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Combined Genetic and Chromosomal Characterization of Wilms Tumors Identifies Chromosome 12 Gain as a Potential New Marker Predicting a Favorable Outcome[1,2]

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Abstract

To identify prognostic factors, array CGH (aCGH) patterns and mutations in *WT1* and 9 other genes were analyzed in 128 unilateral Wilms tumors (WTs). Twenty patients had no aCGH aberrations, and 31 had *WT1* alterations [silent and *WT1* types: relapse-free survival (RFS), 95% and 83%, respectively]. Seventy-seven patients had aCGH changes without *WT1* alterations (nonsilent/non-*WT1* type) and were subtyped into those with or without +12, 11q−, 16q−, or *HACE1* loss. RFS was better for those with than those without +12 ($P = .010$) and worse for those with than those without 11q−, 16q−, or *HACE1* loss ($P = .001$, .025, or 1.2E-04, respectively). Silent and *WT1* type and 8 subtype tumors were integrated and classified into 3 risk groups: low risk for the silent type and +12 subgroup; high risk for the no +12 plus 11q−, 16q−, or *HACE1* loss subgroup; intermediate risk for the *WT1* type and no +12 plus no 11q−, 16q−, or *HACE1* loss subgroup. Among the 27 WTs examined, the expression of 146 genes on chromosome 12 was stronger in +12 tumors than in no +12 tumors, while that of 10 genes on 16q was weaker in 16q− tumors than in no 16q− tumors. Overexpression in 75 out of 146 upregulated genes and underexpression in 7 out of 10 downregulated genes correlated with better and worse overall survival, respectively, based on the public database. +12 was identified as a potential new marker predicting a favorable outcome, and chromosome abnormalities may be related to altered gene expression associated with these abnormalities.

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Abbreviations: WT, Wilms tumor; ROI, retention of imprinting; LOI, loss of imprinting; UPD, uniparental disomy; LOH, loss of heterozygosity; TSG, tumor suppressor gene; RFS, relapse-free survival; OS, overall survival; aCGH, array comparative genomic hybridization; miRNAPG, microRNA processing gene.

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Introduction

Wilms tumor (WT) is one of most common malignancies of childhood, occurring in 1 out of 10,000 children and accounting for 8% of childhood cancers [1,2]. It is considered to result from the malignant transformation of abnormally persistent renal stem cells that retain embryonic differentiation potential [3]. Modern treatments for patients with WT are based on the risk of relapse using variables such as age at diagnosis, histology, and the presence of metastatic disease, and overall survival (OS) rates have reached approximately 90% [2]. However, there are some groups of patients with relapse-free survival (RFS) rates less than 75%, i.e., patients with an anaplastic histology, bilateral tumors, and recurrent tumors of a favorable histology [2]. Furthermore, the high cure rate for WT leads to a new issue in that 25% of survivors have serious chronic health conditions 25 years from their diagnosis [4].

Biomarkers that predict favorable or unfavorable outcomes are needed to stratify patients for further outcome improvements and avoid adverse late effects. The Children's Oncology Group (COG) currently uses a loss of heterozygosity (LOH) at chromosome arms 1p and 16q for the risk stratification of patients; however, a small percentage of WTs show LOH at 1p and/or 16q, and many patients without this marker have unfavorable outcomes [5]. Therefore, novel molecular markers are needed to identify WTs with a favorable histology with favorable or unfavorable outcomes. COG and the International Society of Pediatric Oncology (SIOP) both recently reported that 1q gain may be a good biomarker for predicting unfavorable outcomes, and studies stratifying patients using 1q gain as a biomarker are currently underway [6,7]. In addition, intratumor genetic heterogeneity fosters tumor evolution and may confer resistance to cancer therapy [8]. Cresswell and others reported that 1q+ is heterogeneous in the majority of WTs with this change, with variable evolutionary timing, emphasizing the importance of multi-sampling for reliable evaluation of biomarkers [9].

By analyzing >8200 tumor-normal pairs, Davoli and others found that the distribution and potency of tumor suppressor genes (TSGs), oncogenes, and essential genes on chromosomes predicted the complex patterns of aneuploidy and copy number variation characteristics of cancer genomes [10]. They further demonstrated that somatic copy number alterations (SCNAs) in cancer genomes may be selected during tumor evolution through cumulative haploinsufficiency for deletions and cumulative triplosensitivity for amplifications. Since an array comparable genomic hybridization (aCGH) analysis detects whole genomic aberrations, studies on aCGH and their clinical correlation may have a prognostic impact on cumulative genomic aberrations. Some studies have attempted to identify chromosomal aberrations as biomarkers predicting the outcomes of patients with WT using aCGH; however, the number of patients in each study was 77 or less, and consistent findings have not yet been attained [11–13].

We examined 128 unilateral WTs using single-nucleotide polymorphism (SNP) aCGH, 27 WTs using expression microarrays, and newly identified chromosomal biomarkers predicting favorable or unfavorable outcomes. We also tried to clarify the relationship between gene expression and chromosome abnormalities that may be causally associated with patients' outcomes.

Materials and Methods

Patients and Samples

One hundred and twenty-eight unilateral WT samples were obtained from 128 Japanese patients ranging in age between 2 months and 15 years who underwent surgery or biopsy between December 1987 and August 2015. Of the 128 patients, 42 registered before March 1996 were mostly treated with NWTS-3 or -4 protocol using regimen L or EE for tumors at stage I, regimen K or K-4A for tumors at stage II, and regimen DD or DD-4A with radiotherapy for tumors at stages III/IV [14,15], and 86 registered after March 1996 were treated according to NWTS-5 protocol [5]. Outcomes of the two cohorts of patients were examined and described in the results section.

In addition, 31 *WT1*-mutant bilateral WTs from 23 patients, whose genetic and clinical characteristics were reported previously by our group [16], were included for the *CTNNB1* analyses. Only 128 unilateral WTs were included in the study of the prognostic implications of molecular markers. Specimens were supplied by the tissue bank of the Japan Wilms Tumor Study [17] or directly sent to the Saitama Cancer Center for cytogenetic and molecular genetic analyses from several Japanese institutions. Pathologists in each institution verified that each sample for the molecular genetic analysis contained 70% or more tumor cells. Normal samples were obtained from either peripheral blood or normal renal tissues adjacent to the tumor. The study design was approved by the Ethics Committee of the Saitama Cancer Center. The clinical stage of the disease was assessed at the time of initial surgery or biopsy according to the classification of the Japanese Society of Pediatric Surgeons [18]. The therapeutic strategy was similar to that of the NWTS protocols [2,5,18,19]. As a basic principle, all patients initially underwent nephrectomy, and preoperative chemotherapy was administered after biopsy when the tumor appeared to be unresectable. Postoperative chemotherapy was performed for all but two patients who were younger than 2 years of age with stage I WT of a favorable histology weighing less than 550 g and underwent surgery in 1998.

Three patients at stage IV received open biopsy before preoperative chemotherapy, and their biopsied materials were used for the study. Nine patients at various stages received preoperative chemotherapy, and their tumor samples which showed abnormal aCGH patterns were included in the present study. In addition, 5 patients at stage III or IV who received preoperative chemotherapy and showed a normal aCGH pattern in tumors were not included in 128 patients of the study.

Histological Examination

In all cases, the diagnosis of WT was made with routine hematoxylin and eosin–stained slides by the pathology panel of Japan Wilms Tumor Study or pathologists at each institution according to the classification proposed by the Japanese Pathological Society and/or the NWTS pathology panel [20,21]. Five tumors (3.9%) with an anaplastic histology (diffuse 4, focal 1) were included among 128 tumors for the reason described in the Results section, and the other 123 tumors showed a favorable histology.

Analysis of Copy Numbers and LOH Using SNP Arrays

High-resolution SNP arrays, Affymetrix Mapping 250K-Nsp arrays (Affymetrix, Santa Clara, CA), were used to analyze the chromosomal copy numbers and LOH status of 128 unilateral and 31 bilateral tumors, as described previously [16]. Copy numbers and LOH were calculated using CNAG and AsCNAR programs with paired or anonymous references as controls [22,23].

Analysis of WT1, CTNNB1, WTX, DROSHA, DICER1, DGCR8, SIX1, SIX2,MYCN, and TP53 Abnormalities and the IGF2 Status

We examined *WT1*, *WTX*, *CTNNB1*, and *TP53* abnormalities using MLPA (P118-C1 WT1, MRC-Holland) and/or an SNP array,

and sequencing as previously described [24,25]. Mutations in *DROSHA*, *DICER1*, *DGCR8*, *SIX1*, *SIX2*, and *MYCN* were analyzed by sequencing using the primers listed in Supplementary Table 1. The loss of imprinting (LOI), uniparental disomy (UPD), and retention of imprinting (ROI) of *IGF2* were analyzed as previously described, and all UPDs of *IGF2* were found to be of the paternal origin [24].

Gene Expression Analysis

Samples were hybridized to the Affymetrix GeneChipR Gene 1.0ST Array System for Humans, scanned, subjected to quality control standards, and normalized as previously described [26].

Statistical Analysis

Patients were grouped according to various biological and clinical aspects of the disease. The significance of differences in characteristics between groups was examined using the chi-squared or Fisher's exact test, Student's *t* test, and Welch's *t* test.

RFS was defined as the time from the date of registration until relapse or death due to any cause. OS was defined as the time from the date of registration to death from any cause. Survival functions for RFS and OS were obtained by the Kaplan-Meier method and compared using the log-rank test. We used Statcel 3 software (OMS publisher Co., Tokorozawa, Japan) for the statistical analysis. The influence of various biological and clinical factors on OS rates was estimated using Cox's proportional-hazards model calculated with Stat Flex software for Windows, version 5.0 (Artec Co., Osaka, Japan).

We used the *limma* package to define differentially expressed genes [27]. *P* values were calculated by the eBayes-moderated *t* test and then corrected by the Benjamini-Hochberg method [28]. The criterion of differentially expressed gene was a *q* value <0.3.

Results

Genetic and Chromosomal Abnormalities in 128 Unilateral WTs

Mutations and deletions in 6 WT-associated genes (*WT1*, *CTNNB1*, *WTX*, *MYCN*, *SIX1*, and *SIX2*) were found at various percentages in 128 unilateral WTs (Figures 1-3, Table 1): *WT1* alterations (deletion + mutation) in 31, *CTNNB1* mutations in 28, *WTX* alterations in 34 (32 with deletions and 2 with mutations, p.Q10H/c.30G > T, or p.R353*/c.1057C > T), *MYCN* alterations in 11 (10 with gain and 1 with a mutation, p.P44L/c.131C > T), a *SIX1* (p.Q177R/c.530A > G) mutation in 4, and *SIX2* (p.Q177R/c.530A > G) mutation in 1. miRNA processing genes (miRNAPGs), including *DGCR8*, *DICER1*, *DIS3L2*, and *DROSHA*, were deleted in 11 tumors, and *DROSHA* (p.E1147K/c.3439G > A) was mutated in 2. The miRNA genes *LET7A1*, *LET7A2*, and *LET7A3* were deleted in 3, 15, and 3 tumors, respectively, and 18 tumors had 1 or 2 of these deletions.

Among the 128 WTs, 1q gain was found in 36 tumors, +12 in 34, +7/7q+ in 31, +13 in 20, +20/20q gain in 16, +6/6q gain in 14, 1p– in 12, 11q– in 10, 16q– in 9, 7p– in 8, 17p–/–17 in 6, and a focal deletion including *HACE1* in 4 (Table 1 and Supplementary Figures 1 and 2).

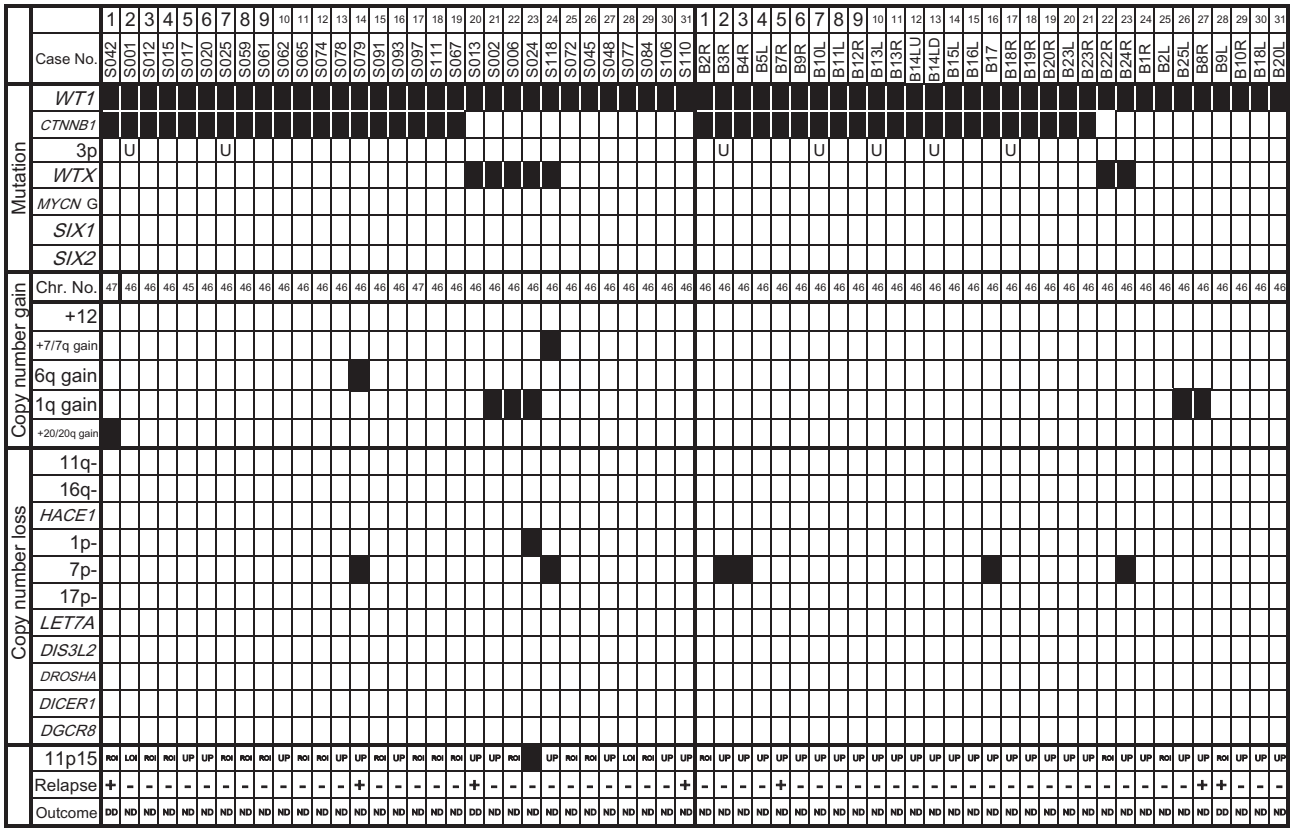

Figure 1. Genetic and chromosomal aberrations in 31 unilateral and 31 bilateral WTs with *WT1* alterations. Black squares indicate the presence of mutations or copy number gains, and gray squares indicate the presence of copy number losses. Ho in the 7p— lane indicates a focal homozygous deletion. Chr. No., chromosome number; U, uniparental disomy; UP, uniparental disomy of 11p; LOI, loss of *IGF2* imprinting; ROI, retention of *IGF2* imprinting; +, the patient relapsed; —, the patient did not relapse; DD, died of disease, ND, no evidence of disease.

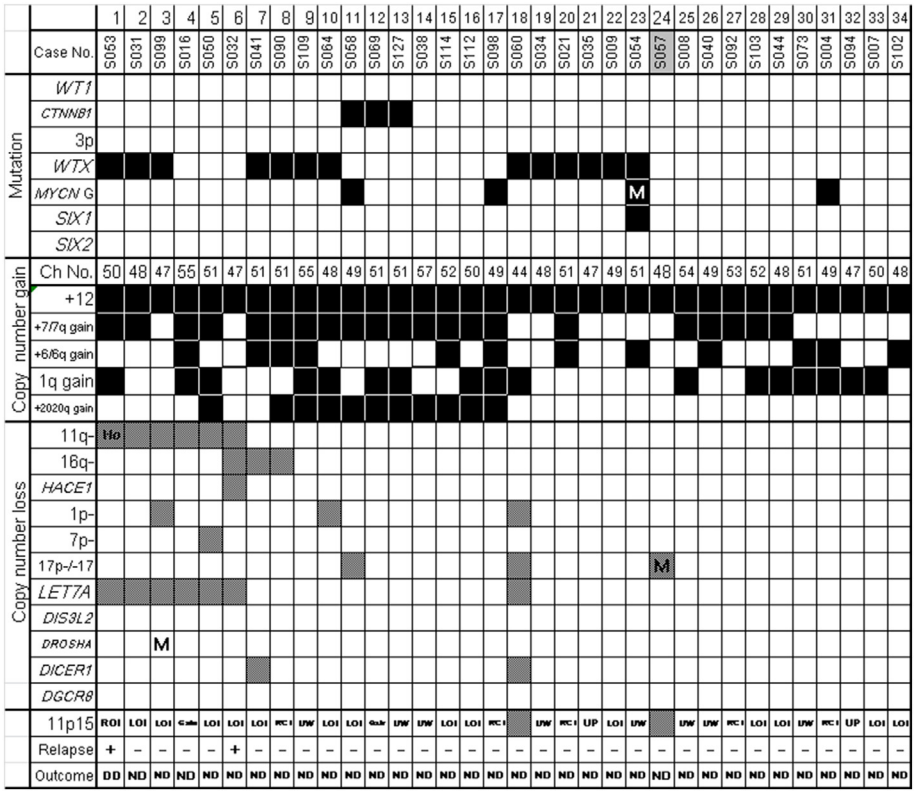

Figure 2. Genetic and chromosomal aberrations in 34 unilateral WTs with +12. A case number in the shaded box indicates that the tumor had a diffuse anaplastic histology. Black squares indicate the presence of mutations or copy number gains, and gray squares indicate the presence of copy number losses. Black squares and M in the *MYCN*-G lane indicate gain and mutation, respectively. Ho in the 11q− lane indicates a focal homozygous deletion. M in the 17p−/−17 lane indicates a *TP53* mutation. UW, uniparental disomy of the whole chromosome 11; UP, uniparental disomy of 11p; ROI, retention of *IGF2* imprinting; LOI, loss of *IGF2* imprinting; Gain, a gain of the 11p15 region; +, the patient relapsed; −, the patient did not relapse; DD, died of disease, ND, no evidence of disease.

We excluded tumors with −11, UPD on whole chromosome 11 (UPD11), or UPD on 11q (UPD11q) from those with 11q− and tumors with −16, UPD16, or UPD16q from those with 16q− because whole chromosome and chromosome arm deletions may be of different biological significance, and loss and UPD may also result in different biological consequences in tumors.

Five out of 128 WTs were classified as having an anaplastic histology (diffuse 4: S036, S057, S089, S122; focal 1: S125, shown in Figures 2 and 3, and Supplementary Table 2). Three tumors with diffuse anaplasia had 17p− or −17, and a sequencing analysis of exons 2 to 10 of *TP53* showed a missense mutation in exon 7 (p.R248W/c.742C > T), another missense mutation in exon 7 (p.D281H/c.841G > C), or a splice site mutation in intron 8 (c. 920-2A > G) in one each. The other two tumors, including one with diffuse anaplasia and one with focal anaplasia, all with normal chromosome 17, showed wild-type *TP53*.

These 5 tumors were included in the present study on 128 tumors because genetic and chromosomal changes, except for the frequent occurrence of *TP53* mutations, in diffuse anaplastic tumors were similar between 5 tumors with an anaplastic histology and 123 tumors with a favorable histology. Besides, the aim of the study is to identify genetic and chromosomal markers that predict outcomes.

RFS and OS Rates in 128 Patients Classified by Clinical, Genetic, and Chromosomal Characteristics

No difference in RFS and OS was found between 42 and 86 patients who were registered before and after May 1, 1996 (P = .990; P = .426), although if we included patients only at stages III and IV for an outcome analysis, OSs were slightly better for patients registered after May 1, 1996, than those before (P = .092) (Table 1).

Patients aged 24 months or older had worse OS than those younger than 24 months (P = .019) (Table 1). Patients at stage IV had worse or slightly worse RFS and OS rates than those at stages I, II, and III (P = .006; P = .065). Significant differences were observed in RFS and OS between 5 patients with anaplastic tumors and 123 with favorable histology tumors (P = .027 and P = 1.2E-05) (Table 1).

Patients with *WTX* alterations in tumors had slightly worse OS rates than those without (P = .070). No significant differences in RFS and OS rates were observed between patients with *WT1* alterations, *CTNNB1* mutations, miRNAPG alterations (*DIS3L2* deletion, *DROSHA* mutation/deletion, *DICER1* mutation/deletion, and *DGCR8* deletion), *MYCN* alterations (gain and mutation), or *SIX1*/*SIX2* mutations and those without the respective alterations.

Patients with 11q− or 16q− in tumors had worse RFS and OS rates than those without (RFS, P = 4.9E-04 and .010; OS, P = 4.4E-06 and .006, respectively). Patients with *HACE1* loss in tumors had worse RFS rates than those without (P = 4.1E-05). Patients with +20/20q gain had worse OS rates than those without (P = .003), although no significant differences were observed in the RFS rate (P = .281). No significant differences were noted in RFS and OS rates between patients with and without 1q gain (P = .515 and .456) (Figure 4, *A* and *B*). Patients with +12 in tumors had slightly better RFS and OS rates than those without (P = .062 and .242). No

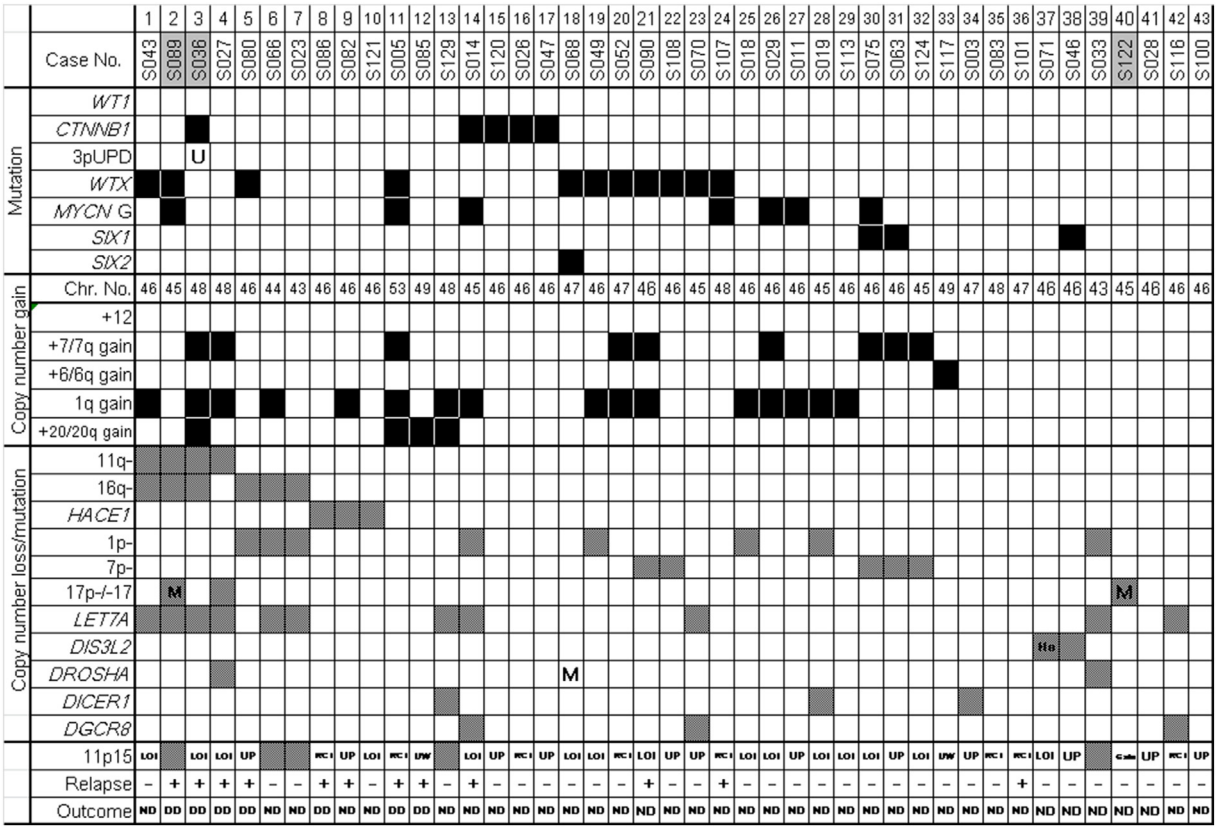

Figure 3. Genetic and chromosomal aberrations in 43 unilateral WTs without *WT1* alterations and +12. Case numbers in the shaded box indicate that the tumors had a diffuse anaplastic histology. Black squares indicate the presence of mutations or copy number gains, and gray squares indicate the presence of copy number losses. M in the 17p−/−17 lane indicates a *TP53* mutation. Ho in the *DIS3L2* lane indicates a focal homozygous deletion. M in the *DROSHA* lane indicates a mutation. U, uniparental disomy; UP, uniparental disomy of 11p; UW, uniparental disomy of the whole chromosome 11; ROI, retention of *IGF2* imprinting; LOI, loss of *IGF2* imprinting; +, the patient relapsed; −, the patient did not relapse; DD, died of disease, ND, no evidence of disease.

significant differences were observed in RFS and OS rates between patients with or without +7/7q gain, those with or without +6/6q gain, those with and without 1p−, and those with or without 7p−.

Three Types and Eight Subtypes of WTs Classified by Genetic and Chromosomal Findings

Among 128 unilateral WTs, 108 tumors had some aCGH abnormalities (gain, loss, and UPD), while the other 20 had no abnormalities (silent type) (Supplementary Table 2). *WT1* is a master gene in kidney development and the most common WT predisposing gene [3,29]. Furthermore, 31 tumors with *WT1* alterations had some aCGH aberrations, and their abnormal patterns were distinct from the other 77 tumors (Supplementary Table 3 and Figures 1-3). Thus, the 108 tumors were subclassified into 31 tumors with *WT1* alterations (*WT1* type) and 77 without (nonsilent/non-*WT1* type).

Among various aCGH aberrations, +12, +20/20q gain, 11q−, 16q−, *HACE1* loss, and 17p−/−17 were associated with better or worse RFS or OS rates (Table 1). In addition, +12, 11q−, 16q−, 17p−/−17, and *HACE1* loss were only found in the 77 nonsilent/non-*WT1* tumors. These 77 tumors were further classified into 4 pairs of 2 subtypes (+12 and no +12, 11q− and no 11q−, 16q− and no 16q−, or *HACE1* loss and no *HACE1* loss) (Supplementary Table 3). The presence or absence of 17p−/−17 was excluded from the subtype analysis because a small number of tumors with 17p−/−17 and the prognostic significance of 17p−/−17, which is causally associated with *TP53* alterations, have been reported previously [30].

Clinical Characteristics of Three types and Eight Subtypes of WTs

The median ages of patients with silent-, *WT1*-, and nonsilent/non-*WT1*–type tumors were 7.5, 18, and 44 months, respectively, and showed a similar male to female ratios (10/10, 15/16, and 39/38, respectively). Regarding the stage distribution, silent-type tumors showed earlier stages than *WT1*- (P = .014) or nonsilent/non-*WT1*– (P = .017) type tumors, and *WT1*- and nonsilent/non–*WT1*-type tumors showed a similar stage distribution (P = .589) (Supplementary Table 2). Regarding the 11p15.5 status, i.e., the LOI, ROI including 11p15.5 loss, and UPD of *IGF2*, including 11p15.5 gain, ROI was more frequent in silent-type tumors than in *WT1*- (P = .024) or nonsilent/non–*WT1*- (P = 3.6E-06) type tumors, LOI was more frequent in nonsilent/non-*WT1*–type tumors than in silent- (P = .035) or *WT1*- (P = 4.0E-04) type tumors, and UPD was not found in silent-type tumors, and its frequency was similar between *WT1*- and nonsilent/non–*WT1*-type tumors (P = .392) (Supplementary Table 4). Thus, silent-type tumors were characterized by a younger age, earlier stages, and frequent ROI, whereas nonsilent/non–*WT1*-type tumors were characterized by an older age and frequent LOI, and *WT1*-type tumors were characterized by an intermediate age between the other two types, a similar stage distribution to nonsilent/non–*WT1*-type tumors, and infrequent LOI.

Table 1. RFS and OS Rates in 128 Patients with Unilateral WTs Classified by Clinical, Genetic, and Chromosomal Characteristics

	RFS				OS			
	No. of Patients (No. of Events)	Survival Rates at the Last Follow-Up	95% CI	*P* Value	No. of Patients (No. of Events)	Survival Rates at the Last Follow-Up	95% CI	*P* Value
All patients	128 (20)	0.82	0.75-0.90		128 (10)	0.88	0.81-0.96	
Age								
Low <24 months	59 (7)	0.88	0.79-0.96	.275	59 (1)	0.98	0.94-1	.019
High ≥24 months	69 (13)	0.78	0.66-0.88		69 (9)	0.80	0.69-0.93	
Stage								
I/II/III	113 (14)	0.86	0.79-0.93	.006	113 (7)	0.91	0.84-0.98	.065
IV	15 (6)	0.54	0.25-0.82		15 (3)	0.72	0.44-0.99	
Stage I/II/III/IV								
Registration period								
1987-Feb/1996	42 (7)	0.83	0.71-0.94	.990	42 (5)	0.86	0.75-0.98	.426
Mar/1996-2015	86 (13)	0.80	0.69-0.92		86 (5)	0.88	0.77-0.99	
Stage I/II								
Registration period								
1987-Feb/1996	26 (1)	0.96	0.8-1	.133	26 (0)	1	1-1	.179
Mar/1996-2015	58 (8)	0.81	0.66-0.96		58 (3)	0.90	0.77-1	
Stage III/IV								
Registration period								
1987-Feb/1996	16 (6)	0.63	0.39-0.86	.154	16 (5)	0.65	0.40-0.91	.092
Mar/1996-2015	28 (5)	0.78	0.60-0.96		28 (2)	0.87	0.67-1	
Histology								
Anaplastic (diffuse 4, focal 1)	5 (2)	0.60	0.17-1	.027	5 (2)	0	0-0	1.2E-05
Favorable	123 (18)	0.83	0.76-0.91		123 (8)	0.90	0.84-0.97	
Array CGH								
No aberration	20 (1)	0.95	0.85-1	.191	20 (0)	1	1-1	.219
Aberrations	108 (19)	0.80	0.72-0.89		108 (10)	0.87	0.78-0.95	
WT1 alterations (mutation + deletion)	31 (5)	0.83	0.70-0.97	.966	31 (2)	0.93	0.83-1	.589
No *WT1* alterations	97 (15)	0.82	0.72-0.91		97 (8)	0.86	0.76-0.96	
CTNNB1								
Mutation	28 (4)	0.84	0.70-0.99	.831	28 (1)	0.96	0.87-1	.326
Wild-type	100 (16)	0.82	0.73-0.90		100 (9)	0.86	0.76-0.95	
WTX alterations (deletion + mutation)	34 (7)	0.75	0.58-0.93	.417	34 (5)	0.79	0.59-0.98	.070
No *WTX* alterations	94 (13)	0.85	0.77-0.93		94 (5)	0.91	0.84-99	
miRNAPG alterations (mutation + deletion)	12 (3)	0.74	0.49-0.99	.350	12 (1)	0.91	0.74-1	.948
No miRNAPG alterations	116 (17)	0.83	0.75-0.91		116 (9)	0.88	0.79-0.96	
SIX1/*SIX2* mutation	5 (1)	0.80	0.45-1	.715	5 (0)	1	1-1	.498
No *SIX1*/*SIX2* mutation	123 (19)	0.82	0.75-0.90		123 (10)	0.88	0.80-0.95	
MYCN alterations (G + mutation)	11 (3)	0.73	0.46-0.99	.326	11 (2)	0.82	0.59-1	.261
No *MYCN* alterations	117 (17)	0.83	0.75-0.91		117 (8)	0.89	0.80-0.97	
1q gain	36 (7)	0.80	0.66-0.93	.515	36 (4)	0.86	0.72-0.99	.456
No 1q gain	92 (13)	0.83	0.73-0.92		92 (6)	0.89	0.79-0.98	
+12	34 (2)	0.94	0.86-1	.062	34 (1)	0.97	0.91-1	.242
No +12	94 (18)	0.78	0.68-0.88		94 (9)	0.85	0.76-0.95	
+7/7q gain	31 (5)	0.81	0.71-0.97	.914	31 (4)	0.77	0.52-1	.122
No +7/7q gain	97 (15)	0.82	0.73-0.91		97 (6)	0.91	0.83-0.98	
+13	20 (4)	0.80	0.62-0.97	.547	20 (3)	0.84	0.68-1	.147
No +13	108 (16)	0.83	0.74-0.91		108 (7)	0.89	0.81-0.97	
+20/20q gain	16 (4)	0.71	0.46-0.96	.281	16 (4)	0.51	0.13-0.90	.003
No +20/20q gain	112 (16)	0.84	0.77-0.92		112 (6)	0.93	0.87-0.99	
+6/6q gain	14 (1)	0.93	0.79-1	.381	14 (0)	1	1-1	.283
No +6/6q gain	114 (19)	0.81	0.73-0.89		114 (10)	0.87	0.79-0.95	
1p−	12 (2)	0.77	0.49-1	.970	12 (1)	0.86	0.60-1	.980
No 1p−	116 (18)	0.84	0.76-0.91		116 (9)	0.89	0.81-0.96	
11q−	10 (5)	0.50	0.19-0.81	4.9E-04	10 (4)	0.47	0.05-0.89	4.4E-06
No 11q−	118 (15)	0.85	0.78-0.92		118 (6)	0.92	0.85-0.99	
16q−	9 (4)	0.44	0.03-0.86	.010	9 (3)	0.53	0.13-0.93	.006
No 16q−	119 (16)	0.85	0.79-0.92		119 (7)	0.92	0.86-0.98	
7p−	8 (2)	0.75	0.45-1	.418	8 (0)	1	1-1	.437
No 7p−	120 (18)	0.83	0.75-0.90		120 (10)	0.88	0.80-0.95	

TABLE 1 (*continued*)

	RFS				OS			
	No. of Patients (No. of Events)	Survival Rates at the Last Follow-Up	95% CI	*P* Value	No. of Patients (No. of Events)	Survival Rates at the Last Follow-Up	95% CI	*P* Value
17p−/−17	6 (2)	0.67	0.29-1	.141	6 (2)	0.67	0.29-1	.001
No 17p−/−17	122 (18)	0.83	0.75-0.91		122 (8)	0.89	0.82-0.97	
HACE1 loss	4 (3)	0.25	0-0.67	4.1E-05	4 (1)	0.75	0.33-1	.283
No *HACE1* loss	124 (17)	0.84	0.77-0.92		124 (9)	0.89	0.81-0.96	

miRNAPG, miRNA processing genes; miRNAPG alterations include mutations and deletions in *DROSHA*, *DICER1*, and *DGCR8* and deletions in *DIS3L2*; *95% CI*, 95% confidence interval.

Regarding the clinical characteristics of patients with 8 subtype tumors, the median age of 34 patients with +12 subtype tumors and 43 with no +12 tumors were 44 and 41 months, respectively, and similar, whereas the 34 patients had a lower male to female ratio than the 43 patients (14/30 vs. 24/19, $P = .024$). The stage distribution was similar between these two subtypes (early stages I + II/advanced stages III + IV; 23/11 vs. 26/17, $P = .341$), and the incidence of the *IGF2* LOI status was also similar between the two subtypes (Supplementary Table 4).

The other 6 subtypes were summarized as 11q−, 16q−, and/or *HACE1* loss group tumors (18 patients) and no 11q−, 16q−, and *HACE1* loss group tumors (59 patients) because the number of each tumor subtype (11q−, 16q−, or *HACE1* loss) was small, and 11q−, 16q−, and *HACE1* loss overlapped in 4 tumors (Supplementary Fig. 2). The 2 groups of patients had the same median age of 44 months, a similar male to female ratio (8/10 vs. 30/29, $P = .282$), a similar stage distribution (early I + II, advanced III + IV; 13/5 vs. 36/23, $P = .282$), and a similar incidence of *IGF2* LOI (Supplementary Table 4).

RFS and OS Rates in Patients with Three Types or Eight Subtypes of Tumors and Those in Three Risk Groups of Patients

All patients with silent-type tumors were alive at the last follow-up without disease, although one patient had relapsed (RFS 95% and OS 100%), those with *WT1*-type tumors had RFS and OS rates of 83 and 93%, respectively, and those with nonsilent/non–*WT1*-type tumors had lower RFS and OS rates of 79 and 83% than the other two types without significance (Table 2). Patients with +12 subtype tumors had better or slightly better RFS and OS rates than those without ($P = .010$ and $P = .075$) (Figure 5, *A* and *B*). Three of 43 patients with no +12 subtype tumors died around 10 years after the diagnosis, two died of WT after late relapse, and one died of secondary leukemia, which may be caused by intensive therapy consisting of CBDCA, etoposide, and doxorubicin and radiotherapy given for the relapsed tumor.

Patients with 11q− subtype tumors had worse RFS and OS rates than those without ($P = .001$ and 9.3E-05) (Supplementary Figures 3, *A* and *B*). Patients with 16q− subtype tumors had worse RFS and OS rates than those without ($P = .025$ and .031) (Supplementary Figures 3, *C* and *D*). Patients with *HACE1* loss subtype tumors had worse RFS than those without, although no significant difference was observed in OS rates between patients with or without *HACE1* loss subtype tumors ($P = 1.2E-04$ and .470) (Supplementary Figures 3, *E* and *F*). Thus, +12 is an exceptional factor, and patients with +12 in tumors had favorable outcomes, whereas those with three other subtypes with chromosomal loss had unfavorable ones.

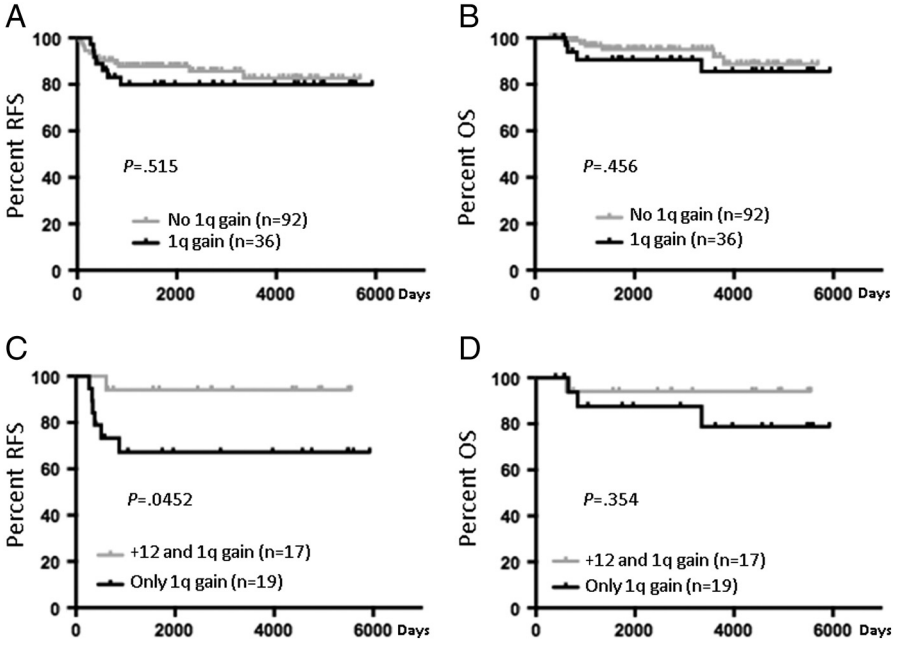

Figure 4. Relapse-free and overall survival curves for 2 groups of patients. Patients were classified by 1q gain (A, B) or 1q gain plus +12 and 1q gain only (C, D).

Table 2. RFS and OS Rates in 128 Patients with Unilateral WTs Classified by 3 Biological Types or 3 Risk Groups

	RFS				OS			
	No. of Patients (No. of Events)	Survival Rates at the Last Follow-Up	95% CI	*P* Value	No. of Patients (No. of Events)	Survival Rates at the Last Follow-Up	95% CI	*P* Value
Biological classification								
Three types				.409				.323
A) *WT1* alterations	31 (5)	0.83	0.70-0.97		31 (2)	0.93	0.83-1	
B) Silent (no genetic or chromosomal abnormalities)	20 (1)	0.95	0.85-1		20 (0)	1	1-1	
C) Non-*WT1*/nonsilent	77 (14)	0.79	0.68-0.89		77 (8)	0.83	0.72-0.95	
The non-*WT1*/nonsilent type (C) was classified into 8 subtypes (D, E, F, G, H, I, J, and K)								
D) +12	34 (2)	0.94	0.86-1	.010 *	34 (1)	0.97	0.91-1	.075 *
E) No +12	43 (12)	0.66	0.49-0.83		43 (7)	0.75	0.57-0.92	
2 types and 2 subtypes (A, B, D, and E)				.024 †				.081 †
F) 11q−	10 (5)	0.5	0.19-0.81	.001	10 (4)	0.47	0.05-0.89	9.3E-05
G) No 11q−	67 (9)	0.83	0.73-0.94		67 (4)	0.89	0.78-0.99	
2 types and 2 subtypes (A, B, F, and G)				.004				2.3E-04
H) 16q−	9 (4)	0.44	0.03-0.86	.025	9 (3)	0.53	0.13-0.93	.031
I) No 16q−	68 (10)	0.84	0.75-0.93		68 (5)	0.89	0.79-0.99	
2 types and 2 subtypes (A, B, H, and I)				.055				.036
J) *HACE1* loss	4 (3)	0.25	0-0.67	1.2E-04	4 (1)	0.75	0.32-1	.470
K) No *HACE1* loss	73 (11)	0.82	0.71-0.92		73 (7)	0.84	0.72-0.96	
2 types and 2 subtypes (A, B, J, and K)				4.5E-04				.390
Risk classification								
3 risk groups				9.1E-06				2.5E-06
L) Low risk (silent type and +12 subgroup)	54 (3)	0.94	0.88-1		54 (1)	0.98	0.94-1	
M) Intermediate risk (*WT1* type and no +12 plus no 11q−, 16q−, or *HACE1* loss subgroup	64 (11)	0.81	0.70-0.91		64 (4)	0.91	0.82-1	
N) High risk (no +12 plus 11q−, 16q−, or *HACE1* loss subgroup)	10 (6)	0.33	0-0.67		10 (5)	0.42	0.07-0.77	

RFS: A vs. B, *P* = .245; A vs. C, *P* = .784; A vs. D, *P* = .181; A vs. E, *P* = .218; B vs. C, *P* = .191; B vs. D, *P* = .933; B vs. E, *P* = .056; L vs. M, *P* = .049; L vs. N, *P* = 8.4E-07; M vs. N, *P* = .001.
OS: A vs. B, *P* = .286; A vs. C, *P* = .425; A vs. D, *P* = .512; A vs. E, *P* = .158; B vs. C, *P* = .192; B vs. D, *P* = .466; B vs. E, *P* = .109; L vs. M, *P* = .281; L vs. N, *P* = 5.9E-04; M vs. N, *P* = 1.1E-04.

* *P* value evaluated from two subtypes of patients;

† *P* value evaluated from two types and two subtypes of patients. Please also see Figure 5 and Supplementary Figure 3.

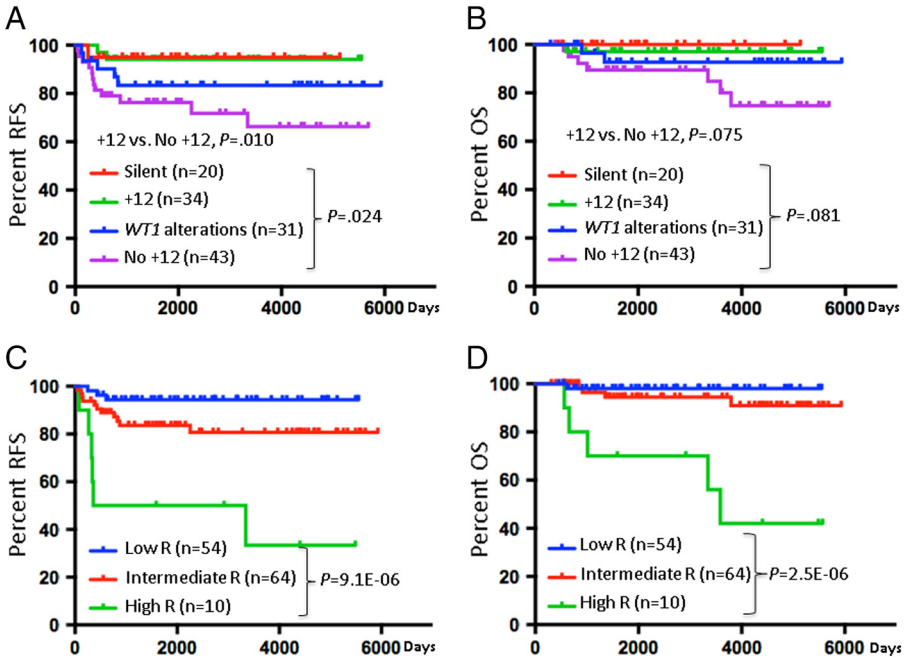

Figure 5. Relapse-free and overall survival curves for 3 or 4 groups of patients.Patients were classified by no CGH aberrations (silent type), *WT1* alterations (*WT1* type), and the presence or absence of +12 (+12 and no +12 subtypes) (A, B) in tumors or by three risk groups (low risk, silent type and +12 subgroup; intermediate risk, *WT1* type and no +12 plus no 11q−,16q−, or *HACE1* loss subgroup; high risk, no +12 plus 11q−,16q−, or *HACE1* loss subgroup) (C, D).

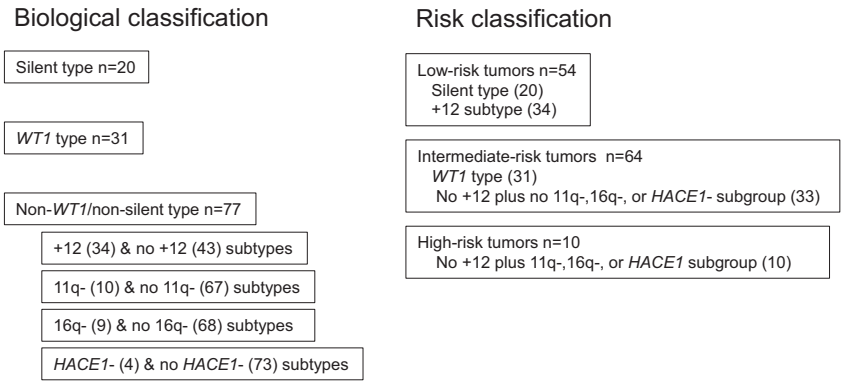

Figure 6. Biological and risk classifications based on genetic and chromosomal characteristics of WT. Silent type, *WT1* type, and each pair (e.g., +12 and no +12 subtypes) of 8 subtype tumors totaled 128 tumors in biological classification. Silent and *WT1* type and eight subtype tumors were integrated and classified into three groups in risk classification.

As mentioned in the previous section, 18 tumors had 11q–, 16q–, and/or *HACE1* loss. Eight of the 18 tumors also had +12 (Supplementary Figure 2). For the risk classification, the 8 tumors were included in +12 subgroup and the remaining 10 tumors were classified as no +12 plus 11q–, 16q–, or *HACE1* loss subgroup. We integrated two types and eight subtypes of tumors, classified them into three risk groups, and examined RFS and OS rates in three risk group of patients. Fifty-four patients with silent type and +12 subgroup of tumors were classified as low risk; 10 with no +12 plus 11q–, 16q–, or *HACE1* loss as high risk; and 64 with *WT1*-type and no +12 plus no 11q–, 16q–, or *HACE1* loss subgroup tumors as intermediate risk (Figure 6). Low-risk patients had better RFS and OS rates than high-risk patients (P = 8.4E-07 and 5.9E-07) and had better RFS rate than intermediate-risk patients (P = .049), whereas low-risk and intermediate-risk patients had comparative OS rates (P = .281). Intermediate-risk patients had better RFS and OS rates than high-risk patients (P = .013 and 1.1E-04) (Table 2 and Figure 5, *C* and *D*).

Multivariate Outcome Analysis of 7 Clinical, Genetic, and Chromosomal Factors in 128 Patients with WT

A multivariate Cox proportional-hazard regression analysis confirmed the relationship between 16q– and a poor outcome after adjustments for age and stage [RFS: hazard ratio (HR) 5.21, P = .007; OS: 5.66, P = .025] (Table 3). The relationship between 11q– or *HACE1* loss and a poor outcome was not evaluable due to collinearity. The relationship between +20/20q gain and a poor outcome was not confirmed after adjustments for age and stage (RFS, HR 1.37, P = .599; OS, HR 2.91, P = .111). The relationship between +12 and a favorable outcome was confirmed or suggested after adjustments for age and stage [RFS: HR 0.23, P = .050; OS: HR 0.19, P = .112], and was confirmed or suggested after adjustments for 11q–, 16q–, or *HACE1* loss, or +20/20q gain in addition to age and stage (RFS: HR 0.096, P = .004; 0.24, P = .057; not evaluable due to collinearity; 0.19, P = .034, respectively; OS: HR not evaluable due to collinearity, 0.19, P = .122; 0.11, P=,075; 0.10, P = .047, respectively).

Ten WTs with 11q–, 9 with 16q–, and 16 with +20/20q gain each were classified into those with or without +12. RFS and OS rates were better in WTs with 11q–, 16q–, or +20/20q gain each plus +12 than in those with 11q–, 16q–, or +20/20q gain only, with or without significance (Supplementary Table 5), and these effects of +12 on favorable outcomes may have contributed to significant P values in the multivariate analyses when each abnormality was added to the three factors (age, stage, and +12) (Table 3). Thirty-six WTs with 1q gain were classified into those with or without +12. RFS and OS rates were better in 17 patients with 1q gain plus +12 than in 19 patients with 1q gain only in tumors with or without significance (P = .045 and P = .358) (Figure 4, *C* and *D*). Therefore, the effect of +12 on a favorable outcome was also identified in WTs with 1q gain.

Differential Gene Expression Profiles Between WTs With or Without +12, and Those With or Without 16q—

We examined the gene expression profiles of 27 WTs and 2 normal kidney tissues; 20 out of 27 tumors were included in the present study on 128 WTs. Of the 27 tumors, 6 had *WT1* alterations, 7 had no aCGH aberrations (silent type), and 14 had the non-*WT1*/nonsilent type; 4 had +12 only, 1 had 16q– only, 3 had both +12 and 16q–, and 6 had neither +12 nor 16q–. The expression of 324 genes was stronger in 7 tumors with than in those without +12; 146 and 178 of the 324 genes were located on chromosome 12 and other chromosomes, respectively (Supplementary Fig. 4). Comparisons of 1198 probes on chromosome 12 and 22,357 probes on other chromosomes revealed that upregulated genes were more likely to be located on chromosome 12 ($P < 10^{-16}$, Fisher's exact test). The expression of 23 genes was weaker in 4 tumors with than in those without 16q–; 10 and 13 genes were located on chromosome arm 16q and other chromosome arms, respectively (Supplementary Fig. 5), indicating that downregulated genes were more likely to be located on 16q ($P < 5.439 * 10^{-12}$, Fisher's exact test).

Relationship between the Overexpression of Each Upregulated Gene in WTs with +12 and Better OS Rates and Between Downregulated Genes on Chromosome Arm 16q or Upregulated Genes on Other Chromosome Arms in WTs with 16q— and Worse OS Rates Based on the TARGET OCG Dataset 148 or 125

Two datasets are available in a public database (R2) (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi) to investigate the relationship between the over- or underexpression of upregulated or downregulated genes in WTs with +12 or 16q– and the better or worse OS rates of patients with WTs, and we firstly used dataset 148 (Tumor Wilms (TARGET) – OCG – 148 – MAS5.0 – u133pa) rather than dataset 125 (Tumor Wilms (TARGET) – OCG – 125 – MAS5.0 – u133p2) because the former

Table 3. Multivariate Analyses of 7 Clinical, Genetic, and Chromosomal Factors in 128 Patients with Unilateral WT

Variable	Comparison	RFS				OS			
		P Value	HR	95%	CI	*P* Value	HR	95%	CI
Age: >24 months	≤24 months	Not evaluable due to collinearity				Not evaluable due to collinearity			
Stage IV	Stage I/II/III								
11q–	No 11q–								
Age: >24 months	≤24 months	.902	1.06	0.41	2.78	.090	6.69	0.75	48.44
Stage IV	Stage I/II/III	.004	4.44	1.57	12.52	.037	4.90	1.09	22.00
16q–	No 16q–	.007	5.21	1.56	17.39	.025	5.66	1.24	25.74
Age: >24 months	≤24 months	Not evaluable due to collinearity				.038	9.49	1.13	78.87
Stage IV	Stage I/II/III					.090	3.24	0.83	12.65
HACE1 loss	No *HACE1* loss					.169	4.85	0.53	38.11
Age: >24 months	≤24 months	.674	1.23	0.46	3.30	.112	5.64	0.67	47.66
Stage IV	Stage I/II/III	.021	3.17	1.18	8.48	.136	2.84	0.72	11.17
+20/20q gain	No+ 20/20q gain	.599	1.37	0.42	4.43	.111	2.91	0.78	10.87
Age: >24 months	≤24 months	.220	1.81	0.70	4.68	.025	10.71	1.34	85.50
Stage IV	Stage I/II/III	.024	3.05	1.53	8.07	.126	2.88	0.74	11.20
+12	No+ 12	.050	0.23	0.05	1.00	.112	0.19	0.02	1.48
Age: >24 months	≤24 months	.755	1.17	0.42	3.32	Not evaluable due to collinearity			
Stage IV	Stage I/II/III	.005	4.28	1.55	11.32				
11q–	No 11q–	<.001	14.42	4.05	51.36				
+12	No+ 12	.004	0.096	0.02	0.43				
Age: >24 months	≤24 months	.449	1.46	0.55	3.87	.046	8.46	1.03	69.29
Stage IV	Stage I/II/III	.007	4.10	1.46	11.56	.050	4.44	0.99	19.94
16q–	No 16q–	.009	4.91	1.47	16.37	.030	5.36	1.17	24.46
+12	No+ 12	.057	0.24	0.05	1.05	.122	0.19	0.02	1.55
Age: >24 months	≤24 months	Not evaluable due to collinearity				.019	23.07	16.5	323.11
Stage IV	Stage I/II/III					.166	2.66	0.67	10.58
HACE1 (6q16) loss	No *HACE1* (6q16) loss					.063	14.40	0.86	239.89
+12	No+ 12					.075	0.11	0.01	1.25
Age: >24 months	≤24 months	.356	1.60	0.59	4.33	.069	7.29	0.85	62.3
Stage IV	Stage I/II/III	.098	2.48	0.84	7.31	.641	1.46	0.30	7.21
+20/20q gain	No+ 20/20q gain	.258	2.14	0.57	8.01	.028	5.03	1.20	26.50
+12	No+ 12	.034	0.19	0.04	0.89	.047	0.10	0.01	0.98

dataset and present study had more similar patient characteristics, including stage distribution and mortality rates, than the latter.

Among the 146 upregulated genes on chromosome 12 in WTs with +12, the higher expression levels of 75 genes were associated with better OS rates based on dataset 148. Furthermore, among the 178 upregulated genes on other chromosomes in WTs with +12, the higher expression levels of 46 genes were associated with better OS rates (Table 4, Supplementary Table 6, and Supplementary Figure 4). Thus, upregulated genes on chromosome 12 were more frequently associated with favorable outcomes than those on the other chromosomes (P = .001). *CDK4* on chromosome 12 was upregulated in WTs with +12; however, no significant P values were obtained based on dataset 148. Because a CDK4 inhibitor is clinically available, we also used dataset 125 and found that higher expression levels of *CDK4* were associated with better OS rates (Table 4).

Some of the upregulated genes in WTs with +12, which were associated with better outcomes when overexpressed in WT according to dataset 148 of a public database (R2), were categorized into 7 groups based on the DAVID analysis: ubiquitination-related, 9 genes; chromatin-related, 12; TP53 pathway-related 11; DNA damage and response, 4; mRNA processing, 11; mitosis and cell division, 5. In addition, four genes were categorized as immune response (Table 5).

As described in the previous paragraph, we initially used dataset 148. Among the 10 downregulated genes on chromosome arm 16q in WTs with 16q–, the lower expression levels of only two genes (*GABARAPL2* and *ATMIN*) were associated with worse OS rates, those of three genes (*FTO*, *CYB5B*, and *AP1G1*) with better OS rates, and those of three genes (*TERF2IP*, *MON1B*, and *MAP1LC3B*) with no significant difference in OS rates. No data existed for the other two genes (*CENPBD1* and *ZFP90*). When we analyzed the three genes with no significant differences in OS rates and two genes with no data using dataset 148, the lower expression levels of these five genes were associated with worse outcomes when we used dataset 125 (Table 6 and Supplementary Figure 5).

In contrast, while no genes on 16q were upregulated in tumors with 16q–, 16 genes on non-16q chromosome arms were upregulated, and the higher expression levels of three genes (*LGALS14*, *INTS1*, and *MMP8*) were associated with worse OS rates. In addition, the higher expression levels of two upregulated genes (*ZBED6CL* and *SLC9C2*) on non-16q arms with no outcome data in dataset 148 were associated with worse OS rates when we used dataset 125 (Table 6).

Homozygous CTNNB1 Mutations Caused by UPD3p

CTNNB1 mutations were found in 27 (21.1%) out of 128 unilateral WTs: 9 (9.3%) out of 97 *WT1*-wild-type tumors and 18 (58.1%) out of 31 *WT1*-mutant tumors (Supplementary Table 7). In addition, we found *CTNNB1* mutations in 20 (64.2%) out of 31 bilateral *WT1*-mutant WTs from 23 patients whose clinical and genetic characteristics were reported previously [16]. Of the 47 unilateral and bilateral tumors with various *CTNNB1* mutations, 10 had the same

Table 4. Upregulated Genes on Chromosome 12 or Other Chromosomes That Are Associated with Better OS When Overexpressed in Patients with +12 in WT

Upregulated Genes on Chromosome 12						Upregulated Genes on Chromosome 12					
			TARGET-OCG Dataset 148						TARGET-OCG Dataset 148		
	Gene s2ymbol	FDR	*P* Value	Bonferroni	Probe Set		Gene Symbol	FDR	*P* Value	Bonferroni	Probe Set
1	*KRAS*	0.23	8.7E-12	1.2E-09	214352_s_at	18	*ZNF268*	0.29	1.7E-04	2.2E-02	209989_at
2	*PRDM4*	0.04	1.3E-08	1.7E-06	218329_at	19	*BAZ2A*	0.24	2.3E-04	3.0E-02	201353_s_at
3	*WBP11*	0.28	6.1E-07	8.1E-05	217822_at	20	*SLC38A1*	0.20	2.8E-04	3.7E-02	218237_s_at
4	*YAF2*	0.10	1.4E-06	1.9E-04	206238_s_at	21	*C2CD5*	0.22	3.0E-04	4.0E-02	212943_at
5	*KANSL2*	0.22	1.9E-06	2.5E-04	221821_s_at	22	*SFSWAP*	0.20	3.2E-04	4.2E-02	202773_s_at
6	*LRP6*	0.20	7.2E-06	9.6E-04	205606_at	23	*CNOT2*	0.15	3.4E-04	4.5E-02	217798_at
7	*KDM5A*	0.28	1.2E-05	1.6E-03	202040_s_at			TARGET-OCG dataset 125			
8	*COL2A1*	0.29	1.9E-05	2.5E-03	217404_s_at		*CDK4*	0.26	3.7E-02	1.0E+00	202246_s_at
9	*CCDC92*	0.22	2.0E-05	2.7E-03	218175_at	Upregulated genes on chromosomes other than chromosome 12					
10	*NOP2*	0.20	2.1E-05	2.8E-03	214427_at				TARGET-OCG dataset 148		
11	*BRAP*	0.24	2.5E-05	2.7E-03	213473_at	1	*RPS26*	0.23	1.4E-06	1.9E-04	217753_s_at
12	*LEMD3*	0.03	3.0E-05	4.0E-03	206967_at	2	*AVL9*	0.20	8.5E-06	1.1E-03	212474_at
13	*ZCCHC8*	0.18	3.4E-05	4.6E-03	218478_s_at	3	*MRM2*	0.24	1.8E-05	2.3E-03	218356_at
14	*CNPY2*	0.29	4.3E-05	5.8E-03	209797_at	4	*LANCL2*	0.26	2.5E-05	3.4E-03	218219_s_at
15	*TDG*	0.22	7.8E-05	1.0E-02	203743_s_at	5	*ZNF79*	0.28	6.5E-05	8.6E-03	214138_at
16	*CAND1*	0.13	1.2E-04	1.6E-02	208839_s_at	6	*TBP*	0.24	3.4E-04	4.5E-02	203135_at
17	*DDX23*	0.20	1.3E-04	1.7E-02	40465_at	7	*EMX1*	0.26	3.5E-04	4.6E-02	215265_at

Upregulated genes with FDR values (<0.3) were identified by the method described in reference [27]. Patients were classified into two groups by two expression levels (higher and lower) of each upregulated gene. OS curves were obtained by the Kaplan-Meier method, and *P* values were obtained by the log-rank test with the Bonferroni correction according to dataset 148 of the public database (R2).

The survival analysis did not show a significant difference between the two expression levels of *CDK4* using dataset 148: therefore, we used dataset 125. Other upregulated genes with better OS rates are listed in Supplementary Table 6.

mutation (c.del133_135TCT, p.del45S), and 7 out of the 10 accompanied UPD3p, including 3p22.1, at which *CTNNB1* is located (Supplementary Figure 6). Of the other 37 WTs with various other *CTNNB1* mutations, 2 had the same *CTNNB1* mutation (c. 121A > G, p.Thr41Ala), 1 of which accompanied UPD3p. These results indicate that the *CTNNB1* mutation is homozygous in some WTs caused by UPD3p and is strongly associated with the specific mutation del45S.

Discussion

We investigated chromosomal, genetic, and epigenetic alterations in 128 unilateral WTs and proposed a biological classification consisting of 3 types: silent, *WT1*, and nonsilent/non-*WT1* types, and 4 sets of 2 subtypes: +12 or no +12, 11q– or no 11q–, 16q– or no 16q–, and *HACE1* loss or no *HACE1* loss (Figure 6). The prognostic implications of silent, 11q–, and 16q– have been previously reported by other groups; however, the favorable outcomes of patients with +12 tumors and unfavorable outcomes of those with *HACE1* loss have never been previously reported [31–33]. +12 was found in 34 (26.6%) out of the 128 WTs in the present aCGH study and in 88 (27%) out of 331 tumors analyzed by the UK cytogenetic group [34] and is the most frequent extra chromosome in WTs.

Davoli and colleagues reported that the distribution and potency of TSGs, oncogenes, and essential genes critical for survival on chromosomes may explain copy number alterations in whole chromosomes and chromosome arms during cancer evolution through a process of cumulative haploinsufficiency and triplosensitivity [10]. The present results that showed significantly higher numbers of upregulated genes on chromosome 12 in WTs with +12 than in those without, and significantly higher numbers of downregulated genes on 16q in tumors with 16q– than in those without, concur with their statement. We speculated that the upregulated genes may have resulted in the favorable outcomes of patients having tumors with +12, and the downregulated genes may have led to the unfavorable outcomes of patients having tumors with 16q–.

The public database provides Kaplan-Meijer survival curves for patients with WT classified by the expression levels of various genes, and we used it to investigate the relationship between each upregulated gene in tumors with +12 and better OS rates, and that between each downregulated gene on chromosome 16q or each upregulated gene on the non-16q chromosome arms in tumors with 16q– and worse OS rates. We found that the higher expression levels of 75 out of 146 upregulated genes on chromosome 12 and those of 46 out of 178 upregulated genes on chromosomes other than chromosome 12, which

Table 5. Groups of Upregulated Genes on Chromosome 12 or Other Chromosomes That Are Associated with Better OS When Overexpressed in Patients with +12 in WT

Biological Function (Gene Nos.)	Upregulated Genes on Chromosome 12	Upregulated Genes on Other Chromosomes
Ubiquitination-related (*n* = 9)	***BRAP***, ***CAND1***. ***KRAS***, *FBXL14, MDM2, MED21, RNF34, UBE3B*	*KBTBD2*
Chromatin-related (*n* = 12)	***BAZ2A***, ***KANSL2***, ***KDM5A***, *TDG, ARID2, SMARCC2, TIMELESS*	*CBX3, H2AFV, PAM, TAF5, TBP*
TP53-related (*n* = 11)	*TDG, MAPKAPK5, MDM2, POLE, RFC5, RNF34, TIMELESS, TRIAP1*	***TBP***, *PAXIP1, TAF5*
DNA damage response (*n* = 4)	***TDG***, *POLE, TIMELESS*	*PAXIP1*
mRNA processing (*n* = 11)	***CNOT2***, ***DDX23***, ***SFSWAP***, ***WBP11***, ***ZCCHC8***, *CPSF6, EIF4B, PAN2, SART3*	*DHX16, SNRPE*
Mitosis and cell division (*n* = 5)	*ASUN, CCNT1, KNTC1, TIMELESS*	*CLTA*
Immune response (*n* = 4)	*SART3, TBK1*	*ICOSLG, LGALS3BP*

Genes in fine print indicate that the overall survival rate of patients with a higher expression level of each gene was significantly better by the log-rank test ($P < .05$). Genes in bold indicate that overall survival rates were significantly better after the Bonferroni correction ($P < .05$).

Table 6. Relationship Between the Underexpression of Downregulated Genes on 16q or the Overexpression of Upregulated Genes on Non-16q Chromosome Arms and Worse OS Rates in Patients with WT

			TARGET-OCG Dataset 148			TARGET-OCG Dataset 125			
	Gene Symbol	FDR	*P* Value	Bonferroni	Probe Set	*P* Value	Bonferroni	Probe Set	Gene Description
Downregulated Genes on Chromosome arm 16q									
1	*GABARAPL2*	0.24	3.70E-02	1.00E+00	209046_s_at	N. R.			GABA type A receptor associated protein like 2
2	*ATMIN*	0.18	4.80E-02	1.00E+00	201855_s_at	N. R.			ATM interactor
3	*TERF2IP*	0.15	N. S.			1.5E-05	1.7e-03	201174_s_at	TERF2-interacting protein
4	*MON1B*	0.23	N. S.			4.8e-06	5.20E-04	203644_s_at	MON1 secretory trafficking family member B
5	*MAP1LC3B*	0.21	N. S.			1.3E-03	0.2	208786_s_at	Microtubule-associated protein 1, light chain 3, beta
6	*CENPBD1*	0.15	Not exist			4.60E-02	1.00E+00	223728_at	CENPB DNA-binding domain containing 1
7	*ZFP90*	0.21	Not exist			1.60E-03	1.78E-01	226124_at	ZFP90 zinc finger protein
Upregulated genes on chromosome arms other than 16q									
1	*LGALS14*	0.22	1.10E-05	1.50E-03	220158_at	N. R.			Galectin 14
2	*INTS1*	0.22	8.40E-04	1.12E-01	212212_s_at	N. R.			Integrator complex subunit 1
3	*MMP8*	0.21	9.30E-03	1.00E+00	207329_at	N. R.			Matrix metallopeptidase 8
4	*ZBED6CL*	0.15	Not exist			1.40E-03	1.56E-01	227598_at	ZBED6 C-terminal like
5	*SLC9C2*	0.24	Not exist			1.60E-02	1.00E+00	1563495_at	Solute carrier family 9 member C2 (putative)

Down- or upregulated genes with FDR values (<0.3) were identified by the method described in reference [27]. Patients were classified into two groups by the two expression levels (higher and lower) of each down- or upregulated gene. OS curves were obtained by the Kaplan-Meier method, and *P* values were obtained by the log-rank test with the Bonferroni correction according to dataset 148 of the public database (R2). When the survival analysis did not show a significant difference between the two expression levels of each gene using dataset 148; therefore, we used dataset 125. *N. R.*, not relevant; *N. S.*, not significant.

may be upregulated by the transcriptional activation of the upregulated genes on chromosome 12, were associated with better OS rates (Table 4, Supplementary Table 6, and Supplementary Figure 4). Some of these genes were grouped as ubiquitination-related (*CAND1*), chromatin-related (*KDM5A*), TP53-related (*TDG*), DNA damage response (*TDG*), mRNA processing (*CNOT2*), mitosis and cell division (*ASUN*), and immune response (*SART3*) (Table 5). The mechanisms by which these groups of upregulated genes contribute to the favorable outcomes of patients have not yet been elucidated.

CDK4 is 1 of the 146 upregulated genes on chromosome 12 in tumors with +12, and an oncogene whose product forms a complex that plays an important role in cell cycle G1/S phase progression [35]. The present study showed that the higher expression level of *CDK4* was associated with better OS rates based on dataset 125 (Table 4). The markedly stronger expression of *CDK4* than *CDK6* was previously reported in WTs [36]; however, the chromosomal status of the tumors was not examined in that study. We speculated that the overexpression of *CDK4* and some other oncogenes promotes the proliferation of WT cells, and these cells are very susceptible to cytotoxic drugs, resulting in a favorable response in and outcome for patients with WTs with +12. The favorable effects of CDK4/6 inhibitors were reported in clinical trials for breast cancer [37]. The substitution of cytotoxic drugs for CDK4/6 inhibitors may be an important subject for the circumvention of adverse effects caused by cytotoxic chemotherapy in the treatment of WT.

The lower expression levels of 7 out of the 10 downregulated genes on 16q and the higher expression levels of 5 out of the 16 upregulated genes on the non-16q chromosome arms were associated with worse OS rates (Table 6 and Supplementary Figure 5). Downregulated genes included *ATMIN* (DNA damage response gene), *GABARAPL2* (autophagy-related), *CENPD1* (control of chromosomal segregation), and *ZFP90* (a negative regulator of NRSF/REST) [38,39]. Upregulated genes, which may be derepressed by the deletion of repressor genes on 16q, included *LGALS14* (a strong inducer of T-cell apoptosis), *INTS1* (RNA polymerase II-associated complex), *MMP8* (matrix metalloproteinase family), *ZBED6CL* (repression of *IGF2* expression), and *SLC9C2* [putative Na(+)/H(+) exchangers] [40]. Matrix metalloproteinases play a pivotal role in tumor growth and the multistep processes of invasion and metastasis [40], and the upregulation of *MMP8* may be causally related to the unfavorable outcomes of patients having WTs with 16q–. The mechanisms by which these downregulated genes on 16q contribute to the unfavorable outcomes of patients having WTs with 16q– need to be clarified.

Whole chromosomal aneuploidy results from errors in the chromosomal segregation of duplicated chromosomes. Our previous study on 10 bilateral WTs with no *WT1* alterations included one tumor with +12 and UPD11, which developed in an infant with premature chromosome separation syndrome [16]. Premature chromosome separation syndrome is caused by biallelic mutations in *BUB1B*, biallelic single nucleotide substitutions in the upstream region of *BUB1B*, or compound monoallelic *BUB1B* mutations and monoallelic single nucleotide substitutions in the *BUB1B* upstream region [41–43]. *BUB1B* is a spindle assembly checkpoint gene, and *RASSF1A* plays some roles at a mitotic checkpoint [44]. We previously reported that *BUB1B* was not mutated in 25 WTs, including 6 with +12, and the expression levels of BubR1, a protein product of *BUB1B*, decreased and *RASSF1A* promoter regions were methylated in hyperdiploid and pseudodiploid WTs but not in diploid WTs [45]. Yost and colleagues recently reported that all six children with biallelic mutations in *TRIP13*, another spindle assembly checkpoint gene, developed WT [46]. These findings suggest that the downregulation of mitotic checkpoint genes may cause hyperdiploid WTs with +12.

Gadd and colleagues recently examined the genetic landscape of 117 WTs and found that genetic alterations preserved the progenitor state and abnormal induction of embryonal kidney cells [47]. They also stated that decreased *LET7A* expression, caused by an *LET7A* deletion or *LIN28B* upregulation and miRNAPG mutations, appears to perpetuate the progenitor state and prevent progenitor cell maturation. *LIN28B* is located at 6q16, and +6/6q gain was almost exclusively found in +12 subtype tumors but rare in no +12 subtype tumors, whereas deletions in *LET7A* and miRNAPGs were frequent in no +12 subtype tumors but rare in +12 subtype tumors (Figures 2 and 3). The upregulation of *LIN28B* by +6/6q gain, *LET7A* deletion, and miRNAPG deletion may result in reduced expression levels of *LET7A* [47]. The present results suggested that +12 and no +12 subtype tumors both preserve the progenitor states through the

decreased expression of *LET7A* either by the overexpression of *LIN28B* or by *LET7A* deletions or miRNAPG alterations; however, we were unable to identify higher expression levels of *LIN28B* in WTs with than in those without +6/6q gain by a microarray analysis.

We identified 11q– and 16q– as markers predicting poor outcomes, and two tumors with 11q– and 16q– were histologically classified as diffuse anaplasia. The other eight tumors with 11q– and seven tumors with 16q– were classified as a favorable histology. The relationship between 11q– and/or 16q– and poor outcomes and an anaplastic histology has been reported by other groups [5,31,32]. Ten genes on 16q were downregulated, and the lower expression levels of seven of them were associated with poor outcomes, indicating the enhanced proliferation or resistance to chemotherapy of tumor cells caused by a haploinsufficiency of possible TSGs on 16q. Unfortunately, there was only 1 tumor with 11q– out of the 27 tumors on which the expression array analysis was performed, and it was not possible to examine the relationship between the lower expression levels of downregulated genes on 11q and poor outcomes in the present study.

We also demonstrated that *HACE1* loss is a marker predicting a poor outcome. This gene is a TSG involved in various cancers [48]. The 6q21 breakpoint of the congenital t(5;6)(q21;q21) translocation in a child with bilateral, young-onset WT disrupted *HACE1* [49], indicating that this gene is one of the WT predisposing genes. Subsequent sequencing revealed *HACE1* mutations in 1 of the 450 WTs, indicating a low incidence of *HACE1* mutations in sporadic WTs. We found focal deletions including *HACE1* in 4 (3.1%) out of 128 WTs. The hypermethylation of CpG islands upstream of *HACE1* and its low expression level were reported in sporadic WTs. The deletion regions of four tumors also included *LIN28B*, and further studies are needed to clarify the role of *HACE1* and *LIN28B* losses in Wilms tumorigenesis.

We found 1q gain in 36 (28.1%) out of 128 WTs, and a similar incidence of 1q gain was reported in COG (28.5%) and SIOP (28.5%) [6,7]. Although EFS and OS rates in patients with or without 1q gain appear to be similar among the three series of WTs, significant differences were observed in EFS and OS rates in the previous two studies but not in the present study (Table 7). We and other investigators reported that the incidence of *IGF2* LOI in WTs was lower in Japanese than that of *IGF2* LOI reported in Caucasians [24,50]. Someone may wonder if the contradictory results in the present study are related to biologic differences between Japanese and Caucasian WTs. Because the percentages of 1q gain or +12 in WTs were similar between Japanese and Caucasians [34], these contradictory results may be caused by the smaller number of patients in the present study than in the other two studies and/or a favorable effect of +12 on tumors with 1q gain in the present study (Figure 4, *A-D*). While the present study examined genetic aberrations in a single tumor from each patient with WT, Cresswell and others examined intratumor genetic heterogeneity in 70 tumor samples from 20 patients with WT [9]. Their data showed 1q gain in 21 tumor samples from 8 patients and +12 in 27 tumor samples from 11 patients, indicating more frequent occurrence of +12 than 1q gain in their WTs. Furthermore, their results indicated that simultaneous occurrence of 1q gain and +12 was found in 14 tumor samples from 6 patients and was the most frequent combination of chromosomal aberrations. Thus, SIOP and COG should examine the favorable effect of +12 on outcomes of patients with WTs with 1q gain.

β-Catenin encoded by *CTNNB1* is a key protein involved in the Wnt signaling pathway that is critical for mesenchymal-epithelial transition [51]. *CTNNB1* mutations in WT are reported to be heterozygous and considered to enhance WT cell proliferation [52]. We showed that 8 out of 47 WTs with *CTNNB1* mutations had homozygous *CTNNB1* mutations due to partial UPD3p covering the *CTNNB1* locus at 3p22.1; 7 out of the 8 WTs had the same *CTNNB1* mutation (Ser45del). The reason why the mutation (Ser45del) was frequently homozygous currently remains unknown. Since *CTNNB1* mutations have a gain of function property, the homozygous mutation may confer a greater proliferative capacity on tumor progenitor cells. Similar findings were reported for the *CBL* gene with gain-of-function mutations, which were duplicated by UPD11q, in myeloid neoplasms [53].

An aCGH analysis revealed no copy number aberrations and no allelic imbalances in 20 (15.6%) out of 128 WTs, although 7 out of the 20 had *WTX* alterations, *CTNNB1* mutations, or LOI of *IGF2*, and these 20 tumors were classified as the silent type. Patients were characterized by a young age, early stage of the disease, frequent epithelial predominant histology, and favorable outcomes. Previous

Table 7. EFS and OS Stratified by 1q Gain in WTs Reported from COG [6], SIOP [7], and the Present Study

COG	No. of Patients	8-Year EFS	95% CI	*P* Value	8-Year OS	95% CI	*P* Value
1q gain	317 (28.5%)	77.0%	72%-81%	<.001	88.0%	83%-91%	<.001
No 1q gain	797	90.0%	88%-92%		96.0%	94%-97%	
	1114	86.0%	84%-88%		94.0%	92%-95%	
The present study							
	No. of Patients	8-Year EFS	95% CI	*P* Value	8-Year OS	95% CI	*P* Value
1q gain	36 (28.1%)	80.5%	62.3%-89.9%	.396	91.6%	73.6%-96.9%	.338
No 1q gain	92	86.9%	75.8%-91.8%		95.6%	87.3%-98.1%	
	128	85.1%	76.1%-89.7%		94.5%	87.6%-97.0%	
SIOP							
	No. of Patients	5-Year EFS	95% CI	*P* Value	5-Year OS	95% CI	*P* Value
1q gain	167 (28.5%)	79.0%	68.5%-82.0%	<.001	88.4%	83.5%-93.6%	.01
No 1q gain	419	88.2%	85.0%-91.4%		94.4%	92.1%-96.7%	
	586						
The present study							
	No. of Patients	5-Year EFS	95% CI	*P* Value	5-Year OS	95% CI	*P* Value
1q gain	36 (28.1%)	80.5%	62.3%-89.9%	.297	91.6%	73.6%-96.9%	.338
No 1q gain	92	88.0%	79.0%-93.1%		95.6%	87.3%-98.1%	
	128	85.9%	78.0%-90.7%		94.5%	87.6%-97.0%	

aCGH studies also reported no chromosomal aberrations in some WTs [11–13]. Subset 1 proposed by Gadd et al. consisted of 11 tumors with an epithelial histology, patient age ranging between 6 and 91 months, and stages I and II, and showed no alterations in *WT1*, *CTNNB1*, and *WTX* or the LOH of 1p and 16q; 1 tumor with LOI of *IGF2* was included in this subset [33]. The favorable outcomes of epithelial predominant WTs were reported by SIOP [54]. Seven out of 20 patients with tumors classified as an epithelial predominant histology in the present study are alive with no relapse. Patients with early-stage WT with an epithelial predominant histology and no aCGH aberration (silent type) may avoid chemotherapy that may cause adverse effects without the risk of relapse.

Conclusions

We newly identified chromosome 12 gain (+12) as a potential marker predicting a favorable outcome and identified or confirmed 11q−, 16q−, and *HACE1* loss as prognostic indicators for poor outcomes [12,13]. Moreover, we reported that the expression of various genes on chromosome 12 was stronger in tumors with than in those without +12, while that of some genes on chromosome arm 16q was weaker in tumors with than in those without 16q−. The higher expression levels of upregulated genes in tumors with +12 and lower expression levels of some downregulated genes in tumors with 16q− predicted favorable and unfavorable outcomes, respectively, based on the public database. These results may lead to the discovery of new targets for avoiding adverse effects and augmenting therapeutic efficacy.

CRediT authorship contribution statement

Masayuki Haruta: Conceptualization, Methodology, Writing - original draft. **Yasuhito Arai:** Methodology & Investigation. **Hajime Okita:** Methodology & Investigation. **Yukichi Tanaka:** Methodology & Investigation. **Tetsuya Takimoto:** Formal analysis. **Ryuichi P. Sugino:** Formal analysis. **Yasuhiro Yamada:** Methodology & Investigation. **Takehiko Kamijo:** Writing - review & editing. Takaharu Oue: Writing - review & editing. **Takaharu Oue:** Writing - review & editing. **Masahiro Fukuzawa:** Writing - review & editing. **Tsugumichi Koshinaga:** Writing - review & editing. **Yasuhiko Kaneko:** Conceptualization, Methodology, Writing - original draft.

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Appendix A. Supplementary Data

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