 2008)
UWTS16	1 year 9 months/M	IV	ROH/ROI (C, P)	Deletion of 4.5 Mb	Deletion spanning exons 2–5	None/None	No. 6 (Haruta *et al*, 2008)
UWTS17	10 months/F	I	ROH/ROI (C, P)	Deletion of 18.5 Mb	ex 3, 588_590dell/P197fs229X	None/None	No. 15 (Haruta *et al*, 2008)
UWTS18	1 year 7 months/M	II	ROH/ROI (C)	Deletion of 2.7 Mb	Deletion spanning exons 1–10	None/None	No. 5 (Haruta *et al*, 2008)
UWTS19	2 years 4 months/M	III	ROH/ROI (C)	Deletion of 10.8 Mb[c]	ex 2, 483_484insCA/H162fs229X	None/None	No. 22 (Haruta *et al*, 2008)
UWTS20	1 year 6 months/F	II	pUPD 11p15 (C)	Deletion of 2.8 Mb	Deletion spanning exons 1–10	None/None	No. 4 (Haruta *et al*, 2008)

Abbreviations: C = methylation analysis of CTCF6 at *H19*-DMR by COBRA; COBRA = combined bisulfite restriction assay; ex = exon; F = female; P = allelic expression analysis on the polymorphic site at exon 9 of *IGF2*; LOI = loss of *IGF2* imprinting; M = male; pUPD = paternal *IGF2* UPD; ROH = retention of heterozygosity on 11p15; ROI = retention of *IGF2* imprinting; UPD = uniparental disomy; WAGR = Wilms tumour–aniridia–genitourinary malformation-mental retardation syndrome. See Table 3 for DNWT1-1 = FWT2-1.

[a]Above UPD indicates that the methylation status of *H19*-DMR was not examined.

[b]Germline origin of the mutation was confirmed.

[c]Somatic origin of the mutation was confirmed.

tumours were examined by the χ^2 or Fisher's exact test. $P<0.05$ (two-sided) was considered statistically significant. The Student's *t*-test or Mann–Whitney test was used to compare mean ages between patients with *WT1*-mutant bilateral WT and those with *WT1*-mutant sporadic and UWT, between patients with sporadic and UWT with a small *WT1* mutation and those with sporadic and UWT with a large *WT1* deletion, or between patients with paternal inheritance of a small *WT1* mutation and those with maternal inheritance of a small *WT1* mutation.

RESULTS

Bilateral *WT1*-mutant WTs with or without pUPD on 11p. Of 45 bilateral tumours from 31 patients, 35 tumours from 25 (81%) patients had *WT1* abnormalities; 30 tumours from 21 patients showed small mutations in 1 *WT1* allele and the same small mutation in the other *WT1* allele, caused by UPD on 11p (Table 1). One tumour (BWT3R) with deletion flanking exons 1–5 also had UPD on 11p, and was added to the above 30 tumours because of the small deletion. Combined bisulfite restriction assay and/or MS-MLPA identified hypermethylation of *H19*-DMR, which indicated the paternal origin of *IGF2* UPD in all 31 tumours except 1 whose tumour DNA was not available (BWT21R). The remaining four tumours from three patients had large deletions encompassing *WT1* in one *WT1* allele and a frame-shift or splice-site mutation (three tumours; BWT2R, 9L and 22R) or a small deletion spanning exons 4–9 (one tumour; BWT2L) in the other *WT1* allele. All four tumours had the retention of heterozygosity (ROH) on 11p, and had normally methylated CTCF6 at *H19*-DMR, indicating retention of *IGF2* imprinting (ROI). The methylation status of CTCF6 at *H19*-DMR in PB was examined in 13 of the 25 patients. All 13 patients showed the normal methylation in PB, indicating somatic origin of UPD on 11p15.

***WT1*-mutant UWTs with or without pUPD on 11p.** To compare *WT1* and *IGF2* statuses between 35 *WT1*-mutant bilateral WTs and 10 *WT1*-mutant syndromic or familial UWTs or 20 *WT1*-mutant sporadic and UWTs, we combined our published and unpublished data on *WT1*-mutant WTs and presented them in Table 2. One (BWT21R) of the 35 WTs and one (UWTG1) of the 10 WTs, in which 11p UPD was identified by SNP array but methylation status of *H19*-DMR was not examined, were included in each group of tumours with small *WT1* mutation and pUPD of *IGF2* because all 30 *WT1*-mutant bilateral WTs with 11p UPD examined in the present study showed the hypermethylation indicating paternal 11p UPD, and the previous study indicated loss of maternal 11p allele in WTs with 11p LOH (Schroeder *et al*, 1987). Of the 10 patients, 5 (UWTG1-5) had small homozygous *WT1* mutations and pUPD on 11p, whereas 5 (UWTG6-10) associated with WAGR syndrome had large deletions in 1 *WT1* allele and small mutations in the other *WT1* allele with *IGF2* ROH/ROI in the tumours (Table 2). Of the 20 patients, 5 (UWTS1-5) had small homozygous *WT1* mutations and pUPD on whole 11p in the tumours, whereas none of the remaining 15 (UWTS6-15) had the small *WT1* mutations and pUPD on 11p; 12 had *IGF2* ROI, 2 had pUPD limited to the 11p15 region and 1 had monosomy 11 of paternal origin in the tumours (Table 2). Thus, a homozygous *WT1* mutation with pUPD on 11p was more frequent in 35 *WT1*-mutant bilateral WTs than in 10 *WT1*-mutant syndromic or familial UWTs ($P=0.017$) or 20 *WT1*-mutant sporadic and UWTs ($P=3.0E-06$).

When we analysed the 3 groups of patients with *WT1*-mutant WTs, the mean age of 25 patients with bilateral WT was 12.4 months, that of 10 patients with syndromic or familial and UWT was 14.3 months and that of 20 patients with sporadic UWT was 25.6 months. The 25 and 10 patients were younger than the 20 patients, respectively ($P=0.001$ and $P=0.006$), whereas no difference in age was found between the 25 and 10 patients ($P=0.286$). When we selected the 20 patients with sporadic and UWTs, the mean age of 5 patients with homozygous *WT1* mutations and paternal 11p UPD was 13.2 months and that of 15 patients with large deletions encompassing *WT1* with or without pUPD limited to the 11p15 region in the tumours was 29.7 months. The 5 patients with homozygous *WT1* mutations and pUPD on 11p were younger than the 15 patients with large 11p13 deletions ($P=0.002$).

***WT1*-wild-type bilateral WTs and the *IGF2* status.** Ten bilateral tumours from six patients had wild-type *WT1*; six tumours had +12, and 2 had 1q+, +6, +8. or pUPD of whole chromosome 11 and one had pUPD of 11p (Table 1). None of the six patients showed characteristics of BWS. Single-nucleotide polymorphisms analysis and COBRA of CTCF6 at *H19*-DMR and/or MS-MLPA revealed pUPD on 11p or whole chromosome 11 in three tumours, ROI of *IGF2* in four and LOI of I*GF2* in three (Table 1). Of three tumours with pUPD on 11p or whole chromosome 11, the corresponding PB showed normal methylation at *H19*-DMR in one (BWT30L) and was not available in two; in one of the two, the contralateral tumour had ROH/ROI (BWT31R), denying the constitutional 11p15 UPD (BWT31L). Of three tumours with LOI of *IGF2*, the corresponding PB showed normal methylation at *H19*-DMR in one (BWT29) and was not available in two bilateral WTs (BWT28L, 28R) from one patient, not denying the possibility of constitutional hypermethylation of maternal *H19*-DMR (Table 1).

Of 27 tumours, whose methylation status at IC2 was examined by MS-MLPA, 26 and 1 showed hypomethylation and normal methylation, respectively; the results were consistent with pUPD and LOI of 11p15, respectively.

Histology of bilateral and FWTs. Of 29 *WT1*-mutant bilateral WTs, which were available for pathological review, 25 and 4 tissue specimens were obtained before and after chemotherapy, respectively. Of the 25 tumours, 12 were classified as the mesenchymal type; 4 and 2 of the 12 also had intra-lobar nephrogenic rests (ILNR) and foetal rhabdomyomatous nephroblastoma, respectively, 11 were classified as the mixed type; 1 also had ILNR, 1 was classified as nephroblastoma with ILNR and 1 was classified as ILNR only. Of 4 specimens obtained after chemotherapy, 3 were classified as the mesenchymal type; one also had ILNR, and one as nephroblastoma with ILNR.

Of seven *WT1*-wild-type bilateral WTs from six patients, which were available for pathological review, five and two specimens were obtained before and after chemotherapy, respectively. Of the five tumours, two (BWT28, and 29) were classified as the epithelial type, two (BWT26 and 30) as the mixed type and one (BWT27) as the mesenchymal type. While the mesenchymal and mixed types were found in both *WT1*-mutant and *WT1*-wild-type bilateral WTs, the epithelial type was only found in *WT1*-wild-type bilateral WTs. Two specimens obtained after chemotherapy showed either the mixed or mesenchymal type. None of the tumour specimens from 31 patients exhibited features of focal or diffuse anaplasia.

The penetrance rates of WTs with inherited or *DNWT1* abnormalities, classified according to parental inheritance and *WT1* abnormality types. We summarised 22 children from 14 families who inherited *WT1* mutation/deletion from their mothers or fathers, and 8 children who had *de novo* large deletion encompassing *WT1* of known parental germ cell origin from the present study and literatures listed in PubMed. The 22 individuals included 3 patients with WTs (FWT4-1, 4-3 and FWT12-1; Figure 1, Table 3); although molecular analyses have not been done in their PB and tumour samples, the pedigrees showed that the 3 patients who developed WT were thought to inherit *WT1* mutations/deletions from their father or mother.

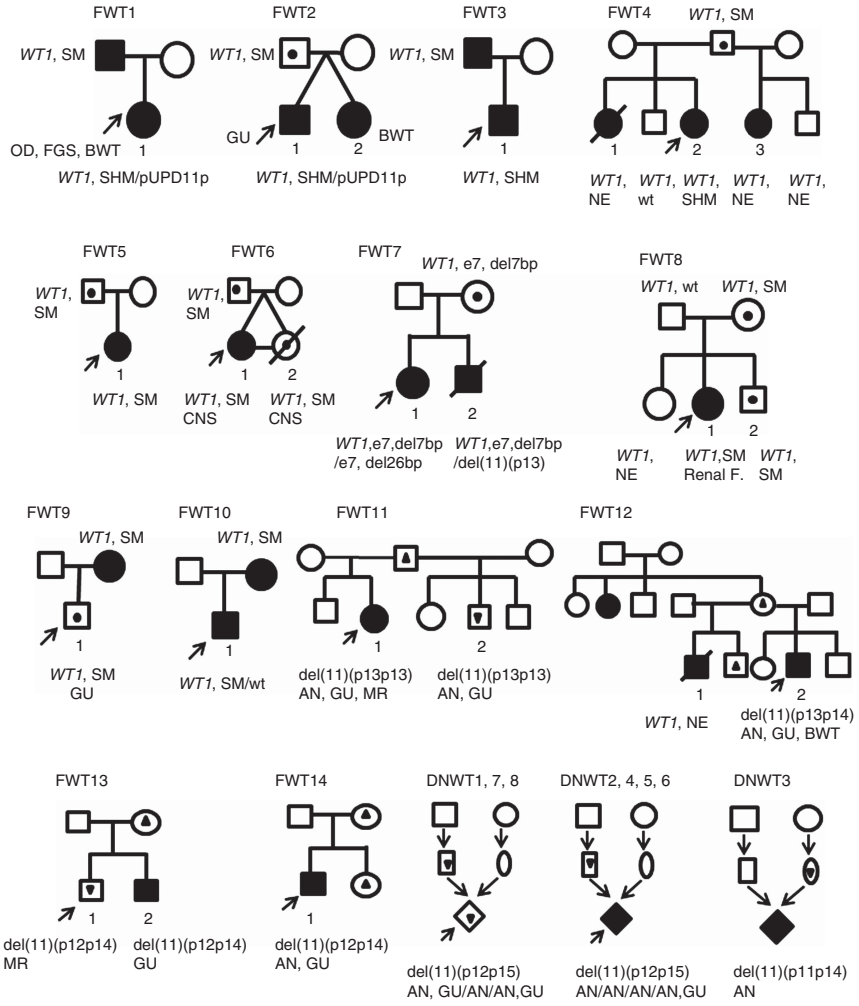

Figure 1. Pedigrees of patients with WTs with small *WT1* germline mutations inherited from fathers (FWT1-6) and mothers (FWT7-10), and those of patients with large 11p13 deletions encompassing *WT1* inherited from fathers (FWT11) or mothers (FWT12-14). DNWT1-8 indicates eight families having the *de novo* deletion of paternal or maternal germ cell origin. Open boxes and circles containing a small circle indicate males and females, respectively, with germinal *WT1* mutations who did not develop WT. Filled boxes and circles indicate males and females, respectively, who developed WT. WT6-2 was excluded from the penetrance analysis because of the reason described in the text (Fencl *et al*, 2012). Open boxes and circles with an upright triangle indicate males and females, respectively, with a balanced chromosomal insertion involving the 11p13 band. Open boxes with an inverted triangle indicate males with the deletion who did not develop WT. Oblongs and ovals indicate paternal and maternal germ cells, respectively. Filled and open diamonds indicate males and females, who developed and did not develop WT, respectively.
Abbreviations: AN = aniridia; CNS = congenital nephrotic syndrome; FGS = focal glomerular sclerosis; GU = genitourinary malformation; MR = mental retardation; NE = not examined; renal F = renal failure; SM = small mutation; SHM = small homozygous mutation; wt = wild type.

Identical twins (DNWT9-1, 2) in this study shared the same nonsense mutation in PB and tumour samples (Table 3). Because their parents had no *WT1* mutation in PB, SNP array analysis was performed on 10 polymorphic markers around *WT1* to identify the parental origin of the *DNWT1* mutation using PB from the parents and twins as well as tumour samples. Because the maternally derived loci identified by SNP markers (SNP A 1946935, 2241668, 4231943 and seven others) were lost in the tumour with UPD on 11p, the paternal germ cell origin of the mutation was determined (Table 3). In addition, two patients (DNWT10, 11) were reported to have small homozygous *WT1* mutations of paternal germ cell origin in tumours (Nordenskjold *et al*, 1994). These four patients with small *DNWT1* mutations of paternal germ cell origin (DNWT9-1, 9-2, 10 and 11) were excluded from the penetrance analysis, because unaffected carriers with small *DNWT1* mutations could not be evaluated. In contrast, eight children with large *de novo* 11p13 deletions (DNWT1-8) were included in the analysis as carriers without development of WT could be evaluated (Figure 1; Huff *et al*, 1990).

All nine patients who inherited a small *WT1* mutation from their fathers developed WT; a girl (FWT6-2) was excluded from the penetrance analysis because she died of renal failure at the age of 23 weeks before the possible development of WT (Pelletier *et al*, 1991; Kaplinsky *et al*, 1996; Jeanpierre *et al*, 1998; Shibata *et al*, 2002; Fencl *et al*, 2012). Of nine WTs with the paternal inheritance of *WT1* mutation, four tumours [FWT1 (BWT23L, 23R), 2-1, 2-2] in this study showed homozygous *WT1* mutations and pUPD on 11p, and two (FWT3, 4-2) showed homozygous *WT1* mutations, suggesting that the six tumours may have had the same *WT1* and *IGF2* abnormalities (Figure 1 and Table 3).

Of 6 individuals who inherited small *WT1* mutations from their mothers, 4 developed WT; in addition to the small germline mutation in 1 allele 1 had a 26 base-pair deletion that differed from the first mutation (FWT7-1), 1 had a large 11p13 deletion (FWT7-2), 1 had a wild-type *WT1* (FWT10) in the other allele in their tumours and the *WT1* status in the tumour was not examined in the last patient (FWT8-1; Pritchard-Jones *et al*, 2000; Zirn *et al*, 2005; Regev *et al*, 2008; Melchionda *et al*, 2013). Thus, all three

Table 3. Genetic and epigenetic alterations in *IGF2* (11p15) and *WT1* (11p13) loci in 34 patients with Wilms tumours of familial or *de novo WT1* mutations

Tumour	Age/sex	*IGF2*	*WT1* allele 1	*WT1* allele 2	Malformations/ laterality	Heredity	References
Children inherited small *WT1* mutations from their fathers ($n=9$)							
FWT1	7 months/F	pUPD (C, M)	ex 9, 1168C>T/R390X	The same as allele 1, UPD11pt-11cen	OD, FGS/BWT	Father had a *WT1* mutation and a history of WT	Present study (BWT23)
FWT2-1	1 year 4 months/M	pUPD (C)	ex 9, 1168C>T/R390X	The same as allele 1, UPD11pt-11cen	GU/UWT	Father had a *WT1* mutation and no history of WT	Present study (UWTG2)
FWT2-2	1 year 7 months/F	pUPD (C)	ex 9, 1168C>T/R390X	The same as allele 1, UPD11pt-11cen	None/BWT	Father had a *WT1* mutation and no history of WT	Present study (BWT25)
FWT3	3 years/M	NE	ex 6, 847delG/H291fs306X	LOH at *WT1* sequences	None/UWT	Father had a *WT1* mutation and a history of WT	Pelletier *et al*, 1991
FWT4-1	<12 months/F	NE	NE	NE	None/UWT	Father had a WT1 mutation and no history of WT	Kaplinsky *et al*, 1996
FWT4-2	4 months/F	NE	ex 8, 1084C>G/R302X	LOH at *WT1* sequences	None/BWT	Father had a *WT1* mutation and no history of WT	Kaplinsky *et al*, 1996
FWT4-3	6 months/F	NE	NE	NE	None/WT	Father had a WT1 mutation and no history of WT	Kaplinsky *et al*, 1996
FWT5	1 year 4 months/F	NE	ex 3, S223N	NE	None/UWT	Father had a *WT1* mutation and no history of WT	Jeanpierre *et al*, 1998
FWT6-1	1 year 1 month/F	NE	ex 8, R366P	NE	CNS/UWT	Father had a *WT1* mutation and no history of WT	Fencl *et al*, 2012
FWT6-2[a]	5 months	NR	ex 8, R366P	NR	CNS	Father had a *WT1* mutation and no history of WT	Fencl *et al*, 2012
Children inherited small *WT1* mutations from their mothers ($n=6$)							
FWT7-1	2 years/F	NE	ex 7, del7bp/H319fs377X	ex 7, 26 bp deletion	None/UWT	Mother had a *WT1* mutation and no history of WT	Pritchard-Jones *et al*, 2000
FWT7-2	4 years/M	NE	ex 7, del7bp/H319fs377X	del(11)(p13p13)	None/UWT	Mother had a *WT1* mutation and no history of WT	Pritchard-Jones *et al*, 2000
FWT8-1	9 years/F	NE	ex 10, 1730A>G/X450W	NE	Renal failure/UWT	Mother had a *WT1* mutation and no history of WT	Zim *et al*, 2005
FWT8-2	17 years/M	NR	ex 10, 1730 A>G/X450W	NR	None	Mother had a *WT1* mutation and no history of WT	Zim *et al*, 2005
FWT9	6 years/M	NR	ex 1, 328C>A/Y109X	NR	GU	Mother had a *WT1* mutation and a history of WT	Regev *et al*, 2008
FWT10	1 year 11 months/M	NE	ex 7, 983delC/P328fs380X	Wild type	GU/UWT	Mother had a *WT1* mutation and a history of WT	Melchionda *et al*, 2013
Children inherited large *WT1* deletions from their fathers or mothers ($n=7$)							
F11-1	2 years/F	NE	del(11)(p13p13)	NE	AN, GU, MR/UWT	Father had ins(14;11)(q23;p12p14)	Lavedan *et al*, 1989
F11-2	4 years/M	NR	del(11)(p13p13)	NR	AN, GU	Father had ins(14;11)(q23;p12p14)	Lavedan *et al*, 1989
FWT12-1	1 year 11 months/M	NE	NE	NE	AN/WT	Mother had ins(2;11)(q23;p12p14)	Yunis and Ramsay, 1980
FWT12-2	1 year 3 months/M	NE	del(11)(p13p14)	NE	AN, GU/BWT	Mother had ins(2;11)(q23;p12p14)	Yunis and Ramsay, 1980
FWT13-1	17 years/M	NR	del(11)(p12p14)	NR	MR	Mother had ins(16;11)(p13;p12p14)	Kousseff and Agatucci, 1981
FWT13-2	6 months/M	NE	del(11)(p12p14)	NE	GU/UWT	Mother had ins(16;11)(p13;p12p14)	Kousseff and Agatucci, 1981
FWT14	NI	NE	del(11)(p12p14)	NE	AN, GU/UWT	Mother had ins(12;11)(p12;p12p14.2)	Nakagome *et al*, 1984
Children with *de novo* large *WT1* deletions of paternal or maternal germ cell origin, included in the penetrance analysis ($n=8$)							
DNWT1	NI	NR	del(11)(p13p14.1)	NR	AN, GU	*de novo* deletion of paternal germ cell origin	Huff *et al*, 1990
DNWT2	NI	NE	del(11)(p13p13)	NE	AN/WT	*de novo* deletion of paternal germ cell origin	Huff *et al*, 1990
DNWT3	NI	NE	del(11)(p11.2p14.1)	NE	AN/WT	*de novo* deletion of maternal germ cell origin	Huff *et al*, 1990
DNWT4	NI	NE	del(11)(p13.4p14.3)	NE	AN/WT	*de novo* deletion of paternal germ cell origin	Huff *et al*, 1990
DNWT5	NI	NE	del(11)(p12p15.1)	NE	AN/WT	*de novo* deletion of paternal germ cell origin	Huff *et al*, 1990
SNWT6	NI	NE	del(11)(p12p15)	NE	AN, GU/WT	*de novo* deletion of paternal germ cell origin	Huff *et al*, 1990
DNWT7	NI	NR	del(11)(p12p15.1)	NR	AN	*de novo* deletion of paternal germ cell origin	Huff *et al*, 1990
DNWT8	NI	NR	del(11)(p13p14.1)	NR	AN, GU	*de novo* deletion of paternal germ cell origin	Huff *et al*, 1990
Patients with *de novo* small *WT1* mutation of paternal germ cell origin, not included in the penetrance analysis ($n=4$)							
DNWT9-1	1 year 6 months/F	UPD[b]	ex 1, 144C>A/Y48X	The same as allele 1, UPD11pt-11cen	None//UWT	*de novo* mutation of paternal germ cell origin	Present study (UWTG1)
DNWT9-2	1 year 8 months/F	pUPD (C, M)	ex 1, 144C>A/Y48X	The same as allele 1, UPD11pt-11cen	None//BWT	*de novo* mutation of paternal germ cell origin	No. 36 (Haruta *et al*, 2008)/BWT24
DNWT10	NI	LOH on 11p15	ex 8, H377R	LOH on 11p13	Drash syndrome/UWT	*de novo* mutation of paternal germ cell origin	Nordenskjold *et al*, 1994
DNWT11	NI	LOH on 11p15	ex 9, D396N	LOH on 11p13	Drash syndrome/UWT	*de novo* mutation of paternal germ cell origin	Nordenskjold *et al*, 1994

Abbreviations: AN=aniridia; BWT=bilateral WT; C=methylation analysis of CTCF6 by COBRA; CNS=congenital nephrotic syndrome; COBRA=combined bisulfite restriction assay; ex=exon; F=female; FGS=focal glomerular sclerosis; FWT=familial WT; GU=genitourinary malformation; LOH=loss of heterogeneity; M=male; M=methylation analysis of *H19*-DMR by MS-MLPA; MR=mental retardation; NI=not informative; NR=not relevant; NE=not examined; OD=ovarial dysgenesis; pUPD=paternal UPD; UPD=uniparental disomy; UWT=unilateral WT; WT=Wilms tumour.

[a]FWT6-2 was excluded from the penetrance analysis because of the reason described in the text.

[b]Above UPD indicates that the methylation status of *H19*-DMR was not examined.

patients who inherited the *WT1* mutation from their mothers and whose *WT1* status in tumours were examined were not likely to have UPD on 11p in their tumours (Figure 1 and Table 3). The mean ages at diagnosis were 14.2 months for the nine patients with the paternal inheritance and 55.3 months for the four patients with the maternal inheritance; the mean age of the nine patients who inherited the mutations from their fathers was younger than that of the four patients who inherited the mutations from their mothers ($P = 0.011$ by Mann–Whitney test).

Fifteen individuals from 12 families were shown to have inherited (FWT11-14) or *de novo* large 11p13 deletions (DNWT1-8) of paternal or maternal origin (Figure 1 and Table 3; Yunis and Ramsay, 1980; Kousseff and Agatucci, 1981; Nakagome *et al*, 1984; Lavedan *et al*, 1989; Huff *et al*, 1990; Nordenskjold *et al*, 1994). All four parents who transmitted large 11p13 deletions had balanced chromosomal insertions involving the 11p13 band. Ten patients developed WTs, on which sequencing analysis of *WT1* was not conducted to identify the status of the other *WT1* allele in the tumours. Thus, the penetrance rate was 100% (9/9) for individuals who inherited small *WT1* mutations from their fathers, and was 67% (14/21) for individuals who inherited small *WT1* mutations from their mothers or large 11p13 deletions or had *de novo* large 11p13 deletions irrespective of the parental origin. Thus, the 9 individuals were more likely to develop WT than the 21 individuals ($P = 0.057$).

DISCUSSION

The incidence of *WT1* abnormalities in the bilateral WTs of Japanese children was 81%, and this incidence was markedly higher than those reported in American, UK and Australian children (the mean percentage of three series, 32%; $P = 9.4E-05$; Table 4; Huff, 1998; Scott *et al*, 2012; Hu *et al*, 2013). The present series included 31 patients and 45 bilateral WTs, and the UK series included 11 patients and 11 bilateral WTs; the statuses of *WT1* and 11p15 were precisely examined in 44 of the 45 and all 11 tumours (Table 4; Scott *et al*, 2012). The incidence of *WT1* abnormality is more frequent in Japanese tumours than in British tumours; however, if we consider that the incidence of WT among Japanese is half of that in Caucasians, the population-based rate of bilateral WT with *WT1* abnormality may be similar between the two populations (Figure 2). Diller *et al* (1998) found constitutional *WT1* abnormalities in 8 of 157 (5%) American children with a history of WT. Likewise, Little *et al* (2004) found constitutional *WT1* abnormalities in 6 (2%) of 282 British children with non-syndromic WT. Unfortunately, no studies on constitutional *WT1* abnormalities have been performed in Japanese children with WT that precluded the comparison of the incidence of constitutional *WT1* abnormalities between the two ethnic populations. In contrast to the equivocal findings in the incidence of *WT1* abnormality, that of *IGF2* LOI was clearly higher in British children with *WT1*-wild-type or *WT1*-wild-type plus *WT1*-mutant bilateral WT than Japanese counterparts (Table 4 and Figure 2; Scott *et al*, 2012). Thus, it is clear that the incidence of *IGF2* LOI in Caucasian bilateral WTs is higher than that in Japanese counterparts, and that the incidence of *WT1* wild-type was low in Japanese bilateral WTs.

The incidence rates of WT are known to vary, being markedly lower in East Asian children than in their Caucasian counterparts (Parkin *et al*, 1988). We previously reported that if only sporadic WTs were included, the frequencies of WT with *WT1* abnormality were similar between East-Asian and Caucasian populations (Haruta *et al*, 2012). Furthermore, we reported a lower incidence of WT with *IGF2* LOI in Japanese children than in American children ($P = 0.041$), and we and others proposed that the lower incidence of *IGF2* LOI may be one of the reasons for the lower incidence of WT in Japan (Fukuzawa *et al*, 2004; Haruta *et al*, 2012). Contrary to the equivocal findings in the incidences of *WT1* abnormality in sporadic or bilateral WTs between the two populations, the difference in the incidence of *IGF2* LOI in bilateral WTs is clear ($P = 0.036$); the relationship is comparative to that of *IGF2* LOI in sporadic WTs between the two populations. Beckwith–Wiedemann syndrome is an imprinting-related growth disorder. Five to 10% of patients with BWS have methylation of *H19*-DMR on both parental chromosomes, resulting in *IGF2* LOI (Cerrato *et al*, 2008). Interestingly, Japanese patients with BWS were shown to have a significantly lower frequency of *H19*-DMR hypermethylation than North American and European patients, whereas the incidences of pUPD on 11p15 were comparable, suggesting that susceptibility to epigenetic alterations differs between the two populations (Sasaki *et al*, 2007). The constitutional 11p15 abnormalities in patients with WT were reported from UK and Netherlands. The UK series included 437 patients with non-syndromic WT and found 11p15 abnormalities in 13 (3%) patients; of the 13 patients 4 had bilateral WT and 6 had hypermethylation of *H19*-DMR (Scott *et al*, 2008). The Netherlands series included 109 patients with syndromic or non-syndromic WTs and found 8 (7.3%) children with 11p15 abnormalities; of the 8 patients 3 had bilateral WT, 4 had BWS and 3 had hypermethylation of *H19*-DMR (Segers *et al*, 2012). Of 13 patients whose methylation status of *H19*-DMR in PB was examined in the present series, all including 1 (BWT29) with *IGF2* LOI in the tumour showed the normal methylation pattern. Thus, the involvement of the constitutional *IGF2* LOI in Japanese bilateral WTs could not be identified. The same decreased susceptibility to the epigenetic change reported in BWS may have also caused the decreased incidence of bilateral WTs with *IGF2* LOI (Sasaki *et al*, 2007). The study for constitutional 11p15 abnormalities in Japanese WTs is needed to prove the hypothesis.

Table 4. Incidence rates of *WT1* and *IGF2* abnormalities in Japanese and British, American or Australian series of bilateral Wilms tumour

	WT1 abn + wt	pUPD	ROI	LOI	*WT1* abn	pUPD	ROI	LOI	*WT1* wt	pUPD	ROI	LOI
A. Japan (the present study)	45[a] (31)	34[a]	8	3	35[a] (25)	31[a]	4	0	10 (6)	3	4	3
B. UK (Scott *et al*, 2012)	11	5	0	6	4	4	0	0	7	1	0	6
Constitutional defect-associated	5	5	0	0	4	4	0	0	1	1	0	0
Sporadic	6	0	0	6	0	0	0	0	6	0	0	6
C. USA (Huff, 1998)	15	NE	NE	NE	4	NE	NE	NE	11	NE	NE	NE
D. Australia (Hu *et al*, 2013)	8	NE	NE	NE	3	NE	NE	NE	5	NE	NE	NE

Abbreviations: abn = abnormality; LOI = loss of *IGF2* imprinting; NE = not examined; pUPD = paternal uniparental disomy; ROI = retention of *IGF2* imprinting; SNP = single-nucleotide polymorphisms; wt = wild type. Numbers indicate numbers of tumours, and those in parentheses indicate numbers of patients in the Japanese series. Numbers in other series indicate both patient and tumour numbers. *WT1* abnormality and *WT1* wild type (patients): A versus B $P = 0.011$; A versus B + C + D $P = 9.4E-05$. *WT1* abnormality and *WT1* wild type (tumours): A versus B $P = 0.012$; A versus B + C + D $P = 5.1E-05$. LOI and UPD + ROI (tumours with *WT1* abnormality and those with *WT1* wild type): A versus B $P = 0.001$. LOI and UPD + ROI (tumours with *WT1* wild type): A versus B $P = 0.036$.

[a]Include one tumour (BWT21R) in which UPD was detected by SNP array analysis but methylation status at *H19*-DMR was not examined.

The present study included six patients having bilateral WTs with wild-type *WT1*, and one of them had PCS syndrome, which was caused by a *BUB1B* mutation and known to be associated with a predisposition to WT (Matsuura *et al*, 2006). The FWT genes, *FWT1* and *FWT2*, were located at 17q21 and 19q13, respectively, and the lack of a linkage to these loci in some WT families was reported previously and indicated the existence of additional FWT genes (Ruteshouser and Huff, 2004). Very recently, Hanks *et al* (2014) identified germline mutation of the *CTR9* gene in 3 of 35 WT families using an exome and sequencing analysis and proposed it as a new WT predisposing gene . These genes may be candidates for germline mutations in the other five patients with *WT1*-wild-type bilateral WTs in the present series.

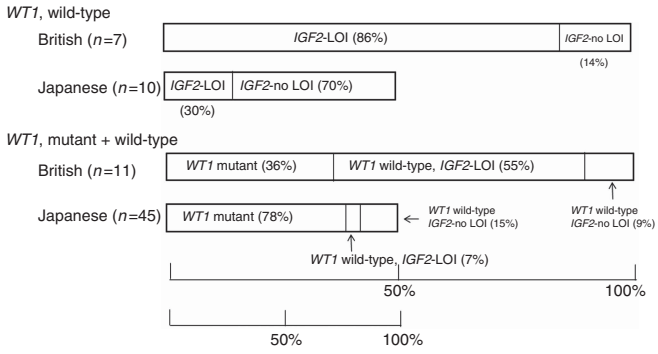

Figure 2. Abnormalities of the *WT1* and *IGF2*-LOI in bilateral WTs in Japanese (the present series) and British children (Scott *et al*, 2012). The bar length for Japanese is half of that for Caucasians because the incidence rate for Japanese is half of that for Caucasians.

A previous study showed that the *WT1* mutations observed in bilateral WT were of germline origin (Huff, 1998). Another study reviewed *WT1* germline mutations in 117 patients with WTs (Royer-Pokora *et al*, 2004). Of the 117 patients, 44 had bilateral WT, indicating that a large proportion of germline *WT1* mutations are associated with bilateral WT, although the inheritance is not known in all patients. The present study included 25 patients with *WT1*-mutant bilateral WTs, and the status of *WT1* in PB was only examined in 3 patients and 6 parents from 3 families; 2 were shown to have inherited the mutation from their father with or without a past history of WT and 1 was identified to have a *DNWT1* mutation of paternal germ cell origin. Thus, mutation analyses of

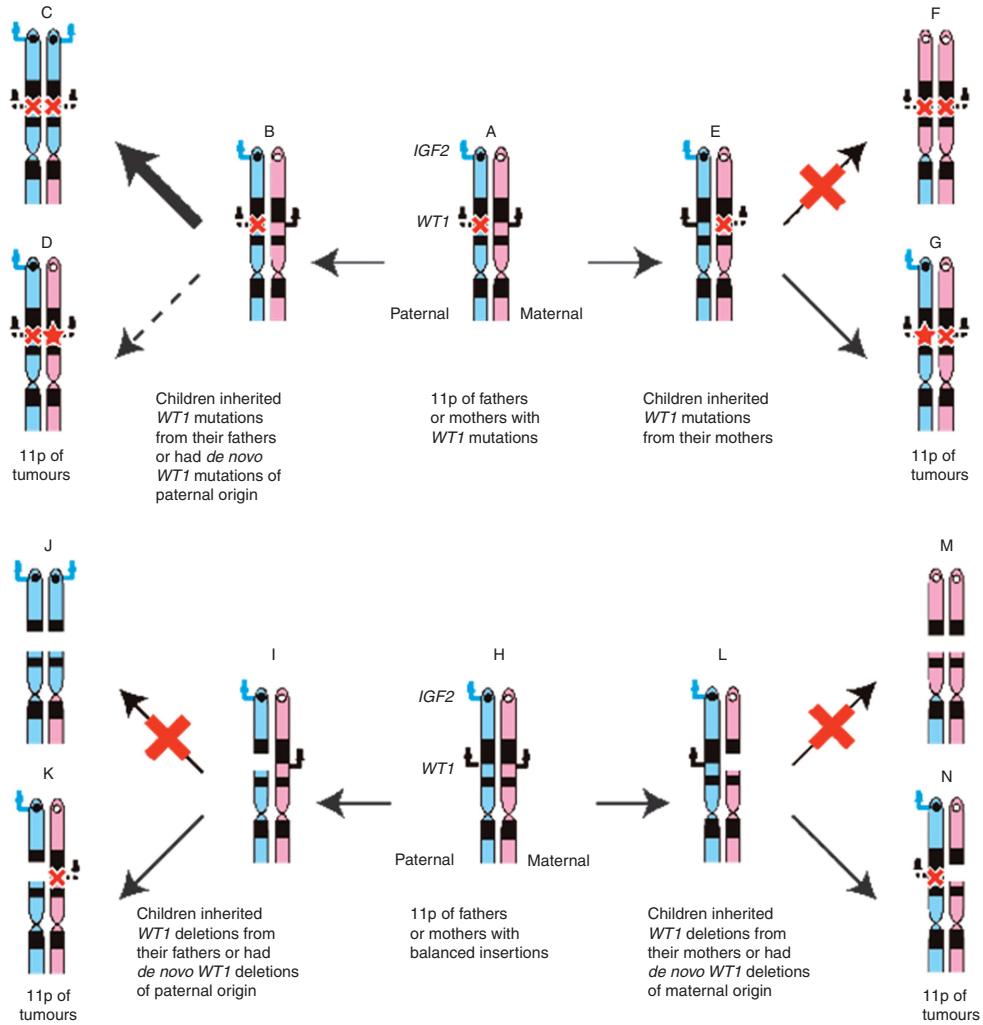

Figure 3. Parental inheritance of small *WT1* mutations and large 11p13 deletion and a model of Wilms tumorigenesis. Black and white circles in terminal 11p represent methylated and unmethylated statuses, respectively, at CTCF6 in the *IGF2-H19* region. Blue arrows indicate the expression of *IGF2*. × and star in the 11p13 region indicate the first and second *WT1* mutations, respectively. Solid and broken arrows in the 11p13 region indicate normal and abnormal *WT1* expression, respectively. Parents had balanced insertions involving the 11p13 band (**H**). A gap in the 11p chromatid (**I** and **L**) indicates a large deletion encompassing *WT1*, and × in the 11p13 region indicates the second *WT1* mutations (**K** and **N**). Explanation for panels **A**–**G**, **J**, and **M** is described in the discussion.

WT1 in the PB of patients and their parents are needed to determine whether the mutation is inherited from the parents or occurred *de novo*.

A previous study examined the parental origin of *de novo RB1* mutations, and found the paternal origin of the mutation in all patients (Dryja *et al*, 1989). Regarding *DNWT1* alterations, we and other researchers found the paternal germ cell origin of the small mutation in four patients and that of the large deletion in seven patients and the maternal germ cell origin of the large deletion in one patient (Huff *et al*, 1990; Nordenskjold *et al*, 1994). No studies have reported the maternal germ cell origin of *de novo* small *WT1* mutations. In a review on human germinal mutations, Crow (2000) described that one marked difference between the human male and female was that there are many more germline cell divisions in the life history of a sperm than that of an egg. In WTs with homozygous *WT1* mutations and paternal *IGF2* UPD, the *IGF2* alteration is thought to be the second genetic event subsequent to the *WT1* alteration that has been shown to occur in the paternal *WT1* allele. The result that pUPD on 11p was found in the great majority of bilateral WTs in the present study further supports the paternal germ cell origin of *de novo* small *WT1* mutations.

We summarised the data of all 30 children from 22 families with hereditary WT, whose inheritance of the *WT1* alteration was described in the present and previous studies (Table 3; Yunis and Ramsay, 1980; Kousseff and Agatucci, 1981; Nakagome *et al*, 1984; Lavedan *et al*, 1989; Pelletier *et al*, 1991; Kaplinsky *et al*, 1996; Jeanpierre *et al*, 1998; Pritchard-Jones *et al*, 2000; Shibata *et al*, 2002; Zirn *et al*, 2005; Regev *et al*, 2008; Fencl *et al*, 2012; Melchionda *et al*, 2013). We classified 30 children into 3 groups based on parental inheritance of the germline mutation and types of *WT1* abnormality, and found that children who inherited small *WT1* mutations from their father were more likely to have the higher penetrance rate than those who inherited small *WT1* mutations from their mothers or inherited the large deletions or had the *de novo* large deletions irrespective of parental origin ($P = 0.057$; Figure 1). Why do parental inheritance and *WT1* abnormality types affect the penetrance rate? Most parents had *DNWT1* mutations of paternal germ cell origin, as shown in (Figure 3A). Children who had a small *WT1* mutation of paternal germ cell origin easily developed WT because pUPD on 11p resulted in homozygous *WT1* mutations and simultaneous over-expression of *IGF2* (Figure 3B and C). Children less frequently developed WT by the second mutation in the maternally derived *WT1* allele because this tumorigenic pathway needs additional genetic and/or epigenetic events (Figure 3D). In contrast, WTs developed in children who inherited the small mutation from their mothers could not take advantage of simultaneous alterations in *WT1* and *IGF2* because maternally derived *IGF2* is imprinted and repressed (Figure 3E and F). Children who inherited the small mutations from their mothers could develop WT if an independent *WT1* mutation occurred in the paternally derived *WT1* allele, which resides on the same 11p expressing *IGF2*; expression even from one *IGF2* allele may be important for the development of embryonic tumours (Figure 3G).

Regarding the large 11p13 deletion, children who inherited the large deletions or had the *de novo* large deletions could develop WT if an independent *WT1* mutation occurred in the paternally or maternally derived W*T1* allele (Figure 3K and N). However, large homozygous deletions in the 11p13 chromosomal region caused by UPD on 11p were unlikely to occur in a nephroblast, because of disadvantage for survival with the loss of a large number of genes (Figure 3J and M). In fact, seven tumours developed in patients with WAGR syndrome, in which both *WT1* and *IGF2* statuses were examined, showed large 11p13 deletions in one *WT1* allele and small mutations in the other allele and ROH on 11pter-11p13 (BWT2L, BWT2R and UWTG6-10; Tables 1 and 2). Furthermore, the patients inherited the small mutations from their fathers were younger than those who inherited the small mutations from their mothers, and the patients with a sporadic and UWT with small homozygous *WT1* mutations and pUPD of *IGF2* were younger than those with a sporadic and UWT with the large deletion. These findings indicate that a small *WT1* mutation with pUPD on 11p is the most efficient mechanism for WT development.

The present result on the *WT1* and *IGF2* statuses in bilateral and FWT led to the hypothesis that individuals who inherited small *WT1* mutations from their fathers may be more likely to develop WT than those who inherited the small mutations from their mothers or inherited large 11p13 deletions or had the *de novo* large deletions irrespective of parental origin. It is obvious that genetic and epigenetic studies in a large number of WT families with *WT1* mutations are needed to confirm the hypothesis. We believe that if confirmed the present findings are useful for the genetic counselling of individuals, including WT survivors, who may inherit *WT1* mutations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

YK, HO, TO, SH and MF designed the research; TK, AY, YO and TT participated in data collection and interpretation; HO, MH, YA, YT and HH participated in the molecular and pathological analysis; YK drafted the manuscript.

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