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Genetic Analysis Reveals the Important Role of the *APC* Gene in Clear Cell Renal Cell Carcinoma

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Abstract. *Background/Aim: Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults. The aim of this study was to elucidate the molecular pathogenesis of sporadic RCC in Taiwan. Materials and Methods: Fifteen patients with RCC were screened for mutations in the von Hippel-Lindau (VHL) gene by PCR and Sanger sequencing. The methylation status of promoters of 24 tumor suppressor genes by methylation sensitive multiplex ligation-dependent probe amplification analysis was also determined. Results: Inactivation of the VHL gene was observed in 5 cases: three missense somatic mutations, one promoter methylation, and one small deletion. In RCCs, methylation was most frequently observed in APC (100%), CDKN2B (92.9%), CASP8, MLH1_167, and KLLN (85.7.4%), but not in FHIT, MLH1_463, DAPK1, or HIC1 (0%). Conclusion: In addition to VHL inactivation, promoter methylation of APC may be a universal pathognomonic event in the tumorigenesis of RCC and a candidate diagnostic and therapeutic biomarker.*

Kidney cancer affects about 300,000 people worldwide and is responsible for 129,000 deaths annually (1). The global age-standardized incidence rate is 4 per 100,000 people per year (2). Moreover, age standardized incidence rate increased from 3.35/100,000 individuals in 2002 to 5.09/100,000 individuals in 2012 in Taiwan (3). Renal cell carcinoma (RCC) is the most common type in adults, accounting for around 90% of all kidney cancers (4). The incidence rates have increased over time in most populations, but mortality rates have levelled off or decreased since the 1990s (5). Based on the 2016 WHO classification, the major subtypes are clear cell, papillary, and chromophobe, which comprise 65-70%, 15-20%, and 5-7% of all RCCs, respectively (6). Clear cell RCC accounts for most kidney cancer-related deaths and is characterized by cells with clear cytoplasm (7).

The genetic feature most closely associated with sporadic clear cell RCC is loss or mutation of the von Hippel-Lindau (*VHL*) tumor suppressor gene (8-10). However, inactivation of *VHL* alone is not sufficient to cause RCC (11, 12). Other genes are likely to be important for its development, including *PBRM1* (29-41% of tumor samples), *SETD2* (8-12%), *BAP1* (6-10%), *KDM5C* (4-7%), and *MTOR* (5-6%) (5). Epigenetic inactivation of tumor suppressor genes by methylation of promoter region CpG dinucleotides has also been implicated in the pathogenesis of RCC (13, 14). Early studies have demonstrated that *VHL*, *CDKN2A/p16INK4a*, and *RASSF1A* tumor suppressor genes are frequently inactivated by methylation in clear cell RCC (14, 15). More recent studies have demonstrated tumor-specific promoter methylation of *BNC1*, *PDLIM4*, *RPRM*, *CST6*, *SFRP1*, *GREM1*, *COL14A1*, and *COL15A1* genes in more than 30% of RCCs (13).

The genetic aspects of RCC have received little attention in Taiwan. Acquired cystic disease-associated RCC has been reported to be associated with frequent abnormalities on chromosome 3 (16). Yano *et al*. noted that the CpG islands of connexin 32 gene are methylated in RCCs of hemodialysis patients (17). The aim of this study was to elucidate the possible etiological role of molecular pathogenesis in sporadic RCCs in Taiwan. A total of 15 patients with RCC were screened for mutations in the *VHL* gene and methylation of 24 tumor suppressor genes. Mutations were identified by PCR and Sanger sequencing. Methylation was determined by methylation sensitive multiplex ligation-dependent probe amplification (MS-MLPA) analysis.

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Key Words: Renal cell carcinoma, von Hippel-Lindau gene, tumor suppressor gene, methylation sensitive multiplex ligation-dependent probe amplification analysis, promoter methylation.

Table I. *Chromosomal locations of the 41 probes in ME001-C2 tumor suppressor-1.*

Length (nt)	Gene	MLPA probe	*HhaI* site	Chromosomal position	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	Yes	9q21.33	09-089.303075
364	*LOC254312*	01234-L00781	-	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
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

Materials and Methods

Study subjects. Fifteen paraffin-embedded tumor and normal tissue samples (Cases 1 to 15, 8 males and 7 females) were provided by the Tumor Tissue Bank of Koo Foundation Sun Yat-Sen Cancer Center which is funded by the National Science and Technology Program for Pharmaceuticals and Biotechnology (#NSC89-2323-B-368-001). The study procedures were approved by the Institutional Review Board of Chung Shan Medical University Hospital (reference number CS2-03052). 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.

DNA extraction. Genomic DNA was extracted from the sections with the QIAamp Tissue Kit (Qiagen GmbH., Hilden, Germany), according to the manufacturer's instructions and finally dissolved in 100 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA concentration of each sample was measured using NanoDrop UV-VIS Spectrophotometer.

Polymerase chain reaction (PCR) and direct sequencing. The three exons of the *VHL* gene were amplified in 7 fragments using published primers and protocols (18). PCR products were purified using QIAquick PCR Purification kits (Qiagen GmbH., Hilden, Germany). The purified PCR products were sequenced *via* the cycle sequencing method with fluorescently labelled dideoxy chain terminators from the ABI Prism kit (Applied Biosystems,

Table II. *RCC patients with and without somatic inactivation of the VHL gene.*

#	Age	Stage	Clear cells	Inactivation of *VHL* gene	*VHL* exon mutation		*VHL* promoter	
					Nucleotide	Protein	Tumor	Tumor/Normal
1	≥50	III	Yes	Yes	677T>G	V155G	-	-
2	<50	I	Yes	ND	ND	ND	ND	ND
3	≥50	III	Yes	Yes	ND	ND	Hypermethylation	1.88
4	≥50	II	Yes	ND	ND	ND	ND	ND
5	≥50	I	Yes	Yes	635A>G	N141S	ND	ND
6	<50	I	Yes	ND	ND	ND	ND	ND
7	≥50	I	Yes	ND	312G>T	ND	ND	ND
8	≥50	III	Yes	ND	ND	ND	ND	ND
9	≥50	I	Yes	Yes	369G>T	E52D	ND	ND
10	≥50	II or III	Yes	ND	ND	ND	ND	ND
11	<50	I	Yes	Partial	ND	ND	CNR 0.478	0.904
12	≥50	I	Yes	Partial	ND	ND	CNR 0.621	1.048
13	≥50	II	Yes	Yes	ND	ND	Deletion	0
14	≥50	I	Yes	ND	ND	ND	CNR 0.734	0.956
15	≥50	I	No	Partial	ND	ND	CNR 0.612	0.728

GenBank accession number NM_000551.3 for nucleotide and NP_000542.1 for amino acid. ND: No somatic changes were detected. Hypermethylation means that 5 normal reference DNA samples were unmethylated and tumor DNA samples were methylated; CNR: copy number rate compared to 5 normal reference DNA samples.

Taipei, Taiwan, ROC) in an ABI Model 377 automated DNA sequencer, according to the distributor's protocol. The sequencing primers were the same as those for the preceding PCRs. When a mutation was detected, the nucleotide sequence was confirmed on both strands.

Copy number and methylation analyses. MS-MLPA analysis was performed using Salsa MS-MLPA kit ME001-C2 Tumor suppressor-1 (MRC-Holland, Amsterdam, Netherlands) 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 status of promoter regions of 24 different tumor suppressor genes by *HhaI* digestion (Table I). MLPA results were analyzed using GeneMarker version 3.2.1 (SoftGenetics, LLC, State College, PA, USA) to determine copy numbers and methylation status of the *Hha*I sites. For copy number, each sample peak area was divided by the nearest control peak areas. Relative copy number was obtained by comparing this ratio with that of a control sample (19). The internal methylation ratio was calculated by comparison of the *Hha*I digested aliquot with the paired undigested aliquot from each sample with intra-sample data normalization according to the manufacturer's instructions (20). Methylation, compared to normal reference, was assessed by comparing the probe methylation percentages obtained for 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 in normal reference. If so, the methylation compared to normal reference was unlimited (∞). If methylation ratios of the test sample and normal reference samples were appropriate, methylation compared to normal reference was around 1.0.

Results

VHL gene inactivation: mutation and promoter methylation. The DNA sequences of *VHL* gene were determined *via* direct sequencing. Four mutations were identified in the exon region of *VHL* gene in the DNA samples prepared from paraffin-embedded tumor specimens (Table II). Among them were three missense mutations. Valine was substituted for glycine *via* heterozygous mutation at codon 155 (V155L) in exon 3, 677T>G in case 1 (Figure 1A); asparagine was substituted for serine *via* heterozygous mutation at codon 141 (N141S) in exon 2, 635A>G in case 5 (Figure 1B); and glutamate was substituted for aspartate *via* heterozygous mutation at codon 52 (E52D) in exon 1, 369G>T in case 9 (Figure 1D). There was one silent mutation, with no change in amino acid sequence, *via* heterozygous mutation at codon 33 in exon 1, 312G>A in case 7 (Figure 1C).

MLPA results were analyzed to determine copy numbers and methylation status of the *Hha*I sites in the promoter region of *VHL* gene located in chromosome 3p25.3 (Table II). In case 3, methylation ratios were unlimited (∞) in both normal and tumor tissue DNA compared to average normal reference (Table II, Figure 2A and B). This indicated that the *VHL* gene is inactivated by methylation of its promoter in both germline and somatic DNA. Copy number ratio of 0 was detected in tumor somatic DNA from case 13 indicating that the *VHL* probe failed to hybridize with its promoter region due to a small deletion (Table II, Figure 2E and F).

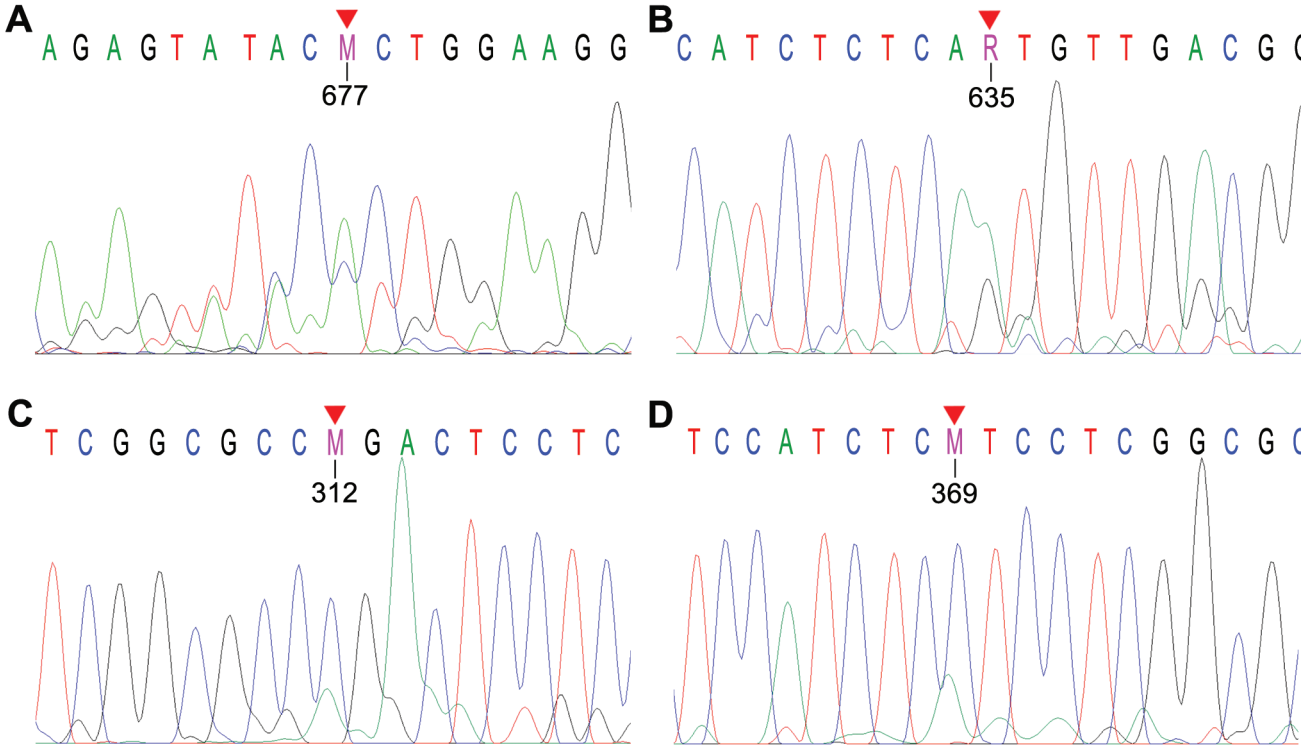

Figure 1. *Partial sequencing chromatograms represent the genetic profiles of fragment 5 in reverse direction from case 1 (A), fragment 4 in forward direction from case 5 (B), and fragment 2 in reverse directions from case 7 (C) and case 9 (D), respectively. The mutated nucleotides are marked with a red arrow.*

In addition, partial inactivation of *VHL* gene was identified due to copy number ratio decreases in cases 11, 12, and 15 in both normal and tumor tissue DNA compared to average normal reference (Table II).

Copy number and methylation analyses. MS-MLPA analysis was performed with DNA from case 2 to case 15 using Salsa MS-MLPA kit ME001-C2 tumor suppressor-1. Increases in copy number ratio of *CADM1* were found in all RCCs. The copy number ratios were 1.38, 1.40, 1.20, 1.29, 1.27, 1.37, 1.42, 1.26, 1.40, 1.30, 1.58, 1.43, 1.54 and 1.22, respectively. For case 13, in addition to the *VHL* gene, copy number ratio of 0 was detected for the *FHIT* gene indicating a small deletion (Figure 2E and F).

Methylation of *APC* (100%) was found in all RCCs (Figure 2, Table III). The second most commonly methylated gene was *CDKN2B* (92.9%). Only case 11 was found to be unmethylated. Methylation of *CASP8* (not in case 11 or 13), *MLH1*_167 (not in case 2 or 11), and *KLLN* (not in case 13 or 14) was found in 12 out of 14 (85.7%) RCCs (Table III). Methylation of *RASSF1*_382 (not in case 2, 4, 6, or 11), *CDH13* (not in case 8, 11, 13, or 14), and *CDKN2A* (not in cases 11 to 14) was found in 10 out of 14 (71.4%) RCCs. Frequencies of 9 genes with medium level of methylation were *ATM* 64.3%, *RASSF1*_328 57.1%, *CD44* 50.0%, *TP73* 42.9%, *RARB*, *ESR1*, and *BRCA1* 35.7%, *TIMP3* and *GSTP1* 28.6%. Moreover, methylation of *CDKN1B* (case 12), *BRCA2* (case 12), and *CADM1* (case 2) was identified in only one (7.1%) case of RCC. Methylation of *CHFR* was identified in only two RCCs, case 3 and case 8. Twenty-one out of 26 MS-MLPA probes showed somatic DNA methylation only. *CDKN2B*, *MLH1*_167, *CDH13*, *RASSF1*_328 and *RARB* demonstrated germline DNA methylation (data not shown). Somatic DNA methylation means that methylation is found in RCC tissue only, not in their corresponding normal tissues. Four of the 24 genes (*FHIT*, *MLH1*_463, *DAPK1*, and *HIC1*) did not show detectable promoter region methylation (Table III).

Patient characteristics in relation to methylations status of tumor suppressor genes. Age (≥50, <50), clear cell type RCC (yes/no), and tumor stage (early, stage I and II; late, stage III to IV) are dichotomous variables based on Moore's work (21). Pathological stage is an important determinant of survival. We found a novel and interesting correlation between methylation of the *CHFR* gene promoter and late

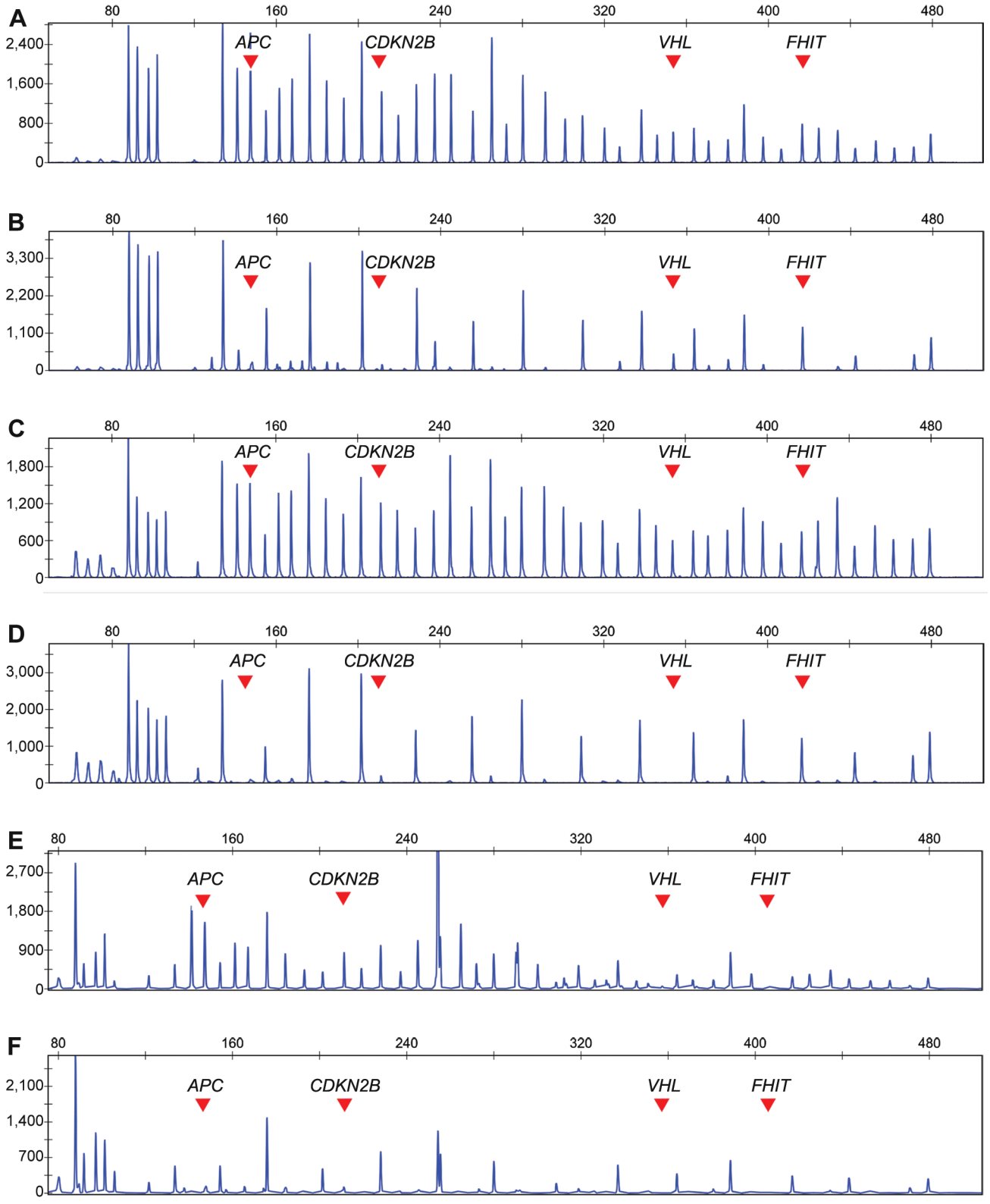

Figure 2. *Detection of the methylation status of 24 different tumor suppressor genes in RCCs by MS-MLPA. The capillary electrophoresis pattern of undigested DNA of case 3 (A), case 7 (C), and case 13 (E) and of the DNA from the same sample digested with HhaI (B, D, F). Red arrows indicate fragment locations of APC, CDKN2B, VHL, and FHIT.*

Table III. *RCC patients with and without methylation in the promoter of 24 tumor suppressor genes.*

Size	Gene	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15
148	*APC*	0.071	0.118	0.112	0.044	0.047	0.072	0.220	0.111	0.111	0.175	0.064	0.303	0.193	0.096
High															
211	*CDKN2B*	0.128	0.107	0.228	0.159	0.261	0.122	0.137	0.174	0.163	0	0.091	0.438	0.182	0.161
265	*CASP8*	0.035	0.040	0.026	0.021	0.020	0.075	0.089	0.097	0.042	0	0.059	0	0.086	0.050
167	*MLH1*	0	0.122	0.080	0.046	0.047	0.097	0.107	0.067	0.070	0	0.165	0.309	0.138	0.074
292	*KLLN*	0.083	0.056	0.063	0.040	0.055	0.060	0.045	0.034	0.032	0.297	0.081	0	0	0.049
382	*RASSF1*	0	0.378	0	0.319	0	0.174	0.183	0.180	0.384	0	0.650	0.376	0.296	1.084
436	*CDH13*	0.077	0.139	0.098	0.048	0.048	0.047	0	0.080	0.065	0	0.221	0	0	0.070
161	*CDKN2A*	0.086	0.107	0.050	0.033	0.047	0.061	0.124	0.064	0.038	0	0	0	0	0.061
Medium															
184	*ATM*	0	0.118	0.083	0	0	0.127	0.057	0.060	0.052	0	0	0.535	0.158	0.096
328	*RASSF1*	0	0.463	0	0.321	0	0.122	2.051	0.180	0.296	0	0.486	0	0	0.805
319	*CD44*	0.135	0	0	0.175	0.147	0	0	0	0.041	0	0.073	0.160	0	0.075
400	*TP73*	0	0	0	0	0	0	0.113	0.075	0.088	0	0.080	0	0	0.131
193	*RARB*	0	0	0.117	0.056	0	0	0.209	0.092	0.038	0	0	0	0	0
373	*ESR1*	0	0	0	0.107	0.097	0	0	0.113	0.189	0	0	0	0	0
246	*BRCA1*	0	0	0	0	0.023	0	0.034	0.043	0	0	0.057	0	0	0
142	*TIMP3*	0	0.217	0	0	0	0	0.154	0.057	0.049	0	0	0	0	0
454	*GSTP1*	0	0	0	0	0	0	0	0.071	0.046	0	0.810	0	0	0.101
Low															
238	*CHFR*	0	0.327	0	0	0	0	0.069	0	0	0	0	0	0	0
274	*CDKN1B*	0	0	0	0	0	0	0	0	0	0	0.090	0	0	0
301	*BRCA2*	0	0	0	0	0	0	0	0	0	0	0.115	0	0	0
427	*CADM1*	0.080	0	0	0	0	0	0	0	0	0	0	0	0	0
409	*FHIT*	0	0	0	0	0	0	0	0	0	0	0	0*	0	0
463	*MLH1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
346	*DAPK1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
220	*HIC1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Data are presented as internal methylation ratio. *Deleted due to copy number equaling zero.

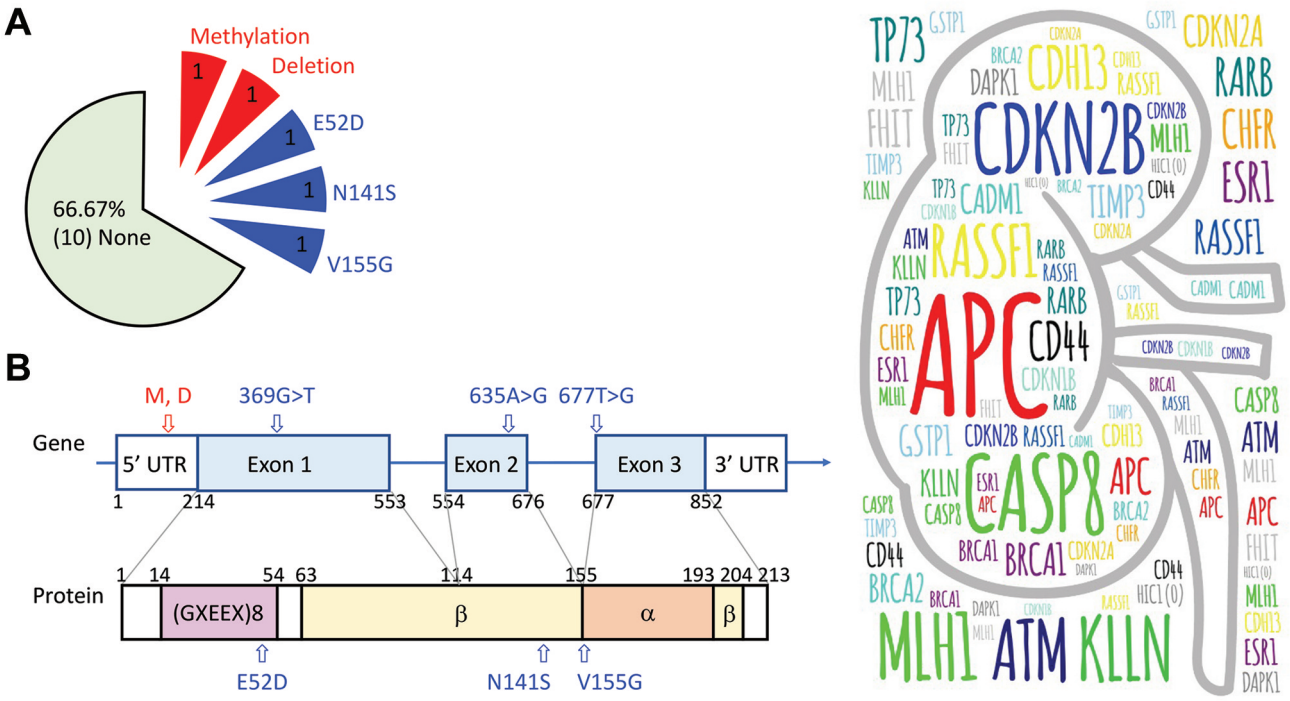

Figure 3. *Pie chart shows that inactivation of the VHL gene was observed in 5 cases (A). Arrows indicate the gene and protein location of the VHL gene inactivation observed in 5 cases (B). Red arrow indicates promoter methylation in case 3 and a small deletion in case 13; blue arrows indicate three missense somatic mutations, V155G in case 1, N141S in case 5, and E52D in case 9. Word cloud art illustrates the extent of methylation of 24 tumor suppressor genes (C).*

stage. No other gene associations were found between promoter methylation and age, or clear cell type RCC. RCC incidence is higher in men than in women (5). However, there was no significant difference in the numbers of males and females in this study (8 males and 7 females).

Discussion

For clear cell RCC, chromosome 3p deletion occurs in at least 95% of the cases and duplication, but not deletion of chromosome 5q, in approximately 30-40% of the cases (22). It has been suggested that the *VHL* tumor suppressor gene is a major gatekeeper gene for clear cell RCC (23). Approximately 50%-80% of sporadic RCCs are shown to have mutations of the *VHL* gene in Western countries (24, 25). In this study, which was conducted in Taiwan, the frequency of *VHL* mutation events for sporadic RCCs was only 20% (3/15), which is much lower than that in Western countries. Our results showed promoter hypermethylation in 1 of 15 (6.6%) tumors. This ratio is also lower than that of a previous study in which silencing of the *VHL* gene by DNA methylation occurred in about 20% of RCCs (10, 24). Recently, it has been reported in the Cancer Genome Atlas that 7% of clear cell RCCs showed epigenetic silencing at *VHL* (7, 26). The discrepancy may be attributed to ethnic effects. However, further studies using larger samples are recommended to verify our results. In the present study, both *FHIT* and *VHL* deletions were found in case 13 (age ≥50, stage II). A previous study has suggested that *FHIT* deletion is an early event and *VHL* deletion as an early and/or late event in RCC (27).

Dulaimi *et al*. reported that the frequencies of hypermethylation in 100 kidney tumors were *RASSF1A* (45%), *APC* (14%), *RARB2* (12%), *CDKN2A/p16INK4a* (10%), and *VHL* (8%) (14). Morris *et al*. noted that RCCs are most frequently methylated at *DAPK* (24%), not at *RARB2* (0%), *CDKN2A/p16INK4a* (0%) or *CDH13* (3%) (28). However, these results were not verified by this study as RCCs were found to be most frequently methylated at *APC* (100%), *CDH13* (71.4%, 10/14), *CDKN2A* (71.4%, 10/14), *RARB2* (35.7%, 5/10), and *VHL* (7.14%, 1/14), not at *DAPK1* (0%). Based on the results of this study, frequencies of promoter methylation in *RASSF1A,* 71.4% (10/14) for *RASSF1*_382 on MV location 03-050.353347, and 57.1% (8/14) for *RASSF1*_328 on MV location 03-050.353298 were much higher than in previous studies in which *RASSF1A* promoter methylation was detected in 56% and 40% of primary clear cell RCCs by Yoon *et al*. and Tokinaga *et al*.,

respectively (29, 30). In this study, neither germline nor somatic DNA methylations in *DAPK1* were identified, which is inconsistent with the findings of a previous study (31). The reasons for this discrepancy are unclear but may be related to the sensitivity of the methods used. With older molecular methods based on radio-labeled primers and polyacrylamide gel electrophoresis, small minor bands may be missed or mistaken. Capillary gel electrophoresis with fluorescence detection allows for the analysis of methylation status with high sensitivity. Dulaimi *et al*. also noted that *RASSF1A* methylation is significantly associated with high-grade tumors (14). Recent studies have highlighted that 16% of RCC cases have loss of *CDKN2A* through mutation, deletion, or promoter hypermethylation (7, 32).

Although there were differential methylation patterns of the 24 tumor suppressor genes among the 14 RCCs, at least two (mean=10.7) genes were methylated in each tumor sample. In this study, all RCCs showed methylation of *APC* specific to RCC, not in normal tissues, which did not change with age. *APC* gene encodes a 312-kDa protein that acts as an antagonist of the Wnt signaling pathway (33). Deregulation of Wnt signaling pathway through *APC* deficiency or loss of heterozygosity has recently been implicated in human RCC (34-36). Aberrant methylation of the *APC* gene promoter has been reported not only in colon (37), but also in breast and lung carcinomas (38). The accumulation of a variety of genetic aberrations is necessary for the initiation and progression of RCCs (39). These results indicated that methylation of *APC* is a universal pathognomonic event in tumorigenesis of RCC and can be a candidate diagnostic and therapeutic biomarker in liquid biopsy as it is found early in the process of carcinogenesis.

In addition to *APC* methylation, there were a variety of other genetic aberrations. *CDKN2B* gene methylation was observed in all RCCs, except for case 11. *CDKN2B* gene on 9p21.3 encodes the $p15^{INK4B}$ protein that binds to and inhibits activation of CDK4 or CDK6 (40). Germline mutations in *CDKN2B* have been identified as a novel cause of familial RCC (41). *CASP8* gene encodes Caspase-8 that is an apoptosis-related cysteine peptidase (42). Methylation at *CASP8* has been demonstrated in 16% of RCCs (28). *MLH1* gene encodes proteins that detect and repair DNA mismatches (43). Expression of mismatch repair MLH1 proteins has been shown to be reduced in 83.7% (118/141) of sporadic RCCs (44). *KLLN* gene encodes the protein killin, which is a p53-regulated nuclear inhibitor of DNA synthesis (45). Bennett *el al*. found germline methylation in 23/41 (56%) RCC patients and somatic methylation in 19/20 (95%) patients with advanced RCC (46). These results indicated that methylation of *APC*, *CDKN2B*, *CASP8*, *MLH1*_167, and *KLLN* is important in the tumorigenesis of RCC, and may have diagnostic, clinical, and therapeutic significance.

In conclusion, inactivation of the *VHL* gene was observed in 5 cases: three missense somatic mutations, V155G in case 1, N141S in case 5, and E52D in case 9, promoter methylation in case 3, and small deletion in case 13 (Figure 3A and B). RCCs were most frequently methylated at *APC* (100%, 14/14), *CDKN2B* (92.9%, 13/14), *CASP8*, *MLH1*_167, and *KLLN* (85.7.4%, 12/14), but not at *FHIT*, *MLH1*_463, *DAPK1*, and *HIC1* (0%) (Figure 3C). The rate of *VHL* inactivation and promoter methylation profile for RCCs in the Taiwanese population differ from those in Western populations. This may be attributed to ethnic effects. However, larger sample size is required to confirm these findings. Moreover, methylation of *APC* may be a universal pathognomonic event in tumorigenesis of RCC and a candidate diagnostic and therapeutic biomarker.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in regard to this study.

Authors' Contributions

Yen-Chein Lai: designed the experiments, performed the experiments, interpreted the results, and drafted the manuscript. Wen-Chung Wang: designed the experiments, interpreted the results, and made critical revisions to the manuscript. All authors have read and approved the final manuscript.

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