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ORIGINAL ARTICLE

Methylation Statuses of H19DMR and KvDMR at WT2 in Wilms Tumors in Taiwan

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Abstract

Wilms tumor is the most common pediatric renal malignancy. Several genetic loci have been shown to be associated with its formation. Genetic or epigenetic aberrations at *WT1* and WT2 loci have been implicated in the etiology of the majority of sporadic Wilms tumors. In our previous study, most Wilms tumors tested negative for both constitutional mutations and somatic mutations in the *WT1* gene. Thus, WT2 may play an important role in these tumors. In the present study, we analyzed the methylation statuses of WT2 at 11p15 using methylation sensitive multiplex ligation-dependent probe amplification in six Wilms tumors. Paternal uniparental disomy at WT2 was observed in two Wilms tumors with epithelial components due to hypermethylation at H19DMR and hypomethylation at KvDMR. Our findings highlight the benefits of testing for 11p15 epigenetic abnormalities to identify Wilms tumors with epithelial components.

Keywords Multiplex ligation-dependent probe amplification · Nephroblastoma · Paternal uniparental disomy · Wilms tumor

Abbreviations

DMR	differentially methylated region
DNA	deoxyribonucleic acid
IC	imprinting center
MAPK	mitogen-activated protein kinase
MS-MLPA	methylation sensitive multiplex ligation-dependent probe amplification
UV-VIS	ultraviolet–visible

Background

Wilms tumor, or nephroblastoma, is an embryonal tumor of the kidney. It is the most frequently occurring solid tumor of childhood, excluding brain tumors [1–3]. The genetic make-up of Wilms tumor is diverse and involves approximately 40 genes [4]. Genes previously implicated in Wilms tumors include *WT1*, *CTNNB1*, *FAM123B*, *DROSHA*, *DGCR8*, *XPO5*, *DICER1*, *SIX1*, *SIX2*, *MLLT1*, *MYCN*, and *TP53* [5]. In addition, *BCOR*, *BCORL1*, *NONO*, *MAX*, *COL6A3*, *ASXL1*,

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MAP3K4, and *ARID1A* have been identified on whole-genome and whole-exome sequencing of 117 Wilms tumors [6]. More recently, four new Wilms tumor predisposition genes have been reported: *TRIM28*, *FBXW7*, *NYNRIN*, and *KDM3B* [7]. Among them, *WT1* is the first to be isolated and is also the most important [8]. A second locus (WT2) of large size (approximately 10 Mb) at chromosome 11p15 has also been described [9], mainly in epithelial and blastemal predominant Wilms tumors [10]. WT2 consists of two domains, each containing several genes. Telomeric domain 1 on chromosome 11p15 is comprised of the insulin-like growth factor 2 (*IGF2*) gene and an imprinted maternally expressed transcript (*H19*), while centromeric domain 2 contains the potassium voltage-gated channel gene (*KCNQ1*), *KCNQ1* opposite strand transcript 1 gene (*KCNQ1OT1*), and cyclin dependent kinase inhibitor 1C gene (*CDKN1C*) (Additional file 1A) [11]. *IGF2* is a paternally derived gene that characterizes the early metanephric mesenchyme gene and is associated with epithelial and blastemal histologies [4, 10, 12]. The most frequent epigenetic alterations in Wilms tumor occur in the WT2 region, secondary to imprinting center mutations or paternal uniparental disomies [13].

From our previous study, most DNA samples from peripheral blood lymphocytes and paraffin-embedded tumor specimens of Wilms tumors test negative for both constitutional mutations and somatic mutations in the *WT1* gene [14]. Furthermore, correlations between histological and array comparative genomic hybridization characterizations of Wilms tumor have been proposed [15]. In the present study, we analyzed the methylation statuses of WT2 at 11p15 using methylation sensitive multiplex ligation-dependent probe amplification (MS-MLPA) in six Wilms tumors that had tested negative for both constitutional mutations and somatic mutations in the *WT1* gene [14].

Methods

Study Subjects

Six patients with Wilms tumor (W7 to W12, 4 males and 2 females) diagnosed in the Department of Pediatrics of National Taiwan University Hospital were enrolled in this study [14]. None of these patients had been diagnosed with Denys-Drash syndrome, Frasier syndrome, or Beckwith-Wiedemann syndrome. Ethics approval of the study procedures was obtained from the Institutional Review Board of Chung Shan Medical University Hospital (reference number CS2–16003). All procedures that involved human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Histological Examination

Paraffin-embedded tumor tissues were cut into 5 um thick sections and stained with hematoxylin and eosin for further pathological examination by two pathologists, authors T-C Hou and C-Y Kuo. The classification of Wilms tumors has been described elsewhere [15].

DNA Extraction

DNA FFPE Tissue Kit (Qiagen) was used to purify genomic DNA from paraffin-embedded tissues as previously described [15]. Genomic DNA was dissolved in 100 ul of TE buffer (10 mM Tris-HCL, pH 8.0, and 1 mM EDTA). The DNA concentration of each sample was determined by NanoDrop UV-VIS spectrophotometer. All samples had tested negative for both somatic and germline mutations in the *WT1* gene in our previous study [14].

Methylation Analysis

Salsa MS-MLPA kit ME030-C3 (MRC-Holland) was used to perform MS-MLPA analysis as previously described [16]. Genomic DNA was first denatured, followed by the addition of MS-MLPA probes and hybridization for 16 h. After ligation and digestion with methylation-sensitive endonuclease, samples were subsequently subjected to capillary electrophoresis on ABI PRISM 3130XL (Applied Biosystems). Automated fragment and data analyses were performed using GeneMarker version 3.2.1 (SoftGenetics, LLC) to determine copy numbers and methylation statuses of the *Hha*I sites in the two imprinted domains H19DMR (IC1) and KvDMR (IC2) located in chromosome 11p15 region (Additional file 1A). Four probes targeting *H19* gene and four probes targeting *KCNQ1OT1* locus contained a recognition site for methylation sensitive *Hha*I enzyme and were located very close to one another in chromosome 11p15 region (Additional file 2). We used the average methylation statuses of these H19DMR and KvDMR probes to determine the methylation statuses of the IC1 and IC2 loci (Table 1).

Microsatellite Analysis

DNA profiles of 15 autosomal STR loci and a sex marker for the amelogenin gene were analyzed based on previously described methods [16]. Based on the manufacturer's recommendations, all loci were amplified by polymerase chain reaction (PCR) using AmpFLSTR® SGM Plus and AmpFLSTR™ Profiler PCR amplification kits (Applied Biosystems) on GeneAmp 9700 PCR System (Applied Biosystems). Capillary electrophoresis size-based separation for dye-labeled PCR products was performed on ABI Prism 3100 Genetic Analyzer (Applied Biosystems) with 37-cm

Table 1 The average copy numbers and methylation ratios of 11p15 KvDMR and H19DMR probes in Wilms tumors

Tumors	Gender	Histopathology	KvDMR Region			H19DMR Region		
			Copy No. Ratio	Methylation Ratio	% methylated	Copy No. Ratio	Methylation Ratio	% methylated
W7	Male	Blastemal	1.034 ± 0.049	0.488 ± 0.039	47.11 ± 1.69	0.912 ± 0.071	0.429 ± 0.050	46.95 ± 2.12
W8	Male	Epithelial	0.676 ± 0.017	0.055 ± 0.024	8.03 ± 3.54	0.780 ± 0.082	0.699 ± 0.098	89.56 ± 6.41
W9	Female	Mixed	1.251 ± 0.198	0.636 ± 0.081	51.24 ± 5.49	1.322 ± 0.236	0.761 ± 0.134	58.08 ± 9.55
W10	Male	Stromal	1.414 ± 0.259	0.681 ± 0.062	49.14 ± 8.36	1.263 ± 0.256	0.647 ± 0.152	51.35 ± 7.85
W11	Male	Stromal	1.031 ± 0.115	0.455 ± 0.041	44.27 ± 2.82	1.084 ± 0.079	0.481 ± 0.056	44.31 ± 2.40
W12	Female	Blastemal/ Epithelial	0.970 ± 0.084	0.104 ± 0.057	11.15 ± 7.06	0.941 ± 0.204	0.892 ± 0.211	94.55 ± 2.52

Data are presented as mean ± SD

capillary and POP-6™ separation medium. Genotyper (Applied Biosystems) was used with fragment size data from GeneScan v3.7 (Applied Biosystems) and GS500 (ROX) as the internal size standard for allele designation.

Results

In this study, we investigated somatic 11p15 methylation patterns of Wilms tumors from 4 males and 2 females. The pathological features, average copy number ratios, and methylation statuses of the H19DMR and KvDMR probes are presented in Fig. 1 and Table 1. For W7, copy number ratios were approximately 1.0 (Fig. 1a). Methylation ratios of the four KvDMR probes were 0.432, 0.500, 0.497, and 0.522, respectively. In the H19DMR region, methylation ratios were 0.487, 0.385, 0.385, and 0.455, respectively. Approximately 50% methylation at both H19DMR and KvDMR loci represented normal methylation status. There were significant changes in the methylation statuses of WT2 at 11p15 in W8. KvDMR showed hypomethylation to approximately 8% and H19DMR showed hypermethylation to approximately 90% (Fig. 1b). Hypermethylation at H19DMR (94%) and hypomethylation at KvDMR (11%) were also observed in W12 (Fig. 1f). In W9, W10, and W11, approximately 50% methylation at both H19DMR and KvDMR loci represented normal methylation status (Fig. 1c–e). In addition, W8 and W12 showed only one allele at TH01 locus on microsatellite analysis (Table 2). The results of W8 and W12 are due to WT2 region paternal uniparental disomy.

Are there any correlations between histological characterizations and WT2 region paternal uniparental disomy of Wilms tumor? In our previous study, W8 demonstrated epithelial predominance with a range of differentiation [15]. Epithelial histology of W8 is shown in detail in Fig. 2a; upper panel is 200X and lower panel is 400X. W9 revealed mixed pattern of three types of cellular components [15]. However, less than one-third was epithelial. W12 was of mixed pattern with blastemal and epithelial cellular components (Fig. 2b; upper panel 200X, lower panel 400X). However, more than one-half was epithelial.

Discussion

WT2 is defined by maternal-specific loss of heterozygosity (LOH) on chromosome 11p15.5 in Wilms tumors [17]. Normally, *KCNQ1OT1* are maternally imprinted genes, with the maternal allele methylated in somatic cells and *H19* are paternally imprinted genes, with the paternal allele methylated in somatic cells (Additional file 1A) [18]. Copy number ratio of 1.0 and methylation ratio of 0.5 were expected in normal somatic DNA at both loci. Methylation ratios were 1.0 in H19DMR and 0 in KvDMR in the presence of paternal uniparental disomy. Hypermethylation at H19DMR and hypomethylation at KvDMR were observed in W8 and W12 (Fig. 1 and Table 1). These findings were consistent with those of Cardoso et al., revealing epigenetic changes associated with Wilms tumor [11]. Over-expression of imprinted genes, such as IGF2, is common in Wilms tumors. This also correlates with loss of imprinting of H19DMR on chromosome 11p15 [19]. From these results, W8 and W12 should be considered type 1B, consistent with the findings of Scott et al. that abnormalities of WT2 are by far the most common genetic defect in sporadic Wilms tumor [20]. Moreover, the epithelial type of Wilms tumor may be associated with WT2 malfunction that is induced by mutation or paternal uniparental disomy. We identified uniparental disomy in epithelial-type Wilms tumor without *WT1* mutations on MS-MLPA analysis.

The largest group of Wilms tumors shows biallelic expression of IGF2 associated with epithelial or blastemal histology [4, 10]. In this study, biallelic expression of IGF2 due to paternal uniparental disomy at WT2 was observed in two Wilms tumors, W8 and W12, which were made up of more than one-half epithelial components. These findings were consistent with the epigenetic

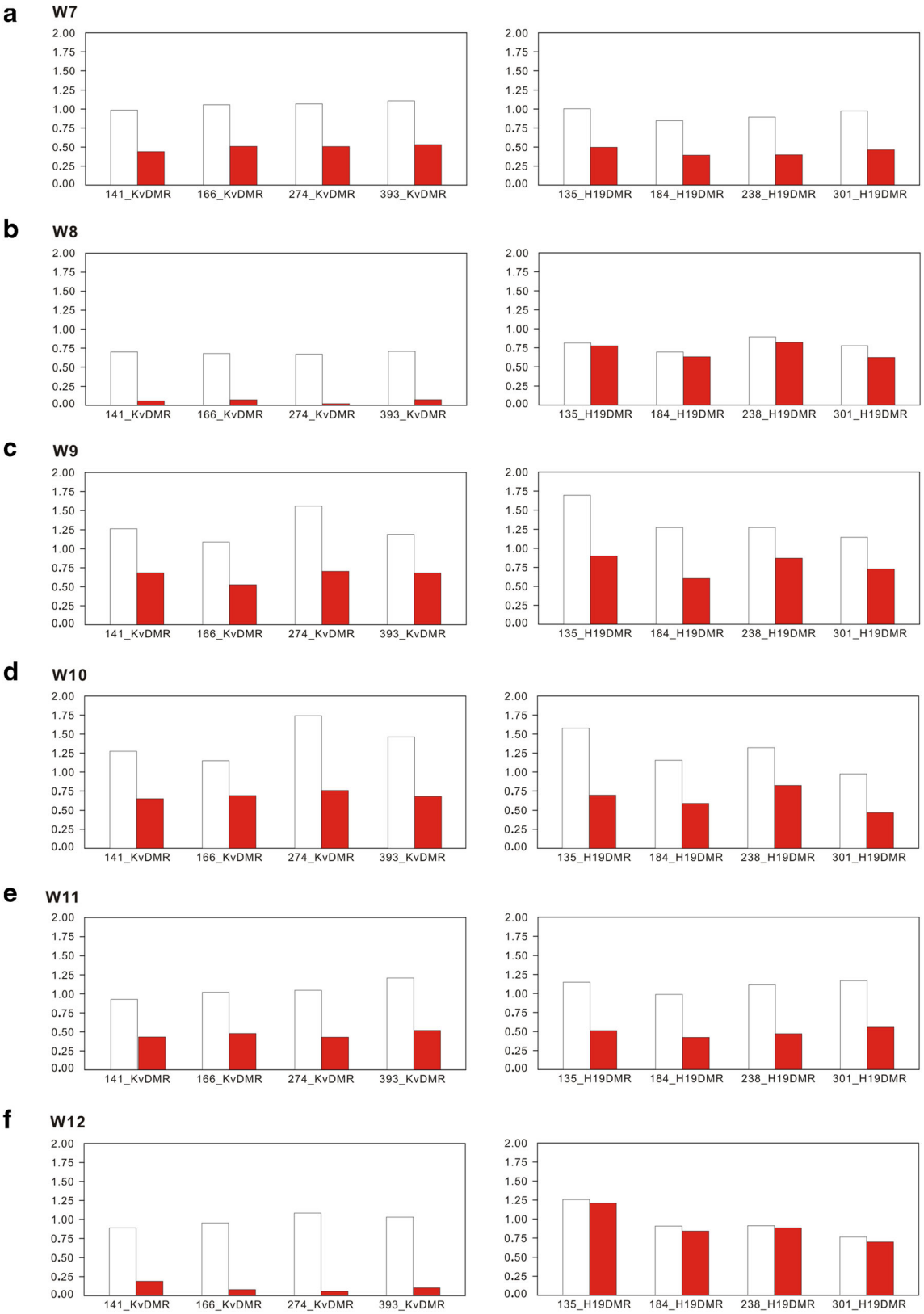

Fig. 1 The copy numbers and methylation ratios of W7 to W12 (a-f) Wilms tumors. Open column (white bar) represents the copy number and solid column (red bar) represents the methylation ratio. Panels: on the left are four 11p15 KvDMR probes; on the right are four 11p15 H19DMR probes

Table 2 The alleles and genotypes of 15 STR loci of Wilms tumors from Taiwanese patients

Gene Loci	Location	W7	W8	W9	W10	W11	W12
TPOX[a]	2p23-2per	8, 9	8, 11	8	8, 11	8, 11	8, 10
D2S1338[b]	2q35–37.1	20	19, 23	18, 24	21, 24	17, 24	22, 23
D3S1358[a, b]	3p21.31	15, 18	15, 17	16, 17	15	15	15, 17
FGA[a, b]	4q28	22, 24	21, 22	23	26, 26.2	21, 23.2	23
D5S818[a]	5q21–31	12, 13	10, 11	10, 13	11	11, 12	10, 12
CSF1PO[a]	5q33.3–34	11, 13	7, 10	11	12, 13	10	12
D7S820[a]	7q11.21–22	11, 12	11, 12	8, 12	8, 11	10, 11	9, 10
D8S1179[b]	8q24.1–24.2	11, 14	13, 14	14, 15	14	10, 14	13, 15
TH01[a, b]	11p15.5	9	9	9, 9.3	9	9	9.3
vWA[a, b]	12p12-pter	14, 18	19	17	14, 19	14, 19	15, 16
D13S317[a]	13q22–31	8, 12	8, 9	8	12	10	10, 12
D16S539[b]	16q24-qter	9, 12	9, 11	9	11, 12	11	9
D18S51[b]	18q21.3	15, 17	14, 15	14, 15	14, 18	14, 16	17, 19
D19S433[b]	19q12–13.1	13, 14	13	14, 15.2	15.2	12, 14	13
D21S11[b]	21q11.2-q21	29, 30	29	28, 32.2	32.2	29	30, 32

[a] STR analysis with AmpFlSTR® Profiler® PCR amplification kit

[b] STR analysis with AmpFlSTR® SGM Plus® PCR amplification kit

changes associated with Wilms tumor identified by Cardoso et al. [11]. Epithelial-type Wilms tumors, which show a range of differentiation, may be associated with paternal uniparental disomy in the WT2 region on 11p15.5, as demonstrated on MS-MLPA analysis of the H19DMR and KvDMR regions. In Wilms tumor, differentiation arrest is incomplete, allowing for maturing lineages of varying proportions [6]. The prognosis for stromal

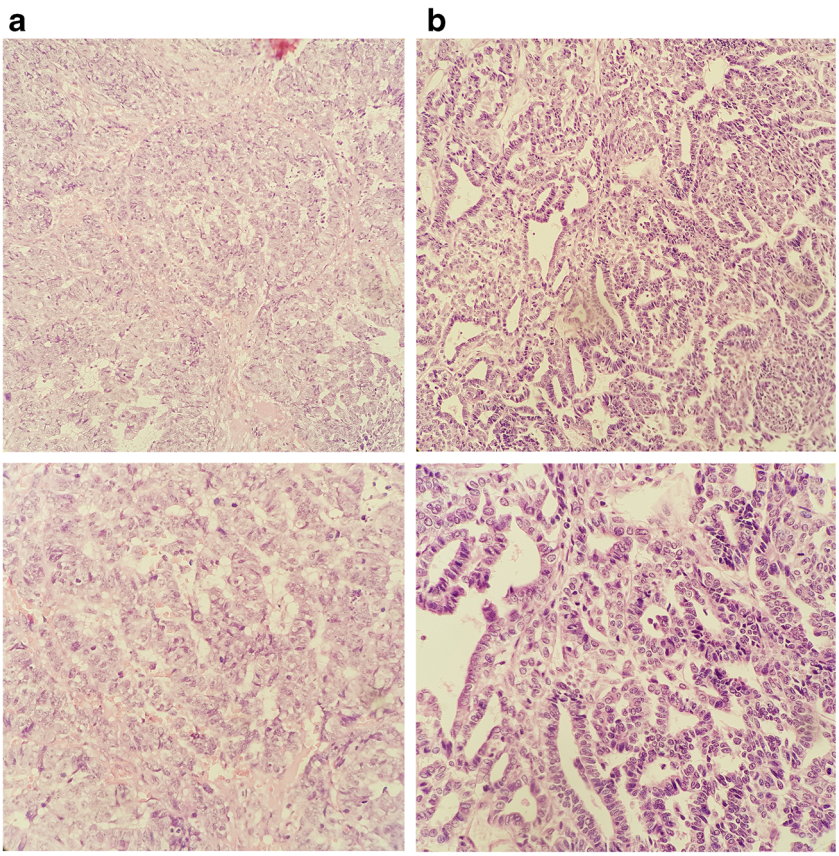

Fig. 2 Histological cross-sections after hematoxylin and eosin staining show predominant epithelial component in W8 (a) and W12 (b). Upper panel is 200X and lower panel is 400X

and epithelial predominant Wilms tumors is excellent [21]. However, stratification of Wilms tumor is not always possible based on morphology alone. MS-MLPA analysis may aid in this regard, highlighting the benefits of combined methylation analysis of the H19DMR and KvDMR regions. With this method, it is possible to detect WT2 methylation or uniparental disomy without serially cutting specimens to determine epithelial-type Wilms tumors.

In this study, the incidence of paternal uniparental disomy was high, two out of six samples. Is there a higher incidence of paternal uniparental disomy in Wilms tumors in Taiwan? To answer this question, additional studies based on larger samples are needed.

Conclusion

In summary, Wilms tumors with epithelia components may be associated with narrow region deletion in the WT2 region at 11p15.5, as demonstrated on MS-MLPA analysis of H19DMR and KvDMR regions. Mixed-type Wilms tumors with epithelial component can be identified by testing 11p15 epigenetic abnormalities, to further refine the molecular classification of Wilms tumors. However, larger sample size is required to provide new insight into the molecular pathogenesis of Wilms tumor. We will keep working to clarify the role of these chromosomal aberrations in the renal tumorigenesis of Wilms tumor.

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Compliance with Ethical Standards

The authors have consulted the journal policy regarding compliance with ethical standards and declare that accepted principles of ethical and professional conduct have been followed. The authors include information regarding sources of funding (previous section) and potential conflicts of interest (financial or non-financial) (next section). As no patients were involved and no personal information was used, informed consent was not applicable.

Conflict of Interest The authors declare that they have no competing interests.

Ethics Approval Ethics approval of the study procedures was obtained from the Institutional Review Board of Chung Shan Medical University Hospital via reference number CS2–16003.

Consent for Publication Not applicable.

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