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, 11 pt-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.

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.

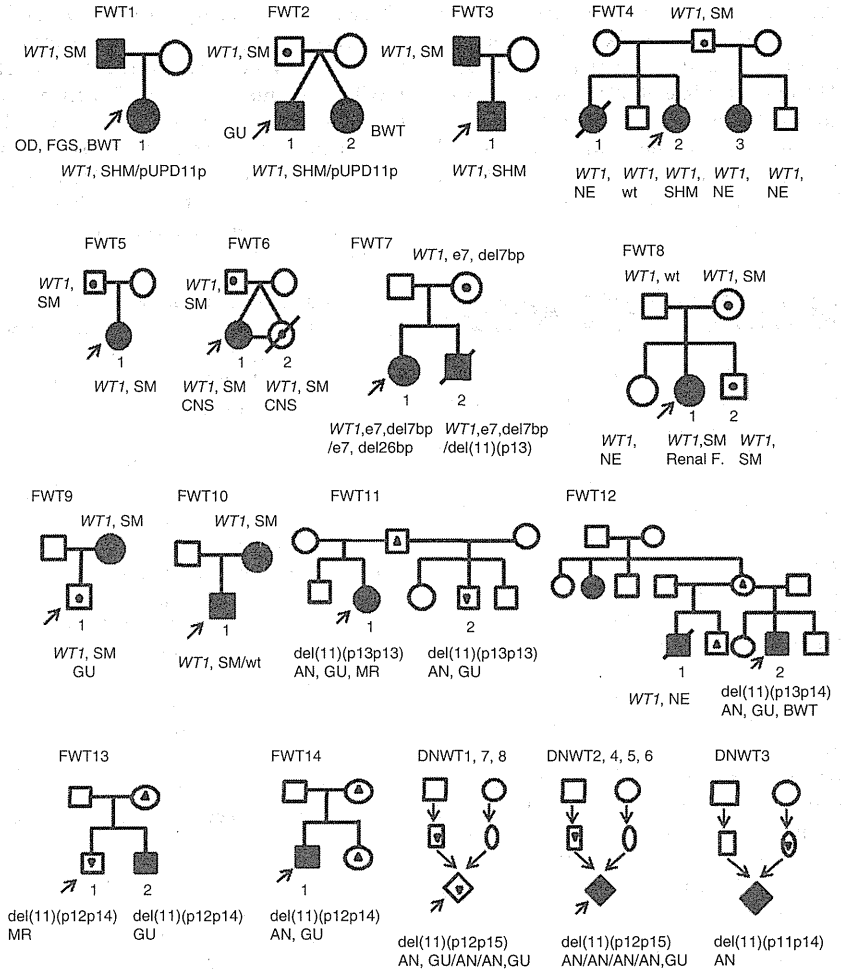

Figure 1. Pedigrees of patients with WTs with small *WT1* germline mutations inherited from fathers (FWT1-6) and mothers (FWT7-10), and those of patients with large 11p13 deletions encompassing *WT1* inherited from fathers (FWT11) or mothers (FWT12-14). DNWT1-8 indicates eight families having the *de 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	Zirn *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	Zirn *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.

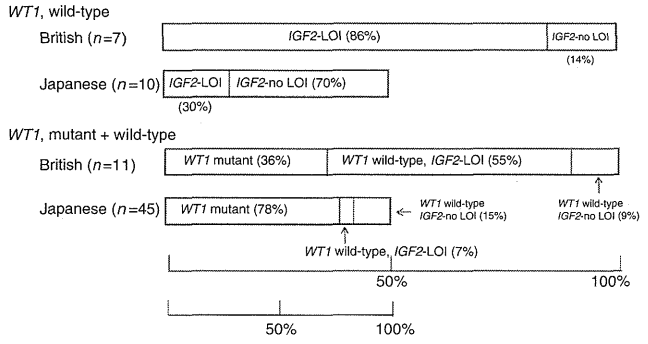

Figure 2. Abnormalities of the *WT1* and *IGF2*-LOI in bilateral WTs in Japanese (the present series) and British children (Scott *et al*, 2012). The bar length for Japanese is half of that for Caucasians because the incidence rate for Japanese is half of that for Caucasians.

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

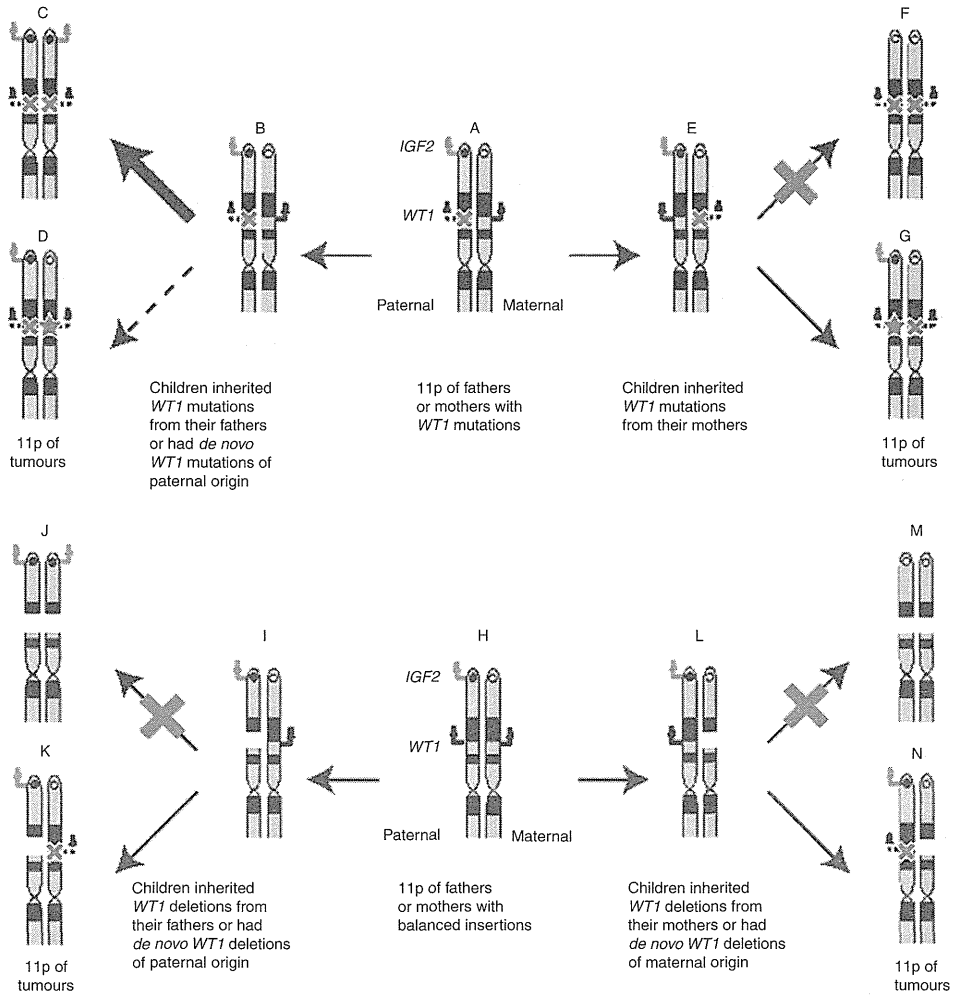

Figure 3. Parental inheritance of small *WT1* mutations and large 11p13 deletion and a model of Wilms tumorigenesis. Black and white circles in terminal 11p represent methylated and unmethylated statuses, respectively, at CTCF6 in the *IGF2-H19* region. Blue arrows indicate the expression of *IGF2*. × 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 *WT1* 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.

REFERENCES

Cerrato F, Sparago A, Verde G, De Crescenzo A, Citro V, Cubellis MV, Rinaldi MM, Boccuto L, Neri G, Magnani C, D'Angelo P, Collini P, Perotti D, Sebastio G, Maher ER, Riccio A (2008) Different mechanisms cause imprinting defects at the *IGF2/H19* locus in Beckwith-Wiedemann syndrome and Wilms' tumour. *Hum Mol Genet* **17**(10): 1427–1435.

Crow JF (2000) The origins, patterns and implications of human spontaneous mutation. *Nat Rev Genet* **1**(1): 40–47.

D'Angio GJ, Breslow N, Beckwith JB, Evans A, Baum H, deLorimier A, Fernbach D, Hrabovsky E, Jones B, Kelalis P, Othersen HB, Tefft M, Thomas PRM (1989) Treatment of Wilms' tumor. Results of the third national Wilms' tumor Study. *Cancer* **64**(2): 349–360.

Diller L, Ghahremani M, Morgan J, Grundy P, Reeves C, Breslow N, Green D, Neuberg D, Pelletier J, Li FP (1998) Constitutional *WT1* mutations in Wilms' tumor patients. *J Clin Oncol* **16**(11): 3634–3640.

Dryja TP, Mukai S, Petersen R, Rapaport JM, Walton D, Yandell DW (1989) Parental origin of mutations of the retinoblastoma gene. *Nature* **339**(6225): 556–558.

Fencl F, Malina M, Stara V, Zieg J, Mixova D, Seeman T, Blahova K (2012) Discordant expression of a new *WT1* gene mutation in a family with monozygotic twins presenting with congenital nephrotic syndrome. *Eur J Pediatr* **171**(1): 121–124.

Foulstone E, Prince S, Zaccheo O, Burns JL, Harper J, Jacobs C, Church D, Hassan AB (2005) Insulin-like growth factor ligands, receptors, and binding proteins in cancer. *J Pathol* **205**(2): 145–153.

Fukuzawa R, Breslow NE, Morison IM, Dwyer P, Kusafuka T, Kobayashi Y, Becroft DM, Beckwith JB, Perlman EJ, Reeve AE (2004) Epigenetic differences between Wilms' tumours in white and east-Asian children. *Lancet* **363**(9407): 446–451.

Hanks S, Perdeaux ER, Seal S, Ruark E, Mahamdallie SS, Murray A, Ramsay E, Del Vecchio Duarte S, Zachariou A, de Souza B, Warren-Perry M, Elliott A, Davidson A, Price H, Stiller C, Pritchard-Jones K, Rahman N (2014) Germline mutations in the PAF1 complex gene *CTR9* predispose to Wilms tumour. *Nat Commun* **5**: 4398.

Haruta M, Arai Y, Sugawara W, Watanabe N, Honda S, Ohshima J, Soejima H, Nakadate H, Okita H, Hata J, Fukuzawa M, Kaneko Y (2008) Duplication of paternal *IGF2* or loss of maternal *IGF2* imprinting occurs in half of Wilms tumors with various structural *WT1* abnormalities. *Genes Chromosomes Cancer* **47**(8): 712–727.

Haruta M, Arai Y, Watanabe N, Fujiwara Y, Honda S, Ohshima J, Kasai F, Nakadate H, Horie H, Okita H, Hata J, Fukuzawa M, Kaneko Y (2012) Different incidences of epigenetic but not genetic abnormalities between Wilms tumors in Japanese and Caucasian children. *Cancer Sci* **103**(6): 1129–1135.

Hu M, Fletcher J, McCahon E, Catchpoole D, Zhang GY, Wang YM, Algar EM, Alexander SI (2013) Bilateral Wilms tumor and early presentation in pediatric patients is associated with the truncation of the Wilms tumor 1 protein. *J Pediatr* **163**(1): 224–229.

Hu Q, Gao F, Tian W, Ruteshouser EC, Wang Y, Lazar A, Stewart J, Strong LC, Behringer RR, Huff V (2011) *Wt1* ablation and *Igf2* upregulation in mice result in Wilms tumors with elevated ERK1/2 phosphorylation. *J Clin Invest* **121**(1): 174–183.

Huff V (1998) Wilms tumor genetics. *Am J Med Genet* **79**(4): 260–267.

Huff V (2011) Wilms' tumours: about tumour suppressor genes, an oncogene and a chameleon gene. *Nat Rev Cancer* **11**(2): 111–121.

Huff V, Meadows A, Riccardi VM, Strong LC, Saunders GF (1990) Parental origin of de novo constitutional deletions of chromosomal band 11p13. *Am J Hum Genet* **47**(1): 155–160.

Jeanpierre C, Denamur E, Henry I, Cabanis MO, Luce S, Cecille A, Elion J, Peuchmaur M, Loirat C, Niaudet P, Gubler MC, Junien C (1998) Identification of constitutional *WT1* mutations, in patients with isolated diffuse mesangial sclerosis, and analysis of genotype/phenotype correlations by use of a computerized mutation database. *Am J Hum Genet* **62**(4): 824–833.

Kaplinsky C, Ghahremani M, Frishberg Y, Rechavi G, Pelletier J (1996) Familial Wilms' tumor associated with a *WT1* zinc finger mutation. *Genomics* **38**(3): 451–453.

Kousseff BG, Agatucci A (1981) Aniridia-Wilms tumor association. *J Pediatr* **98**(4): 676–678.

Lavedan C, Barichard F, Azoulay M, Couillin P, Molina Gomez D, Nicolas H, Quack B, Rethore MO, Noel B, Junien C (1989) Molecular definition of de novo and genetically transmitted WAGR-associated rearrangements of 11p13. *Cytogenet Cell Genet* **50**(2-3): 70–74.

Leiderman YI, Kiss S, Mukai S (2007) Molecular genetics of *RB1*–the retinoblastoma gene. *Semin Ophthalmol* **22**(4): 247–254.

Little MH, Dunn R, Byrne JA, Seawright A, Smith PJ, Pritchard-Jones K, van Heyningen V, Hastie ND (1992) Equivalent expression of paternally and maternally inherited *WT1* alleles in normal fetal tissue and Wilms' tumours. *Oncogene* **7**(4): 635–641.

Little SE, Hanks SP, King-Underwood L, Jones C, Rapley EA, Rahman N, Pritchard-Jones K (2004) Frequency and heritability of *WT1* mutations in nonsyndromic Wilms' tumor patients: a UK Children's Cancer Study Group Study. *J Clin Oncol* **22**(20): 4140–4146.

Matsuura S, Matsumoto Y, Morishima K, Izumi H, Matsumoto H, Ito E, Tsutsui K, Kobayashi J, Tauchi H, Kajiwara Y, Hama S, Kurisu K, Tahara H, Oshimura M, Komatsu K, Ikeuchi T, Kajii T (2006) Monoallelic *BUB1B* mutations and defective mitotic-spindle checkpoint in seven families with premature chromatid separation (PCS) syndrome. *Am J Med Genet A* **140**(4): 358–367.

Melchionda F, Spreafico F, Ciceri S, Lima M, Collini P, Pession A, Massimino M, Radice P, Perotti D (2013) A novel *WT1* mutation in familial Wilms tumor. *Pediatr Blood Cancer* **60**(8): 1388–1389.

Nakagome Y, Ise T, Sakurai M, Nakajo T, Okamoto E, Takano T, Nakahori Y, Tsuchida Y, Nagahara N, Takada Y, Ohsawa Y, Sawaguchi S, Toyosaka A, Kobayashi N, Matsunaga E, Saito S (1984) High-resolution studies in patients with aniridia-Wilms tumor association, Wilms tumor or related congenital abnormalities. *Hum Genet* **67**(3): 245–248.

Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, Kurokawa M, Chiba S, Bailey DK, Kennedy GC, Ogawa S (2005) A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res* **65**(14): 6071–6079.

Nordenskjold A, Friedman E, Anvret M (1994) *WT1* mutations in patients with Denys-Drash syndrome: a novel mutation in exon 8 and paternal allele origin. *Hum Genet* **93**(2): 115–120.

Oue T, Fukuzawa M, Okita H, Mugishima H, Horie H, Hata J, Saito M, Nozaki M, Chin M, Nakadate H, Hinotsu S, Koshinaga T, Kaneko Y, Kitano Y, Tanaka Y (2009) Outcome of pediatric renal tumor treated using the Japan Wilms Tumor Study-1 (JWiTS-1) protocol: a report from the JWiTS group. *Pediatr Surg Int* **25**(11): 923–929.

Parkin DM, Stiller CA, Draper GJ, Bieber CA (1988) The international incidence of childhood cancer. *Int J Cancer* **42**(4): 511–520.

Pelletier J, Bruening W, Li FP, Haber DA, Glaser T, Housman DE (1991) *WT1* mutations contribute to abnormal genital system development and hereditary Wilms' tumour. *Nature* **353**(6343): 431–434.

Pritchard-Jones K, Rahman N, Gerrard M, Variend D, King-Underwood L (2000) Familial Wilms tumour resulting from *WT1* mutation: intronic polymorphism causing artefactual constitutional homozygosity. *J Med Genet* **37**(5): 377–379.

Ravenel JD, Broman KW, Perlman EJ, Niemitz EL, Jayawardena TM, Bell DW, Haber DA, Uejima H, Feinberg AP (2001) Loss of imprinting of insulin-like growth factor-II (*IGF2*) gene in distinguishing specific biologic subtypes of Wilms tumor. *J Natl Cancer Inst* **93**(22): 1698–1703.

Regev M, Kirk R, Mashevich M, Bistritzer Z, Reish O (2008) Vertical transmission of a mutation in exon 1 of the *WT1* gene: lessons for genetic counseling. *Am J Med Genet A* **146A**(18): 2332–2336.

Rivera MN, Haber DA (2005) Wilms' tumour: connecting tumorigenesis and organ development in the kidney. *Nat Rev Cancer* **5**(9): 699–712.

Royer-Pokora B, Beier M, Henzler M, Alam R, Schumacher V, Weirich A, Huff V (2004) Twenty-four new cases of *WT1* germline mutations and review of the literature: genotype/phenotype correlations for Wilms tumor development. *Am J Med Genet A* **127A**(3): 249–257.

Royer-Pokora B, Weirich A, Schumacher V, Uschkereit C, Beier M, Leuschner I, Graf N, Autschbach F, Schneider D, von Harrach M (2008) Clinical relevance of mutations in the Wilms tumor suppressor 1 gene *WT1* and the cadherin-associated protein beta1 gene *CTNNB1* for patients with Wilms tumors: results of long-term surveillance of 71 patients from International Society of Pediatric Oncology Study 9/Society for Pediatric Oncology. *Cancer* **113**(5): 1080–1089.

Ruteshouser EC, Huff V (2004) Familial Wilms tumor. *Am J Med Genet C Semin Med Genet* **129C**(1): 29–34.

Sasaki K, Soejima H, Higashimoto K, Yatsuki H, Ohashi H, Yakabe S, Joh K, Niikawa N, Mukai T (2007) Japanese and North American/European patients with Beckwith-Wiedemann syndrome have different frequencies of some epigenetic and genetic alterations. *Eur J Hum Genet* **15**(12): 1205–1210.

Schroeder WT, Chao LY, Dao DD, Strong LC, Pathak S, Riccardi V, Lewis WH, Saunders GF (1987) Nonrandom loss of maternal chromosome 11 alleles in Wilms tumors. *Am J Hum Genet* **40**(5): 413–420.

Scott RH, Douglas J, Baskcomb L, Huxter N, Barker K, Hanks S, Craft A, Gerrard M, Kohler JA, Levitt GA, Picton S, Pizer B, Ronghe MD, Williams D, Cook JA, Pujol P, Maher ER, Birch JM, Stiller CA, Pritchard-Jones K, Rahman N (2008) Constitutional 11p15 abnormalities, including heritable imprinting center mutations, cause nonsyndromic Wilms tumor. *Nat Genet* **40**(11): 1329–1334.

Scott RH, Murray A, Baskcomb L, Turnbull C, Loveday C, Al-Saadi R, Williams R, Breatnach F, Gerrard M, Hale J, Kohler J, Lapunzina P, Levitt GA, Picton S, Pizer B, Ronghe MD, Traunecker H, Williams D, Kelsey A, Vujanic GM, Sebire NJ, Grundy P, Stiller CA, Pritchard-Jones K, Douglas J, Rahman N (2012) Stratification of Wilms tumor by genetic and epigenetic analysis. *Oncotarget* **3**(3): 327–335.

Segers H, Kersseboom R, Alders M, Pieters R, Wagner A, van den Heuvel-Eibrink MM (2012) Frequency of *WT1* and 11p15 constitutional aberrations and phenotypic correlation in childhood Wilms tumour patients. *Eur J Cancer* **48**(17): 3249–3256.

Shibata R, Hashiguchi A, Sakamoto J, Yamada T, Umezawa A, Hata J (2002) Correlation between a specific Wilms tumour suppressor gene (*WT1*) mutation and the histological findings in Wilms tumour (WT). *J Med Genet* **39**(12): e83.

The committee on histological classification of childhood tumors. The Japanese Society of Pathology (2008) *Tumors of the Kidney*, 1st edn. Kanahara Shuppan: Tokyo, Japan.

Watanabe N, Nakadate H, Haruta M, Sugawara W, Sasaki F, Tsunematsu Y, Kikuta A, Fukuzawa M, Okita H, Hata J, Soejima H, Kaneko Y (2006) Association of 11q loss, trisomy 12, and possible 16q loss with loss of imprinting of insulin-like growth factor-II in Wilms tumor. *Genes Chromosomes Cancer* **45**(6): 592–601.

Yamamoto G, Nannya Y, Kato M, Sanada M, Levine RL, Kawamata N, Hangaishi A, Kurokawa M, Chiba S, Gilliland DG, Koeffler HP, Ogawa S (2007) Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. *Am J Hum Genet* **81**(1): 114–126.

Yunis JJ, Ramsay NK (1980) Familial occurrence of the aniridia-Wilms tumor syndrome with deletion 11p13-14.1. *J Pediatr* **96**(6): 1027–1030.

Zirn B, Wittmann S, Gessler M (2005) Novel familial *WT1* read-through mutation associated with Wilms tumor and slow progressive nephropathy. *Am J Kidney Dis* **45**(6): 1100–1104.

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ORIGINAL ARTICLE

Histological features of primary tumors after induction or high-dose chemotherapy in high-risk neuroblastoma

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Abstract
Purpose In the recent years in Japan, an increasing number of patients with neuroblastoma (NB) are being treated by the "delayed local treatment (DL)" policy, undergoing surgery after the completion of high-dose chemotherapy with hematopoietic stem cell rescue (HDC). We reviewed the histopathological findings of second-look operations, including those of patients treated with DL.
Patients From 1998 to 2013, 26 patients with high-risk NB underwent radical operation following chemotherapy. Surgery was performed after induction chemotherapy in 17 cases (standard; STD), whereas 9 cases completed induction chemotherapy and HDC before undergoing tumor resection (DL). The amount of necrosis and the degree of differentiation within the post-treatment tumor were assessed.
Results Eighty-eight percent of the tumors showed necrosis in more than 1/3 of the specimen. Two DL cases showed complete disappearance of viable tumor cells. Amount of necrosis did not affect the prognosis of the patient. Tumors with immature, poorly differentiated phenotypes showed an extremely aggressive thereafter. Though not statistically proven, ^{123}I-MIBG (metaiodobenzylguanidine) uptake may be correlated with the amount of viable cells remaining within the tumor, but not with the degree of differentiation.
Conclusions Our results support the previous reports advocating that tumors that sustain unfavorable histology after chemotherapy behave aggressively thereafter.

Keywords Neuroblastoma · Histology · Chemotherapy · Differentiation

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Introduction

Neuroblastoma (NB) is the most frequent extracranial solid tumor in childhood and originates from the sympathoadrenal lineage derived from the neural crest [1]. Despite the development of multimodal treatment including induction chemotherapy, surgical resection, high-dose chemotherapy with hematopoietic stem cell rescue (HDC), and radiotherapy, the outcome of patients with advanced NB remains poor [2, 3].

A series of clinical and biologic prognostic markers has been adopted for risk stratification of NB, and patients are generally treated following the risk-adapted therapeutic strategy. Tumor histology classification at diagnosis using the international neuroblastoma pathology classification

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(INPC) [4, 5] is the mainstay of stratification, along with age [6], *MYCN* status [7], clinical stage by international neuroblastoma staging system (INSS) [8] and DNA index [9]. INPC categorizes the patients based on the pathological findings into favorable histology (FH) group and unfavorable histology (UH) group by applying the concept of age-dependent (age-appropriate) normal ranges of morphologic features. In addition to this, the classification itself regardless of age is shown to serve as a powerful tool for independently predicting the prognosis of the patient [10]. However, contrarily to the significant influence of pretreatment histological features in the treatment of NB, little has been discussed on the histology of post-treatment tumors [11–14].

In the recent decade in Japan, a unique treatment strategy following the "delayed local treatment" concept has been carried out as pilot studies in many institutions. Aiming to intensify the "dose per period" of the systemic chemotherapy regimen, the local therapy including second-look surgery and local radiotherapy is postponed until the completion of systemic chemotherapy, including HDC [15]. We have adopted this strategy as well, and found that most of the tumors remain viable at the time of surgery, leaving radiotherapy as the only postoperative treatment. This fact motivated us to investigate the post-treatment histological status within the primary tumors and its relevance to the outcome of the patient. Thus, we conducted this retrospective study including high-risk NB cases that were treated in the recent 15 years, in which patients were treated with similar induction chemotherapy protocols. The cohort included cases that underwent tumor resection at the conventional timing (after induction chemotherapy), or after the completion of HDC, to enable comparison of the different timings of surgery and the histological findings.

^{123}I-metaiodobenzylguanidine (MIBG) scintigraphies are not only valuable as a diagnostic tool [16], but also are currently implicated in the response evaluation during the treatment courses [17]. It has been reported that aggressive tumors with immature phenotype and high mitosis rate show increased ^{123}I-MIBG uptake in untreated NB tumors [18]. However, little is known about ^{123}I-MIBG uptake within primary tumors after chemotherapy and its correlation with the histological features of the tumor. In this retrospective study, we also assessed the relevance of ^{123}I-MIBG uptake prior to the radical resection of the tumor and the post-treatment histological features.

Materials and methods

Medical records of patients with high-risk NB treated with preoperative chemotherapy followed by elective surgery in two local pediatric oncology centers within the period from January 1998 to December 2013 were reviewed. Criteria of entry for this retrospective review were 1 year or older with either stage 4 disease or stage 3 with *MYCN* amplification, or under 1 year with stage 4 disease with *MYCN* amplification. A total of 26 cases met these criteria. There were eight boys and 18 girls. Median age at diagnosis was 39 months (ranging from eight to 124 months; one was under 1 year of age and the others were over one). Stage of disease (international NB staging system; INSS) was stage 3 in two patients, and stage 4 in 24 patients. The site of the original tumor was abdominal in 23 cases and mediastinal in three. All patients were initially treated with a multi-agent protocol regimen including cyclophosphamide, vincristine, pirarubicin and cisplatin. Radical resection of the primary tumor was performed in between courses of induction chemotherapy in 17 cases (standard group; STD). In 9 cases, surgery was postponed until after the completion of the entire chemotherapy regimen, including HDC (delayed local therapy group; DL). This retrospective analysis was approved by the Institutional Review Boards of the two participating institutions.

We reviewed hematoxylin–eosin-stained histologic slides of both the biopsied specimens before treatment and the post-treatment tumors for the study patients. Biopsied specimens before treatment were evaluated and classified by the international neuroblastoma pathology classification (INPC) [4, 5]. Briefly, tumors were divided into four major categories, i.e., (1) NB (schwannian stroma-poor), (2) Ganglioneuroblastoma (GNB), intermixed (schwannian stroma-rich), (3) ganglioneuroma (GN) (Schwannian stroma-dominant), and (4) GNB nodular (composite schwannian stroma-rich/stroma-dominant and stroma-poor). NB was further subdivided into undifferentiated NB, poorly differentiated NB, and differentiating NB, and GN was subdivided into GN maturing and GN mature [4].

Using the post-treatment tumor specimen, the effect of chemotherapy was evaluated in two measures: (1) amount of necrosis (including granulation tissue and fibrosis) within the entire tumor sample (2) maturation of viable neuroblasts and surrounding stroma. The amount of necrosis was quantified using the Histologic Criteria for Effectiveness of Treatment in Pediatric Solid Tumors (established by the Committee on Histological Classification of Childhood Tumors, The Japanese Society of Pathology). In the criteria, the effect of treatment is classified into four grades according to the proportion of necrosis within the tumor (Table 1). Maturation of the tumor tissue was assessed on the basis of INPC. As mentioned above, INPC is originally designed to classify tumors based on the histologic features of primary tumors in untreated patients. To adopt this system into post-treatment tumor tissue, we focused on the viable cellular components of the resected tumor and excluded the area

Table 1 Histologic criteria for effectiveness of treatment in pediatric solid tumors (established by the Committee on Histological Classification of Childhood Tumors, The Japanese Society of Pathology)

Criteria	Histological findings
Ef0	No or minimal effect
Ef1a	Mild effect: necrosis of tumor cells is observed in less than approximately one-third of the tumor volume
Ef1b	Mild to moderate effect. Necrosis is observed in one-third to two-thirds of the tumor volume
Ef2	Moderate effect. Necrosis is observed in more than two-thirds of the tumor volume
Ef3	The entire tumor is replaced with necrosis

within the tumor consisting of necrotic tissue. The morphological changes during preoperative chemotherapy were assessed by comparing INPC at the time of biopsy and at the time of radical operation.

Finally, to clarify the correlation between positive signals at the tumor site in preoperative ^{123}I-MIBG scintigraphy studies and the histological features of the subsequently removed tumor, scans obtained immediately prior to the radical operation were reviewed.

Overall survival was defined as the time from diagnosis to death. The prognostic value was evaluated by a log-rank test. Correlation of ^{123}I-MIBG scintigraphy uptake with the histological features was evaluated using Fischer's exact test.

Results

Background of patients and treatment

All patients underwent biopsy at diagnosis. Initial histological classification of the biopsied specimens before treatment according to the INPC was unfavorable histology in all cases. Pretreatment histological subtype was undifferentiated NB in three cases, poorly differentiated NB in 19, differentiating NB in one, and GNB nodular in three, according to the revised version of INPC [5]. In the three cases that were diagnosed GNB nodular, the histological diagnosis of primary tumors were GN maturing, but bone marrow aspiration revealed vivid tumor cells compatible to poorly differentiated NB, thus leading to the clinical diagnosis of GNB nodular [5].

Preoperative chemotherapy consisted of at least three cycles of induction chemotherapy, including cyclophosphamide, pirarubicin, cisplatin and vincristine, following the group study protocols in which each patient had been enrolled [19]. Five of the 26 cases received various second-line treatments before radical surgery for either stable/progressive diseases or side effects caused by initial induction chemotherapy. The median of the number of preoperative conventional chemotherapy courses given to the patients in the STD group was five (range: three to eighteen). The patients in the DL group received five or six courses of conventional induction chemotherapy followed by HDC, using either of the following preconditioning regimen: (1) melphalan, etoposide and carboplatin, (2) melphalan, etoposide and cisplatin, followed by total body irradiation, (3) mephalan and busulfan. The sources of hematopoietic stem cell were autologous peripheral blood in all cases but for two, in which allogenic bone marrow from related donors were used.

Radical surgery was aimed to obtain gross total resection in all cases, with maximal effort to preserve vital organs and major vessels. As a result, gross total resection was performed in 17 cases, subtotal resection in eight, and partial resection in two cases. In the DL group, surgery was performed during the period from day 30 to 60 post-transplantation.

Radiotherapy was used in a total of 20 cases. These included 18 cases for which local radiotherapy were used postoperatively. Three cases in the STD group received total body irradiation as a part of the preconditioning regimen for hematopoietic stem cell transplantation. None of the cases underwent preoperative radiotherapy, thus the treatment effect observed in the tumor specimens of delayed radical resection is purely a result of preoperative chemotherapy.

At the time of evaluation, eight patients were alive without disease, four were alive with disease, twelve died of the disease, and two died from treatment-related complications. The average follow-up period of the living patients was 63.5 months in the STD group, and 32.6 months in the DL group.

Histological cytotoxic effects in tumor specimens obtained in radical resection

The summarized results of the histological review of cytotoxic effects using the Histologic Criteria for Effectiveness of Treatment in Pediatric Solid Tumors are shown in Table 2. There were no tumors in the Ef0 category, suggesting that every case showed at least signs of necrosis. Tumors that were categorized into the Ef1b or Ef2 criteria accounted for 80.8 % of the total number. Fifteen cases (61.5 %) had recurrence after radical surgery. Recurrence evenly occurred in cases categorized into Ef1, Ef2a and Ef2b, suggesting that the amount of necrotic change of the primary tumor caused by preoperative chemotherapy are not correlated to a good prognosis.

Interestingly, the two cases that showed Ef3 response (complete disappearance of viable tumor cells) both belonged to the DL group. Tumors categorized either into the Ef2 or the Ef3 criteria accounted for 70 % of the DL cases,

Table 2 Cytotoxic effect measured by amount of necrosis in tumors after treatment and recurrence

Effect criteria	STD ($n = 17$)			DL ($n = 9$)		
	Total	Recurrence		Total	Recurrence	
		Local	Metastatic		Local	Metastatic
Ef0 ($n = 0$)	0	–	–	0	–	–
Ef1a ($n = 3$)	2	0	1	1	1	0
Ef1b ($n = 10$)	8	3	4	2	0	0
Ef2 ($n = 11$)	7	0	6	4	0	0
Ef3 ($n = 2$)	0	–	–	2	0	0

whereas only 44 % fell into these categories in the STD group. Although we could not draw any firm conclusion from the limited number of the cohort, the difference between the STD group and the DL group may owe to the intensified therapeutic impact of the HDC. Furthermore, the DL group appears to have less recurrence, but since the follow-up period of this subgroup is shorter than the STD group, validation of this tendency requires further follow-up.

Next, we investigated the estimated overall survival rate of patients in each effect criteria. As mentioned above, the follow-up period of the DL group was shorter than that of the STD group, and furthermore, the overall survival rate of the STD group and the DL group was not significantly different. Thus, we combined the STD group and the DL group together in the survival analyses. The overall survival of the patients also did not correlate with the histologic cytotoxic change within the primary tumors after chemotherapy. As shown in Fig. 1, the effect criteria had no relevance to the probability of survival. The two cases that showed Ef3 effects were both *MYCN*-amplified cases. Both cases are alive without disease after 74 and 37 months from diagnosis, respectively.

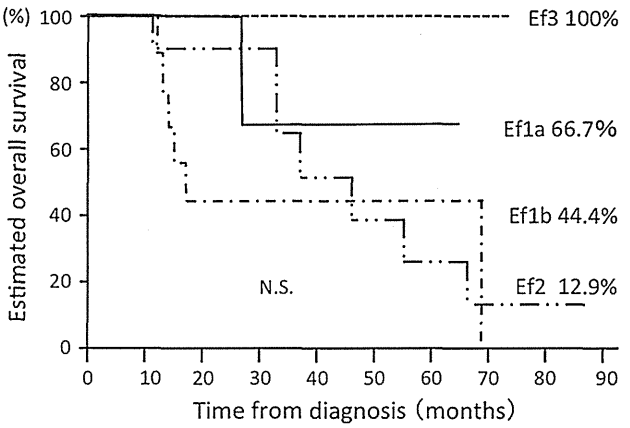

Fig. 1 Effect criteria and overall survival of patients

Histological maturation in tumor specimens obtained in delayed radical resection

The degree of maturation/differentiation of the tumor at diagnosis and at the time of radical resection was assessed based on the INPC criteria (Table 3). As expected, tumors that originally had features of differentiation at diagnostic biopsy tended to shift towards a further maturated phenotype at the time of second-look surgery, featured by enriched Schwannian stroma and maturing ganglion-like cells. On the other hand, those that originally had immature features (i.e., undifferentiated NB and poorly differentiated NB) responded to chemotherapy variously. Among the 22 tumors in these criteria, 18 tumors shifted up to either of differentiated NB, ganglioneuroblastoma (GNB) intermixed, or GN maturing. However, there were four cases that remained in the undifferentiated or poorly differentiated NB subgroup, indicating that there were no or minimum effect of differentiation/maturation within the primary tumor despite the use of preoperative chemotherapy. Table 4 shows the summary of posttreatment INPC category and the pattern of recurrence. Out of the four cases that remained in the undifferentiated or poorly differentiated NB subgroup, three were in the STD group, which all relapsed shortly after the surgery during the postoperative treatment courses (3, 5 and 7 months from surgery to relapse). Of these three patients, one underwent intraoperative radiotherapy and was subsequently treated with postoperative conventional chemotherapy, when the tumor relapsed. The other two were scheduled to receive local radiotherapy after HDC but never had the chance to do so, since local recurrence occurred shortly after HDC. The case in the DL group did not suffer recurrence but was continuously being treated for stable disease at the time of data collection. Relapse also occurred in cases that showed moderate to high degree of differentiation (i.e., differentiating NB, GNB intermixed and GN maturing categories). Twelve out of 20 cases in these categories had tumor recurrence. It is worth noting that local recurrence as the first postsurgical event was rare in this group of patients, with only one in the DL group. The first site of relapse was bone marrow or distant lymph nodes in the remaining 11 cases, which was in contrast to the relapse pattern in the poorly differentiated tumors, in which recurrence tended to occur locoregionally.

Table 3 Pre- and post-treatment tumor phenotype based on INPC criteria

Pretreatment histology of primary tumor from biopsy	Post-treatment histology of resected specimen					
	Undifferentiated NB ($n = 2$)	Poorly differentiated NB ($n = 2$)	Differentiating NB ($n = 7$)	GNB intermixed ($n = 10$)	GN maturing ($n = 3$)	No viable cell ($n = 2$)
Undifferentiated NB ($n = 3$)	1	0	0	1	1	0
Poorly differentiated NB ($n = 19$)	1	2	7	6	1	2
Differentiating NB ($n = 1$)	0	0	0	1	0	0
GN maturing ($n = 3$)	0	0	0	2	1	0

Table 4 Tumor phenotype based on INPC criteria in tumors after treatment and recurrence

Post-treatment INPC category	STD ($n = 17$)			DL ($n = 9$)		
	Subtotal	Recurrence		Subtotal	Recurrence	
		Local	Metastatic		Local	Metastatic
Undifferentiated NB ($n = 2$)	2	2	0	0	–	–
Poorly differentiated NB ($n = 2$)	1	1	0	1	0	0
Differentiating NB ($n = 7$)	5	0	4	2	1	0
GNB intermixed ($n = 10$)	8	0	6	2	0	0
GN maturing ($n = 3$)	1	0	1	2	0	0
No viable tumor cell ($n = 2$)	0	–	–	2	0	0

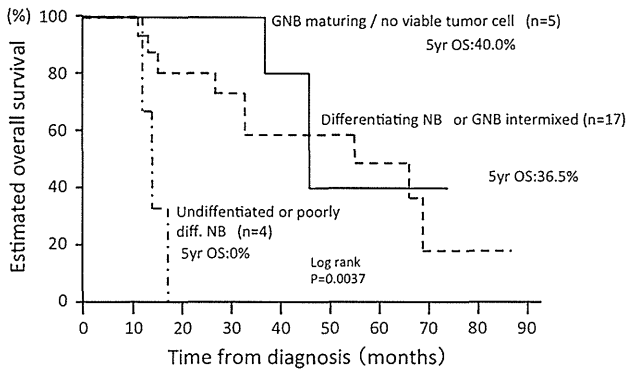

Fig. 2 Post-treatment INPC category and overall survival of patients

Overall survival of the patients in each category is shown in Fig. 2. Due to the shortage of the number of cases, each category was combined into three groups; i.e., undifferentiated/poorly differentiated NB group, differentiating NB/GNB intermixed group, and GN maturing and no viable cell group. The estimated 5-year overall survival rate of the undifferentiated/poorly differentiated NB group was 0 %, as compared to 36.5 and 40 % in the differentiating NB/GNB intermixed group and GN maturing and no viable cell group, respectively. The survival rate of the undifferentiated/poorly differentiated NB group was significantly lower than the other two groups (log-rank test; $P = 0.0037$).

Histology and ^{123}I-MIBG uptake

To clarify the correlation between preoperative imaging studies and histology of the resected tumor, we evaluated the clinical response of the local and metastatic tumors by retrospectively assessing the ^{123}I-MIBG (metaiodobenzylguanidine) scintigraphy findings. Five early cases in which ^{123}I-MIBG had not been applied routinely were excluded, and 21 cases were subjected for assessment (Table 5). Although not statistical, the cytotoxic effect and the ^{123}I-MIBG-positive rates appeared to be in an adverse relationship. Tumors that showed Ef1a or Ef1b effect tended to be ^{123}I-MIBG positive as compared to Ef2 and Ef3. On the other hand, the degree of differentiation had no apparent correlation to preoperative ^{123}I-MIBG-positive rates. The data suggests that the ^{123}I-MIBG uptake is rather dependent on the amount of viable NB cells within the tumor, regardless of the differentiation status of the NB component.

Discussion

Histological evaluation of the primary tumor in untreated advanced stage NB is mandatory for risk stratification in the current standard treatment strategies. Besides being a well-established and universally approved pathological classification, INPC also serves as a powerful predictor of

Table 5 Result of preoperative ^{123}I-MIBG scintigraphy and histological findings in tumors after treatment ($n = 21$)

	^{123}I-MIBG uptake	
	Positive	Negative
Effect criteria		
Ef1a	2	0
Ef1b	6	2
Ef2	4	5
Ef3	0	2
Resected tumor INPC		
Undifferentiated NB	1	1
Poorly diff. NB	2	0
Differentiated NB	4	1
GNB intermixed	4	4
GN maturing	1	1
No viable tumor cell	0	2

prognosis of the patients [10]. In contrast, there are only several reports in the literature focusing on the clinical value of tumor histology after the use of chemotherapy [11–14].

In the current study, we aimed to clarify the clinical impact of the histological characteristics of tumors resected at second-look surgeries. The prominent feature of our cohort is that it includes patients treated with the "delayed local treatment" policy, a multimodal treatment strategy in which the radical resection of the tumor is withheld until after the completion of all courses of chemotherapy including HDC. Local radiotherapy follows the surgery, but as a general rule, no postoperative chemotherapy courses are given. The strategy aims to avoid the prolongation of the chemotherapy intervals that could possibly be caused by performing surgery and/or radiotherapy in between the chemotherapy courses, and to consequently intensify the "dose per period" of the systemic treatment [15]. An increasing number of institutions in Japan, including ours, have adopted this strategy during the recent decade, and a nationwide multicenter group JNBSG is currently conducting a clinical study to test the feasibility and the effect of this treatment strategy. However, the strategy has raised two novel questions that had not been discussed in the past: (1) is a tumor left behind after HDC still viable? (2) if so, how would it behave afterwards? To answer these questions, we analyzed the histological features in a cohort that included patients treated with the conventional (STD) and the novel (DL) approach to compare the treatment outcomes between the two groups.

Tumor necrosis was seen in a various degree in our study, but the amount of necrosis did not correlate to the outcome of the patient. There have been reports demonstrating that increased tumor necrosis in response to preoperative chemotherapy correlates to the favorable prognosis of the patient in childhood solid tumors, such as Ewing sarcoma [20, 21], Wilms tumor [22] and osteosarcoma [23]. In neuroblastoma, the amount of necrosis in the tumor after treatment has been reported to be indicative of a better prognosis by some investigators [11, 12, 14], while Bomken et al. [13] stated that patients with tumors showing <90 % necrosis did better than those with >90 % necrosis in the post-treatment tumor. The authors indicated the possible contribution of *MYCN* amplification, which was in close correlation with post-treatment necrosis [13]. George et al. [14] commented in their recent publication that *MYCN* amplification was positively correlated with >10 % tumor necrosis at resection in their study, but these patients had an improved prognosis as compared to those with <10 % tumor necrosis. Interestingly, in our study there were eleven out of 26 cases with *MYCN* amplification, and the only two tumors that showed Ef3 effect (complete eradication of viable neuroblasts) were *MYCN* amplified. Five *MYCN*-amplified tumors showed Ef2 effect. Thus, it seems apparent that *MYCN*-amplified tumors have a trend to show more necrotic change within the post-treatment tumors, which is in agreement with the previous studies [13, 14]. Whether the amount of necrosis in the resected tumor is prognostic or not remains controversial, owing to a number of factors, including the difference in treatment intensity, timing of operation, and the cutoff of evaluation of necrotic tissue. The true value of amount of necrosis remains to be clarified by analyzing a larger cohort in a prospective manner.

Maturation of neuroblastomas into ganglioneuromatous tumors in response to chemotherapy is a well-known phenomenon. We evaluated the maturation status of post-treatment tumors adopting the INPC criteria. As a result of chemotherapy, there was an upshift toward a more differentiated phenotype in 19 of the 26 tumors. There were only four cases in which the post-chemotherapeutic phenotype was undifferentiated NB or poorly differentiated NB. Three of the four cases had an extremely poor clinical course after the surgery despite the continuation of standard postoperative multimodal therapy. This suggests that, rather than the amount of necrotic tissue within the tumor, the differentiation status of the remaining clusters of neuroblasts within the tumor tissue was more valuable as a prognostic factor in our cohort. The current study contrasts with findings in the report by George and colleagues, in which the degree of differentiation within the resected tumor (undifferentiated/poorly differentiated NB versus differentiating NB) did not have significant impact on the survival of the patients [14]. It is notable though, that in their study, 15 out of 40 cases remained in the undifferentiated/poorly differentiated NB categories after induction chemotherapy. The discrepancy between our study and theirs may have

been caused by the different periods that the patients were treated, or the different induction protocols, or the number of courses used before the surgery. Despite the negative results regarding tumor differentiation, George and colleagues found that the mitosis-karyorrhexis index (MKI) is strongly correlated to the prognosis of the patients [14]. The authors' finding, along with ours, suggests that the phenotype of the remaining tumor cell cluster after induction chemotherapy is critical for predicting the further clinical course of the patients, and additional or substantial treatment might be necessary for those that have aggressive phenotypes remaining, since these cells are likely to represent chemoresistance.

Tumors in the DL group had a trend to have a larger amount of necrosis as compared to the STD group. The intensity of chemotherapy is reported to be proportional to the amount of necrosis from previous studies [11, 12]. Our result suggests that the megadose treatment further added cytotoxic effects against the remaining tumor. However, in seven out of nine cases in the DL group, viable tumor cells were still evident. This indicates that local treatment including surgery and radiotherapy is mandatory in most cases despite the intensification of systemic chemotherapy. There is a concern that the presence of viable tumor cells at the end of chemotherapy would be a high risk of recurrence. However, the recurrence rate as well as the overall survival was not inferior in the DL group as compared to the STD group in the current study. This is in agreement with the previous report by Hashii and colleagues reporting a favorable outcome of patients treated with the "delayed local treatment" policy [15]. At this point, we do not have any firm data to know whether presence of viable tumor cells left after HDC should be a target of further treatment, but from the results of the current study, we would suggest that at least those with undifferentiated/poorly differentiated NB phenotypes after HDC may be at a high risk of recurrence and should undergo further treatment. The results of a large-scale study are warranted to clarify the clinical impact of the features of tumors resected after HDC.

Finally, we investigated the relation between preoperative ^{123}I-MIBG uptake and histological findings. Our results revealed a discrepancy in between the ^{123}I-MIBG uptake and the histological maturation of the tumor (Table 5). The uptake was evenly observed in roughly half of tumors regardless of the degree of differentiation. On the other hand, ^{123}I-MIBG uptake appeared to be correlated with the cytotoxic effect evaluated by the amount of necrosis within the tumor, though this trend was not statistically supported. It is likely that in post-treated tumors, the gross uptake decreases significantly compared to pretreated tumors, owing to the shrinkage of the tumor and the increased necrosis within the tumor. In this situation, we consider that the visible uptake would be significantly affected by the volume of the tumor and the number of viable tumor cells remaining within the tumor. Caution is therefore needed when evaluating the tumor with ^{123}I-MIBG scintigraphies after chemotherapy, since negative uptake may not guarantee the disappearance of tumor cells with unfavorable phenotypes, and the persistence of ^{123}I-MIBG-uptaking tumors not necessarily indicates the presence of aggressive tumor cells.

The weakness of the current study is that, as a nature of a retrospective institutional review, the details of treatment vary and the statistical power is low. Our findings require verification by precisely analyzing the histology of the post-treatment tumor specimens of high-risk neuroblastoma treated in a multicenter prospective clinical trial.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Bolande RP (1974) The neurocristopathies: a unifying concept of disease arising in neural crest maldevelopment. Hum Pathol 5:409–429
2. Kreissman SG, Seeger RC, Matthay KK, London WB, Sposto R, Grupp SA, Haas-Kogan DA, Laquaglia MP, Yu AL, Diller L, Buxton A, Park JR, Cohn SL, Maris JM, Reynolds CP, Villablanca JG (2013) Purged versus non-purged peripheral blood stem-cell transplantation for high-risk neuroblastoma (COG A3973): a randomised phase 3 trial. Lancet Oncol 14:999–1008. doi:10.1016/S1470-2045(13)70309-7
3. Pearson AD, Pinkerton CR, Lewis IJ, Imeson J, Ellershaw C, Machin D (2008) High-dose rapid and standard induction chemotherapy for patients aged over 1 year with stage 4 neuroblastoma: a randomised trial. Lancet Oncol 9:247–256
4. Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B (1999) Terminology and morphologic criteria of neuroblastic tumors: recommendations by the International Neuroblastoma Pathology Committee. Cancer 86:349–363
5. Peuchmaur M, d'Amore ESG, Joshi VV, Hata J, Roald B, Dehner LP, Gerbing RB, Stram DO, Lukens JN, Matthay KK, Shimada H (2003) Revision of international neuroblastoma pathology classification: confirmation of favorable and unfavorable prognostic subsets in ganglioneuroblastoma, nodular. Cancer 98:2274–2281
6. Evans AE, D'Angio GJ, Propert K, Anderson J, Hann HW (1987) Prognostic factors in neuroblastoma. Cancer 59:1853–1859
7. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM (1984) Amplifications of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224: 1121–1124
8. Brodeur GM, Pritchard J, Berthold F et al (1993) Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 11:1466–1477
9. Look AT, Hayes FA, Shuster JJ, Douglass EC, Castleberry RP, Bowman LC, Smith EI, Brodeur GM (1991) Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a pediatric oncology group study. J Clin Oncol 9:581–591
10. Sano H, Bonadio J, Gerbing RB, London WB, Matthay KK, Lukens JN, Shimada H (2006) International neuroblastoma

pathology classification adds independent prognostic information beyond the prognostic contribution of age. Eur J Cancer 42:1113–1119
11. Miyauchi J, Matsuoka K, Oka T, Kamii Y, Honna T, Bessho F, Sasaki S, Melanowska J, Tsuchida Y (1997) Histopathologic findings of advanced neuroblastoma after intensive induction chemotherapy. J Pediatr Surg 32:1620–1623
12. Tsuchida Y, Miyauchi J, Kuroiwa M, Suzuki N, Sakamoto J, Suzuki M, Shitara T (2005) Histologic survey of neuroblastomas after intensive induction chemotherapy. Pediatr Blood Cancer 45:656–662
13. Bomken S, Davies B, Chong L, Cole M, Wood KM, McDermott M, Tweddle DA (2010) Percentage tumor necrosis following chemotherapy in neuroblastoma correlates with MYCN status but not survival. Pediatr Hematol Oncol 28:106–114. doi:10.3109/08880018.2010.526684
14. George RE, Perez-Atayde AR, Yao X, London WB, Shamberger RC, Neuberg D, Diller L (2012) Tumor histology during induction therapy in patients with high-risk neuroblastoma. Pediatr Blood Cancer 59:506–510. doi:10.1002/pbc.24013
15. Hashii Y, Kusafuka T, Ohta H, Yoneda A, Osugi Y, Kobayashi Y, Fukuzawa M, Hara J (2008) A case series of children with high-risk metastatic neuroblastoma treated with a novel treatment strategy consisting of postponed primary surgery until the end of systemic chemotherapy including high-dose chemotherapy. Pediatr Hematol Oncol 25:439–450. doi:10.1080/08880010802104601
16. Shulkin BL, Shapiro B (1998) Current concepts on the diagnostic use of MIBG in children. J Nucl Med 39:679–688
17. Yanik GA, Parisi MT, Shulkin BL, Naranjo A, Kreissman SG, London WB, Villablanca JG, Maris JM, Park JR, Cohn SL, McGrady P, Matthay KK (2013) Semiquantitative mIBG scoring as a prognostic indicator in patients with stage 4 neuroblastoma: a report from the Children's oncology group. J Nucl Med 54:541–548. doi:10.2967/jnumed.112.112334
18. Fendler WP, Melzer HI, Walz C, von Schweinitz D, Coppenrath E, Schmid I, Bartenstein P, Pfluger T (2013) High ^{123}I-MIBG uptake in neuroblastic tumours indicates unfavourable histopathology. Eur J Nucl Med Mol Imaging 40:1701–1710. doi:10.1007/s00259-013-2491-y
19. Kaneko M, Tsuchida Y, Uchino J, Takeda T, Iwafuchi M, Ohnuma N, Mugishima H, Yokoyama J, Nishihira H, Nakada K, Sasaki S, Sawada T, Kawa K, Nagahara N, Suita S, Sawaguchi S (1999) Treatment results of advanced neuroblastoma with the first Japanese study group protocol. Study Group of Japan for Treatment of Advanced Neuroblastoma. J Pediatr Hematol Oncol 21:190–197
20. Wunder JS, Paulian G, Huvos AG, Heller G, Meyers PA, Healey JH (1998) The histological response to chemotherapy as a predictor of oncological outcome of operative treatment of Ewing sarcoma. J Bone Joint Surg Am 80:1020–1033
21. Picci P, Rougraff BT, Bacci G, Neff JR, Sangiorgi L, Cazzola A, Baldini N, Ferrari S, Mercuri M, Ruggieri P, Caldora P, Benassi MS, Fabbri N, Monti C, Campanacci M (1993) Prognostic significance of histopathological response to chemotherapy in nonmetastatic Ewing's sarcoma of the extremities. J Clin Oncol 11:1763–1769
22. Boccon-Gibod L, Rey A, Sandstedt B, Delemarre J, Harms D, Vujanic G, De Kraker J, Weirich A, Tournade MF (2000) Complete necrosis induced by preoperative chemotherapy in Wilms tumor as an indicator of low risk: report of the international society of paediatric oncology (SIOP) nephroblastoma trial and study 9. Med Pediatr Oncol 34:183–190
23. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, Zoubek A, Jürgens H, Winkler K (2002) Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20:776–790

Cisplatin-induced Hearing Loss: The Need for a Long-term Evaluating System

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Summary: Cisplatin is an effective chemotherapeutic agent against pediatric cancers; however, ototoxicity is a concern. This study describes the frequency, severity, and clinical course of hearing loss in Japanese pediatric patients treated with cisplatin-based multimodal therapy. A total of 55 children who received cisplatin-based therapy from 1983 to 2012 underwent audiologic evaluations. Data were analyzed to determine the onset, time-to-progression, and severity of hearing loss. Thirty-five patients, 12 of 16 older patients (4 y or older), and 23 of 39 younger patients (under 4 y), including 7 of 8 patients treated with cisplatin, carboplatin, and radiotherapy, developed hearing loss. Ten of 18 patients who received a cumulative cisplatin dose of $<360\,mg/m^2$ developed hearing loss at a minimum dose of $200\,mg/m^2$. Median time to onset after the last cisplatin dose was 71 days; 6 patients developed hearing loss after ≥ 2 years. Four patients required hearing aids, 6 patients developed progressive hearing loss with time, and 4 patients exhibited persistent hearing failure at low frequencies. Risk factors for acquired hearing loss and its severity may be associated with a combination of factors such as cisplatin and carboplatin therapy, radiotherapy, age at diagnosis, and genetic background. Our results suggested that all pediatric patients treated with cisplatin would have their hearing evaluated regularly, irrespective of the cumulative cisplatin dose as a suggestion, and that further prospective studies regarding ototoxicity including genetic polymorphisms analysis were required.

Key Words: cisplatin, carboplatin, hearing loss, radiation, solid tumor

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Although successful treatment of pediatric malignancy by multimodal therapy has improved the outcome of children with cancer, we need to pay attention to the late effects of anticancer therapies. Cisplatin is an effective chemotherapeutic agent against pediatric cancers and has contributed to the increased long-term survival of children with cancer.[1] Particularly, cisplatin plays an important role in the treatment of neuroblastoma, hepatoblastoma, osteosarcoma, germ-cell tumor, brain tumors, and retinoblastoma. However, the use of cisplatin causes adverse effects including nephrotoxicity and ototoxicity. Although the nephrotoxicity of cisplatin can be decreased by hyperhydration,[2] ototoxicity is usually permanent with no treatment or preventive measures established to-date. Hearing loss can influence speech and language development, educational attainment, and socialization, particularly in young children.[3] The incidence of cisplatin-induced hearing loss in children ranges from 22% to 70%.[4–7] Cisplatin-induced ototoxicity is characterized by bilateral sensorineural hearing loss.[3,4,7] Initially, hearing at high frequencies (4 to 8 kHz) is affected, and this can subsequently progress toward hearing loss at lower frequencies (1 to 2 kHz). Moreover, there is some evidence that hearing loss may be progressive, even after cisplatin administration has been terminated.[8] Among patients treated with cisplatin, the major risk factors associated with hearing loss are younger age, higher cumulative cisplatin dose (above 360 to $400\,mg/m^2$),[9,10,11] central nervous system tumors, and concomitant central nervous system radiation.[3] Carboplatin is also reported to cause ototoxicity when administered in combination with cisplatin or in high doses.[5,7,12,13] In contrast, cisplatin-induced ototoxicity exhibits significant individual variability; the audibility of some patients treated with cisplatin is not affected.[14,15]

Recently, Japanese patients were reported to be more susceptible to cisplatin-induced ototoxicity.[16] To evaluate the course, incidence, and severity of hearing loss after cisplatin treatment, we conducted a retrospective study of cisplatin-induced ototoxicity in children with cancer treated at the Saitama Children's Medical Center. To the best of our knowledge, this is the largest pediatric case study reported to date in Japan.

PATIENTS AND METHODS

The medical records of pediatric patients diagnosed with cancer and treated with cisplatin-based chemotherapy between 1983 and 2012 at the Saitama Children's Medical Center were retrospectively reviewed. Patients were excluded if age at diagnosis was over 18 years or if audiograms had not been performed. Fifty-five patients fulfilled the study's inclusion criteria.

Audiometric assessment was performed by pediatric otolaryngologists. The method of evaluation was selected on the basis of age, developmental status of patients, ability to cooperate, and status of the disease. Conventional audiometry, conditioned play audiometry, and visual reinforcement audiometry were used to measure pure tone thresholds. Bone conduction audiometry was performed for patients with an abnormal air conduction threshold if the patient's cooperation was obtained. Frequency-specific auditory brainstem response testing or the measurement of distortion product otoacoustic emissions were also carried out as adjunctive tests for very young or very ill patients.

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