Identification of Molecular Biomarkers in Taiwanese Patients with Wilms Tumor Using a Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA)-Based Approach

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Research

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Abstract

Background: Wilms tumor is a solid tumor that frequently occurs in children. Genetic or epigenetic aberrations in *WT1* and *WT2* loci are implicated in its etiology. Moreover, tumor suppressor genes are frequently silenced by methylation in this tumor.

Methods: In the present study, we analyzed the methylation statuses of promoter regions of 24 different tumor suppressor genes using a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)-based approach in six Wilms tumors.

Results: All six Wilms tumors showed methylation of *RASSF1* specific to tumors, not in normal tissues. Moreover, methylated *HIC1* was identified in stromal type Wilms tumors and methylated *BRCA1* was identified in epithelial type Wilms tumors. Unmethylated *CASP8*, *RARB*, *MLH1*_167, *APC*, and *CDKN2A* were found only in blastemal predominant type Wilms tumors.

Conclusions: Our results indicated that methylation of *RASSF1* is the essential event in the tumorigenesis of Wilms tumor, which may inform its clinical and therapeutic management. In addition, mixed type Wilms tumors may be classified according to epithelial, stromal, and blastemal components via MS-MLPA-based approach.

Background

Wilms tumor, also known as nephroblastoma, is an embryonal malignant tumor of the kidney. It is the most commonly occurring solid tumor in children, excluding brain tumors [1–3]. The etiology of Wilms tumor is still poorly understood, although a number of associated genes and loci have been identified [4, 5]. The genetic changes in Wilms tumors are diverse and involve approximately 40 genes [6]. Genes that have been previously implicated include *WT1*, *CTNNB1*, *FAM123B*, *DROSHA*, *DGCR8*, *XPO5*, *DICER1*, *SIX1*, *SIX2*, *MLLT1*, *MYCN*, and *TP53*. Whole-genome sequencing and whole-exome sequencing have led to the addition of *BCOR*, *BCORL1*, *NONO*, *MAX*, *COL6A3*, *ASXL1*, *MAP3K4*, and *ARID1A* [7]. Recently, four new predisposing genes have been identified - *TRIM28*, *FBXW7*, *NYNRIN*, and *KDM3B* [5].

Hypermethylation of CpG islands upstream of tumor suppressor genes has been reported for which methylation status is altered in one or more types of tumors [8, 9]. It is interesting to note that Wilms tumor develops mainly through alterations in epigenetic regulation triggered by dedifferentiation [10]. However, the risk of Wilms tumor conferred by epigenetic changes associated with tumor suppressor genes is poorly characterized. In the present study, we investigated the methylation statuses of promoter regions of 24 different tumor suppressor genes in six Wilms tumors using a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)-based approach.

Methods

Study subjects

The six subjects have been described elsewhere [11–13]. Their paraffin-embedded Wilms tumor tissue samples were provided by the Department of Pediatrics of National Taiwan University Hospital. These patients had no history of Denys-Drash syndrome, Frasier syndrome, or Beckwith-Wiedemann syndrome. The study procedures were approved by 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

The tumor tissue samples (W7 to W12) were embedded in paraffin wax and cut into 5 um-thick slices. Then, sections were stained with hematoxylin and eosin and reviewed by two of the authors (T-C Hou and C-Y Kuo) under a microscope to confirm their diagnostic classifications. Typing of Wilms tumor has been described in detail elsewhere [11, 13]. W7 is of blastemal type and W8 is of epithelial type. In addition, W10 and W11 are of stromal type. W9 is a triphasic Wilms tumor comprised of three components: blastema, stroma, and epithelium, while W12 is of mixed blastemal and epithelial type.

DNA extraction

Genomic DNA was purified from paraffin-embedded tissues with the DNA FFPE Tissue Kit (Qiagen), according to the manufacturer's instructions and dissolved in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) as previously described [11, 13]. UV-Vis measurements of DNA concentration of each sample were obtained using NanoDrop UV-VIS Spectrophotometer (Thermo Scientific Nano-Drop 2000c).

Methylation analysis

MS-MLPA analysis was performed using Salsa MS-MLPA kit ME001-C2 Tumor suppressor-1 (MRC-Holland) according to the manufacturer's instructions. Samples were then subjected to capillary electrophoresis on an ABI PRISM 3130XL (Applied Biosystems). Twenty-six MS-MLPA probes were used to detect the methylation statuses of promoter regions of 24 different tumor suppressor genes by *HhaI* digestion (Table 1). MLPA results were analyzed using GeneMarker version 3.2.1 (SoftGenetics, LLC) to determine copy numbers and methylation statuses of the *Hha*I sites. The internal methylation ratio was obtained by comparison of the *Hha*I digested aliquot (Fig. 1B) with the paired undigested aliquot (Fig. 1A) from each sample with intra-sample data normalization according to the manufacturer's instructions [14]. Methylation was assessed by comparing the probe methylation percentages of the test sample with the percentages of the 5 normal reference samples. Copy number ratio of 1.0 and methylation ratio of 0 were expected in most genes on normal reference, meaning that the methylation compared to normal reference was unlimited (∞). If methylation ratios of test sample and normal reference samples were appropriate, methylation compared to normal reference was around 1.0.

Table 1
Chromosomal locations of the 41 probes in ME001-C2 Tumor suppressor-1.

Length (NT)	Gene	MLPA probe	*Hha*I site	Chromosomal location	MV location (hg18)
400	TP73	04050-L01263	yes	1p36.32	01-003.558977
265	CASP8	02761-L02210	yes	2q33.1	02-201.830871
353	VHL	03810-L01211	yes	3p25.3	03-010.158426
193	RARB	04040-L01698	yes	3p24.2	03-025.444559
167	MLH1	01686-L01266	yes	3p22.2	03-037.009621
463	MLH1	02260-L01747	yes	3p22.2	03-037.010000
475	CTNNB1	03984-L03251	-	3p22.1	03-041.241066
328	RASSF1	02248-L01734	yes	3p21.31	03-050.353298
382	RASSF1	03807-L02159	yes	3p21.31	03-050.353347
409	FHIT	02201-L01699	yes	3p14.2	03-061.211918
483	CASR	02683-L02148	-	3q21.1	03-123.485226
148	APC	01905-L01968	yes	5q22.2	05-112.101357
373	ESR1	02202-L01700	yes	6q25.1	06-152.170883
154	PARK2	03366-L02750	-	6q26	06-162.126766
310	CDK6	03184-L02523	-	7q21.3	07-092.085391
161	CDKN2A	01524-L01744	yes	9p21.3	09-021.985276
211	CDKN2B	00607-L00591	yes	9p21.3	09-021.998808
346	DAPK1	01677-L01257	-	9q21.33	09-089.303075
364	LOC254312	01234-L00781	yes	10p14	10-011.017023
136	CREM	00981-L00566	-	10p12.1	10-035.517225
292	KLLN	02203-L08261	yes	10q23.3	10-089.612348
319	CD44	03817-L01731	yes	11p13	11-035.117389
454	GSTP1	01638-L01176	yes	11q13.2	11-067.107774
184	ATM	04044-L03849	yes	11q22.3	11-107.599044
427	CADM1	03819-L03848	yes	11q23.3	11-114.880585
175	TNFRSF1A	00554-L13516	-	12p13	12-006.321241
444	CD27	00678-L00124	-	12p13.31	12-006.430685
274	CDKN1B	07949-L07730	yes	12p13.1	12-012.761863
229	PAH	02334-L01820	-	12q23.2	12-101.795401
238	CHFR	03813-L03753	yes	12q24.33	12-131.974372

Length (NT)	Gene	MLPA probe	*Hha*I site	Chromosomal location	MV location (hg18)
301	BRCA2	04042-L03755	yes	13q12.3	13-031.787722
418	BRCA2	01617-L01199	-	13q13.1	13-031.851548
202	MLH3	01245-L00793	-	14q24.3	14-074.578836
281	TSC2	01832-L01397	-	16p13.3	16-002.061786
337	CDH1	02416-L01862	-	16q22.1	16-067.424755
436	CDH13	07946-L07727	yes	16q23.3	16-081.218219
220	HIC1	03804-L00949	yes	17p13.3	17-001.905107
246	BRCA1	05162-L04543	yes	17q21.31	17-038.530811
256	BCL2	00587-L00382	-	18q21.33	18-058.946868
390	KLK3	00713-L00108	-	19q13.33	19-056.050014
142	TIMP3	02255-L03752	yes	22q12.3	22-031.527795

Results

The methylation statuses of the *Hha*I sites in 24 different tumor suppressor genes were determined by 26 MS-MLPA probes on MS-MLPA analysis (Fig. 1). If the target site was unmethylated, the DNA–probe complex was digested to prevent exponential amplification. No signal was detected after fragment analysis (Fig. 1B).

Methylation of *RASSF1* and *CDKN2B* was found in all six Wilms tumors (Fig. 1A, Table 2). Methylation was observed in both probes in *RASSF1* gene. Internal methylation ratio was not 0 and methylation compared to normal reference was unlimited (∞). *CDKN2B* gene was methylated in normal reference. In comparison with normal reference, methylation was not unlimited (∞). W7 to W11 methylation was 3.72, 8.85, 2.58, 3.15, and 5.40-fold that of normal reference, respectively. However, W12 methylation was only 1.05-fold that of normal reference.

Table 2
Methylated and unmethylated genes identified in six Wilms tumors

Size (NT)	Gene	Cytoband	W7	W8	W9	W10	W11	W12
All								
328	*RASSF1*	3p21.31	0.84/∞	0.17/∞	0.74/∞	0.74/∞	0.28/∞	0.63/∞
382	*RASSF1*	3p21.31	0.94/∞	0.22/∞	0.74/∞	0.71/∞	0.33/∞	0.70/∞
211	*CDKN2B*	9p21.3	0.18/3.72	0.54/8.85	0.15/2.58	0.13/3.15	0.26/5.40	0.073/1.05
None								
353	*VHL*	3p25.3	0/0	0/0	0/0	0/0	0/0	0/0
463	*MLH1*	3p22.2	0/0	0/0	0/0	0/0	0/0	0/0
409	*FHIT*	3p14.2	0/0	0/0	0/0	0/0	0/0	0/0
238	*CHFR*	12q24.33	0/0	0/0	0/0	0/0	0/0	0/0
301	*BRCA2*	13q12.3	0/0	0/0	0/0	0/0	0/0	0/0
Stroma only								
220	*HIC1*	17p13.3	0/0	0/0	0.05/∞	0.06/∞	0.04/∞	0/0
Epithelium only								
246	*BRCA1*	17q21.31	0/0	0.04/∞	0.03/∞	0/0	0/0	0.03/∞
Blastema only								
265	*CASP8*	2q33.1	0/0	1.01/∞	0.79/∞	0.58/∞	0.55/∞	0.79/∞
193	*RARB*	3p24.2	0/0	0.05/9.28	0.10/11.75	0.12/23.95	0.06/8.18	0.09/10.25
167	*MLH1*	3p22.2	0/0	0.03/2.82	0.09/4.62	0.06/3.863	0.03/2.79	0.05/2.96
148	*APC*	5q22.2	0/0	0.06/∞	0.15/∞	0.09/∞	0.08/∞	0.15/∞
161	*CDKN2A*	9p21.3	0/0	0.05/∞	0.06/∞	0.09/∞	0.04/∞	0.07/∞
Others								
184	*ATM*	11q22.3	0/0	0.04/∞	0.10/∞	0.10/∞	0.05/∞	0.18/∞
400	*TP73*	1p36.32	0/0	0.09/∞	0.12/∞	0.13/∞	0/0	0.10/∞
454	*GSTP1*	11q13.2	0/0	0.07/∞	0.09/∞	0.09/∞	0/0	0.09/∞
292	*KLLN*	10q23.3	0.13/∞	0.07/∞	0.07/∞	0/0	0.05/∞	0/0
436	*CDH13*	16q23.3	0/0	0.08/∞	0.09/∞	0.11/∞	0/0	0.10/∞
373	*ESR1*	6q25.1	0/0	0.08/∞	0/0	0.13/∞	0/0	0/0
319	*CD44*	11p13	0.34/∞	0/0	0/0	0.07/∞	0/0	0/0
142	*TIMP3*	22q12.3	0/0	0.04/∞	0.05/∞	0/0	0/0	0/0

Data are presented as internal methylation ratio/methylation compared to normal reference.

Size (NT)	Gene	Cytoband	W7	W8	W9	W10	W11	W12
346	*DAPK1*	9q21.33	0/0	0.041/∞	0/0	0/0	0/0	0/0
427	*CADM1*	11q23.3	0/0	0.06/∞	0/0	0/0	0/0	0/0
274	*CDKN1B*	12p13.1	0/0	0.04/∞	0/0	0/0	0/0	0/0
Data are presented as internal methylation ratio/methylation compared to normal reference.								

VHL, *MLH1*_463, *FHIT1*, *CHFR*, and *BRCA2* were unmethylated in all six Wilms tumors (Fig. 1B). Moreover, there was methylation of *HIC1* in the Wilms tumors of stromal type (W9, W10, and W11) and methylation of *BRCA1* in the Wilms tumors of epithelial type (W8, W9, and W12). *CASP8*, *RARB*, *MLH1*_167, *APC*, and *CDKN2A* were unmethylated only in W7, which was of blastemal predominant type (Table 2). In other words, there was methylation of *CASP8*, *RARB*, *MLH1*_167, *APC*, and *CDKN2A* in stromal and epithelial type Wilms tumors, but not in blastemal type Wilms tumors.

In addition to these 13 tumor suppressor genes (15 MS-MLPA probes), there were differential methylation patterns for the other 11 tumor suppressor genes among the six Wilms tumors. The six Wilms tumors are listed in order from minimum number to maximum number of these methylated genes: W7 and W11 (2), W12 (4), W9 and W10 (6), and W8 (10). Only two tumor suppressor genes, *KLLN* and *CD44*, were methylated in W7. Only one tumor suppressor gene, *CD44*, was unmethylated in W8.

Discussion

Traditional methods of methylation detection involve modifying DNA by methylation-specific restriction enzymes or sodium sulfite, combined with Sanger sequencing, PCR, or hybridization analysis. This process is cumbersome and difficult, with low reproducibility and insufficient sensitivity. MLPA is a PCR amplification reaction. ME001 can simultaneously detect changes in the degree of methylation of up to 24 different genes in a single reaction tube. Due to MLPA's easy operation, low cost, and wide range of applications, our team has published several studies based on this method [13, 15, 16]. However, MLPA analysis of tumor samples only provides information regarding the “average” situation in the cells from which the DNA samples were purified [14].

The results of this study regarding methylation of *RASSF1* gene are consistent with the findings of previous studies [17, 18]. *RASSF1* gene on 3p21.31 encodes a cytosolic RASSF1A protein similar to Ras effector proteins [19]. De novo methylation of the *RASSF1* promoter is one of the most frequent epigenetic inactivation events in human cancer and leads to silencing of *RASSF1* expression [20, 21]. Association of *RASSF1* promoter methylation with Wilms tumor has been reported [22]. The methylation status of *RASSF1* might be a novel biomarker for predicting outcome of Wilms tumor patients [23]. It appears that methylation of *RASSF1* is the essential event in the tumorigenesis of Wilms tumor, which may inform its diagnostic, clinical, and therapeutic management. *CDKN2B* gene hypermethylation was observed in W7 to W11, but not in W12. *CDKN2B* gene on 9p21.3 encodes the $p15^{INK4B}$ protein that binds to CDK4 or CDK6 and inhibits its activation [24]. Hypermethylation of *CDKN2B* CpG islands occurs in the majority of leukemia patients [25].

Hypermethylation at CpG islands in the 5' ends of tumor suppressor genes is controversial and difficult to interpret. *HIC1* gene on 17p13.3 encodes a transcriptional repressor for p21 [26]. Hypermethylation of *HIC1* gene is found in 3% of Wilms tumors [27]. *CASP8* gene on 2q33.1 encodes Caspase-8 that is an apoptosis-related cysteine peptidase [28]. In 43% of Wilms tumors there is methylation at *CASP8* [22]. *MLH1* gene on 3p22.2 encodes proteins that detect and repair DNA mismatches [29]. A small proportion of Wilms tumors might be associated with the presence of microsatellite instability [30]. *CDKN2A* gene on 9p21.3 encodes two proteins that regulate two critical cell cycle regulatory pathways, the p53 pathway and the RB1 pathway [31]. Arcellana-Panlilio *et al*. demonstrated methylation of the CpG island in the 5' region of *CDKN2A* (p16) in seven out of seven Wilms tumors exhibiting decreased *CDKN2A* expression [32]. This is inconsistent with

the finding of negligible methylation of the 5' CpG island of *CDKN2A* by Erlich *et al.* [21]. *BRCA1* gene on 17q21.31 encodes a nuclear phosphoprotein that plays a role in maintaining genomic stability [33]. However, *BRCA1*, which demonstrates promoter hypomethylation, is over-expressed in Wilms tumor [34]. *RARB2* gene on 3p24.2 encodes retinoic acid receptor beta that is a type of nuclear receptor activated by all-trans retinoic acid and 9-cis retinoic acid [35]. Our results were inconsistent with those of Morris *et al*. in which promoter methylation was absent at *RARB2* [22]. *APC* gene on 5q22.2 encodes a 312-kDa protein that acts as an antagonist of the Wnt signaling pathway [36]. Activation of the Wnt/β-catenin pathway is common in Wilms tumor, but rarely through β-catenin mutation and *APC* promoter methylation [37]. An important finding of this study is the possibility to further classify mixed type Wilms tumors using genetic results of epithelial, stromal, and blastemal components based on MS-MLPA-based approach. In particular, the methylation statuses of these 9 genes make them candidate molecular markers of metastasis in Wilms tumors.

In Wilms tumor, the differentiation arrest of renal progenitor cells is not complete, allowing for maturing lineages of varying proportions [7]. The outcome for stromal and epithelial predominant Wilms tumors is generally excellent [38]. Histological classification of Wilms tumor is not always possible based on morphology alone [39]. From our results, methylation of *HIC1* in stroma, *BRCA1* in epithelium, and *CASP8*, *RARB*, *MLH1*_167, *APC*, and *CDKN2A* in either stroma or epithelium can be used to identify stromal predominant and epithelial predominant Wilms tumors which are associated with a good outcome [38]. In the situation that the subtype of Wilms tumor is difficult to diagnose, unmethylated *CASP8*, *RARB*, *MLH1*_167, *APC*, and *CDKN2A* are potential diagnostic markers to diagnose blastemal type Wilms tumors which are associated with a poor outcome [40]. Stratification of Wilms tumor by epigenetic analysis of these genes highlights the benefits of methylation status analysis of important tumor suppressor genes. Is it possible that epigenetic modifications in Wilms tumor provide potential therapeutic options? To answer this question, additional studies based on a larger number of cases and tissue microdissection are necessary.

Conclusions

In summary, all six Wilms tumors showed methylation of *RASSF1* specific to tumors, not in normal tissues. Moreover, methylation of *HIC1* was identified in the Wilms tumors of stromal type and methylation of *BRCA1* was identified in the Wilms tumors of epithelial type. Unmethylated *CASP8*, *RARB*, *MLH1*_167, *APC*, and *CDKN2A* was found only in the Wilms tumor that was of blastemal predominant type. Our results indicated that methylation of *RASSF1* is the essential event in the tumorigenesis of Wilms tumor, which may inform its clinical and therapeutic management. In addition, mixed type Wilms tumors may be classified using genetic results of epithelial, stromal, and blastemal components based on MS-MLPA-based approach. However, a larger number of cases are necessary to further refine the molecular classification and pathogenesis of Wilms tumors.

Abbreviations

APC: adenomatous polyposis coli; *BRCA1*: breast cancer 1; *BRCA2*: breast cancer 2; *CASP8*: caspase 8; *CDKN2A*: cyclin dependent kinase inhibitor 2A; *CDKN2B*: cyclin dependent kinase inhibitor 2B; *CHFR*: Checkpoint with Forkhead and Ring Finger Domains; DNA: deoxyribonucleic acid; *FHIT*: fragile histidine triad protein; *HIC1*: hypermethylated-in-cancer 1; *KLLN*: killin; *MLH1*: mutL homolog 1; MS-MLPA: methylation-specific multiplex ligation-dependent probe amplification; NT: nucleotide; *RARB2*: retinoic acid receptor B2; *RASSF1*: ras-association domain family member 1; UV-VIS: ultraviolet–visible; *VHL*: von Hippel-Lindau.

Declarations

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Authors' contributions

MYL provided samples and clinical data. TCH and CYK reviewed tumor sections. YCL designed the experiments, performed the experiments, interpreted the results, and drafted the manuscript. WWC designed the experiments, interpreted the results and made critical revisions to the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval of the study procedures was obtained from the Institutional Review Board of Chung Shan Medical University Hospital via reference number CS2-16003. As no patients were involved and no personal information was used, informed consent was not applicable.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Figures

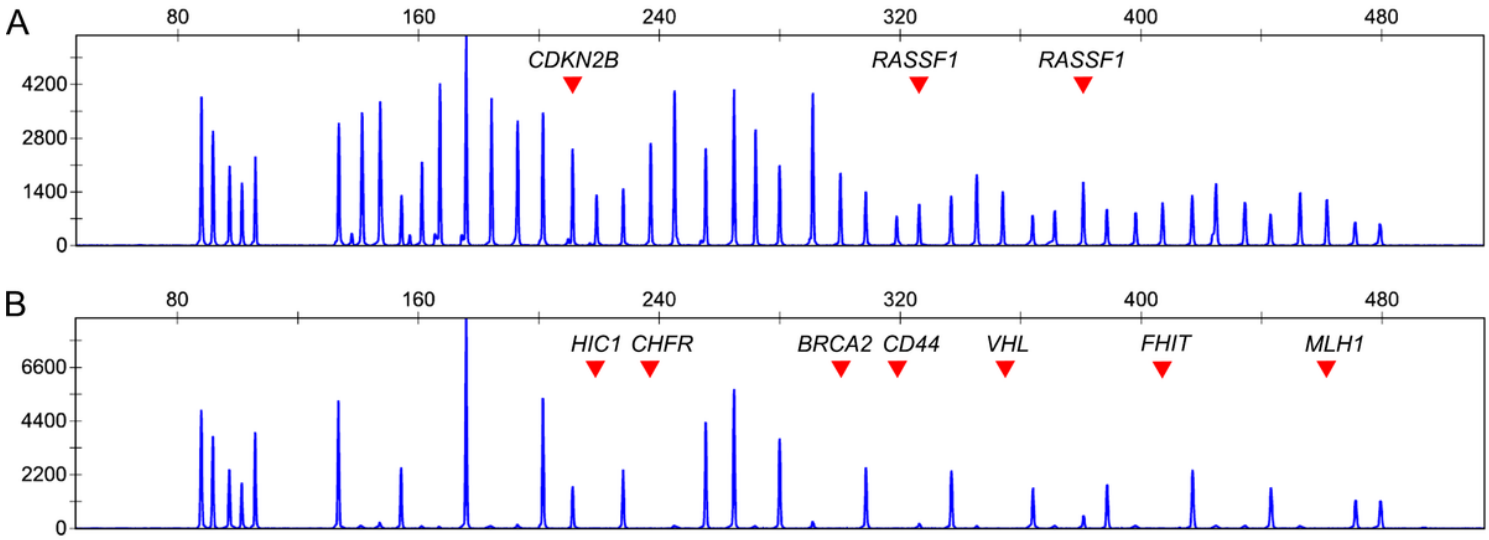

Figure 1

Detection of the methylation statuses of 24 tumor suppressor genes in W8 by MS-MLPA. (A) The capillary electrophoresis pattern from undigested DNA. Red arrows indicate methylation of RASSF1 and CDKN2B in all six Wilms tumors. (B) The capillary electrophoresis pattern from the same sample but digested with HhaI site. Red arrows indicate that all DNA is unmethylated. Therefore, after MS-MLPA, no signal was generated from the MS-MLPA probes.