Methylation of adenomatous polyposis coli (*APC*) gene promoter is a generalized feature on molecular pathology tests of sporadic renal cell carcinomas in Taiwan

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Research article

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Abstract

Background: Renal cell carcinoma (RCC) is the most common form of kidney cancer in adults. Approximately 50% to 80% of sporadic RCCs are associated with mutations in the von Hippel-Lindau (*VHL*) gene in Western countries. The aim of this study is to elucidate the possible etiological role of molecular pathogenesis in sporadic RCCs in Taiwan.

Methods: Fifteen patients with RCC were screened for mutations in the *VHL* gene and methylation statuses of promoters of 24 tumor suppressor genes. Mutations were identified by PCR and Sanger sequencing. Methylation statuses were determined on methylation sensitive multiplex ligation-dependent probe amplification (MS-MLPA) analysis.

Results: Inactivation of *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. 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*, or *HIC1* (0%).

Conclusions: In addition to *VHL* inactivation, promoter methylation of *APC* and *CDKN2B* may play important roles in the tumorigenesis of RCC. Methylation of *APC* may be a very early pathognomonic event in tumorigenesis of RCC and a candidate diagnostic and therapeutic biomarker.

Background

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 form in adults, accounting for around 90% of all kidney cancer [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 in 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 of 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 genes *BNC1*, *PDLIM4*, *RPRM*, *CST6*, *SFRP1*, *GREM1*, *COL14A1*, and *COL15A1* 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 is 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 statuses in 24 tumor suppressor genes. Mutations were identified by PCR and Sanger sequencing. Methylation statuses were determined on methylation sensitive multiplex ligation-dependent probe amplification (MS-MLPA) analysis.

Methods

Study subjects

Fifteen paraffin-embedded tumor and normal tissue samples (Cases 1 to 15, 8 males and 7 females, Table 1) 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.

Table 1
RCC patients with and without somatic inactivation of *VHL* gene

				Inactivation of *VHL* gene	*VHL* Exon Mutation		*VHL* Promoter	
#	Age	Stage	Clear cells		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.								

DNA extraction

Genomic DNA was extracted from the sections with the QIAamp Tissue Kit (Qiagen), 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 with published primers under published conditions (Additional File 1) [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 ABI Prism kit (Applied Biosystems, Taipei, Taiwan) 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) 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 (Additional File 2). MLPA results were analyzed using GeneMarker version 3.2.1 (SoftGenetics, LLC) to determine copy numbers and methylation statuses 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 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 from paraffin-embedded tumor specimens (Table 1). 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 (Fig. 1A); asparagine was substituted for serine via heterozygous mutation at codon 141 (N141S) in exon 2, 635A > G in case 5 (Fig. 1B); and glutamate was substituted for aspartate via heterozygous mutation at codon 52 (E52D) in exon 1, 369G > T in case 9 (Fig. 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 (Fig. 1C).

MLPA results were analyzed to determine copy numbers and methylation statuses of the *Hha*I sites in the promoter region of *VHL* gene located in chromosome 3p25.3 (Table 1). In case 3, methylation ratios were unlimited (∞) in both normal and tumor tissue DNA compared to average normal reference (Table 1, Fig. 2A and 2B). This indicated that the *VHL* gene is inactivated by its promoter methylation in both germline and somatic DNA. Copy number ratio of 0 was detected in tumor somatic DNA from case 13, and meaning that the *VHL* probe failed to hybridize with its promoter region due to a small deletion (Table 1, Fig. 2E and 2F). 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 1).

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. 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 in the *FHIT* gene indicating a small deletion (Fig. 2E and 2F).

Methylation of *APC* (100%) was found in all RCCs (Fig. 2, Table 2). 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 2). 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%) 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.

Table 2
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.

Four of the 24 genes (*FHIT*, *MLH1*_463, *DAPK1*, and *HIC1*) did not show detectable promoter region methylation (Table 2).

Patient characteristics in relation to methylations statuses in 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 stage. No other gene associations for promoter methylation were found for 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

It has been suggested that the *VHL* tumor suppressor gene is a major gatekeeper gene for clear cell RCC [22]. About 50%-80% of sporadic RCCs are shown to have mutations of the *VHL* gene [23, 24]. 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 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, 23]. Recently, it has been reported in the Cancer Genome Atlas that 7% of clear cell RCCs showed epigenetic silencing at *VHL* [7, 25]. 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 [26].

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%) [27]. 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*, 76.9% (10/13) for *RASSF1A*_382, and 61.5% (8/13) for *RASSF1A*_382, in 8/14 cases were much higher than in previous studies in which *RASSF1A* promoter methylation was detected in 56% and 40% of primary clear cell RCCs, respectively [28, 29]. In this study, neither germline nor somatic DNA methylations in *DAPK1* were identified, which is inconsistent with the findings of a previous study [30]. 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, 31].

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 [32]. Deregulation of Wnt signal pathway through *APC* deficiency or loss of heterozygosity has recently been implicated in human RCC [33–35]. Aberrant methylation of the *APC* gene promoter has been reported not only in colon [36], but also in breast and lung carcinomas [37]. The accumulation of a variety of genetic aberrations is necessary for the initiation and progression of RCCs [38]. These results indicated that methylation of *APC* is a very early pathognomonic event in tumorigenesis of RCC and can be a candidate diagnostic and therapeutic biomarker 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 [39]. Germline mutations in *CDKN2B* have been identified as a novel cause of familial RCC [40]. *CASP8* gene encodes Caspase-8 that is an apoptosis-related cysteine peptidase [41]. Methylation at *CASP8* has been demonstrated in 16% of RCCs [27]. *MLH1* gene encodes proteins that detect and repair DNA mismatches [42]. Expression of mismatch repair proteins MLH1 has been shown to be reduced in 83.7% (118/141) of sporadic RCCs [43]. *KLLN* gene encodes the protein killin, which is a p53-regulated nuclear inhibitor of DNA synthesis [44]. Bennett *el al.* found germline methylation in 23/41 (56%) RCC patients and somatic methylation in 19/20 (95%) advanced RCC patients [45]. These results indicated that methylation of *APC*, *CDKN2B*, *CASP8*, *MLH1*_167, and *KLLN* is important in the tumorigenesis of RCC, which may inform its diagnostic, clinical, and therapeutic management.

Conclusions

Inactivation of *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. 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%). 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 finding. Moreover, methylation of *APC* may be a very early pathognomonic event in tumorigenesis of RCC and a candidate diagnostic and therapeutic biomarker.

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; *KDM6A*: lysine (K)-specific demethylase 6A; *MLH1*: mutL homolog 1; MS-MLPA: methylation-specific multiplex ligation-dependent probe amplification; NT: nucleotide; *PBRM1*: polybromol; *RARB2*: retinoic acid receptor B2; *RASSF1*: ras-association domain family member 1; RCC: renal cell carcinoma; *SETD2*: SET domain containing 2; UV-VIS: ultraviolet–visible; *VHL*: von Hippel-Lindau.

Declarations

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Authors' contributions

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.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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 CS-03052. 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

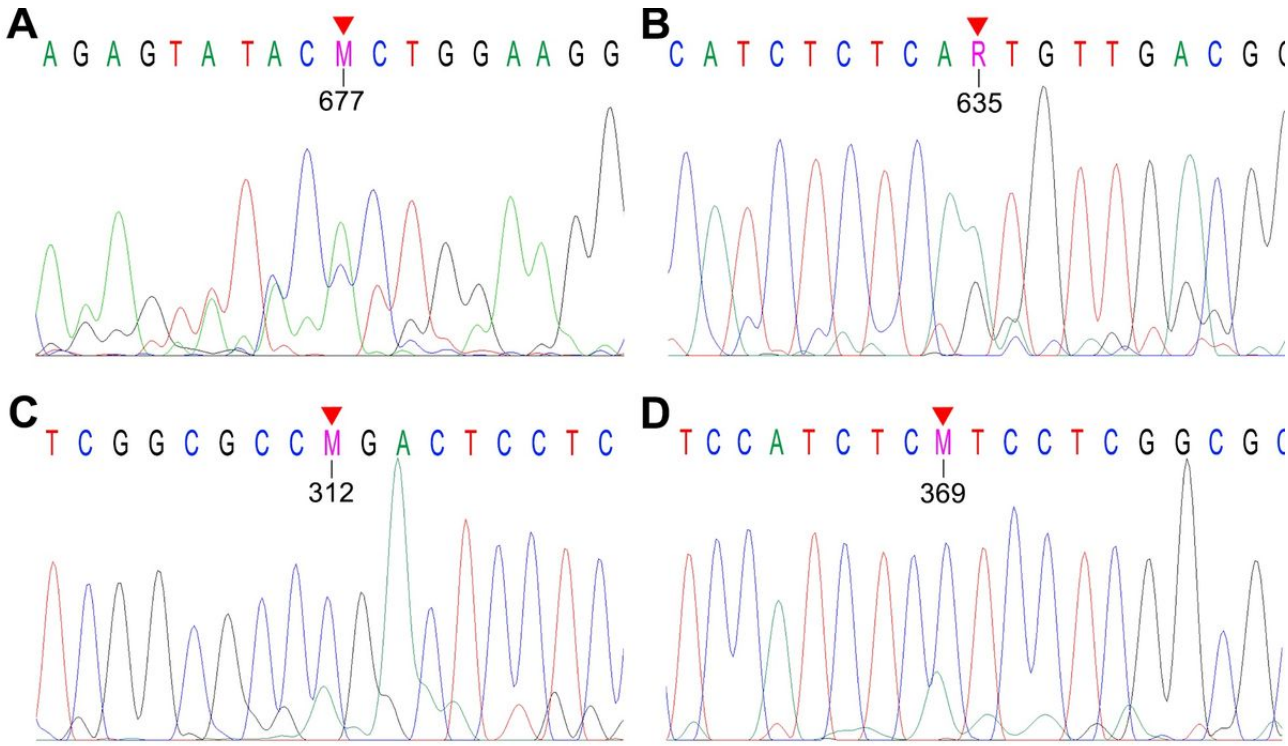

Figure 1

Partial sequencing chromatograms represent the genetic profiles of fragment 5 in reverse directions from case 1 (A), fragment 4 in forward directions 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.

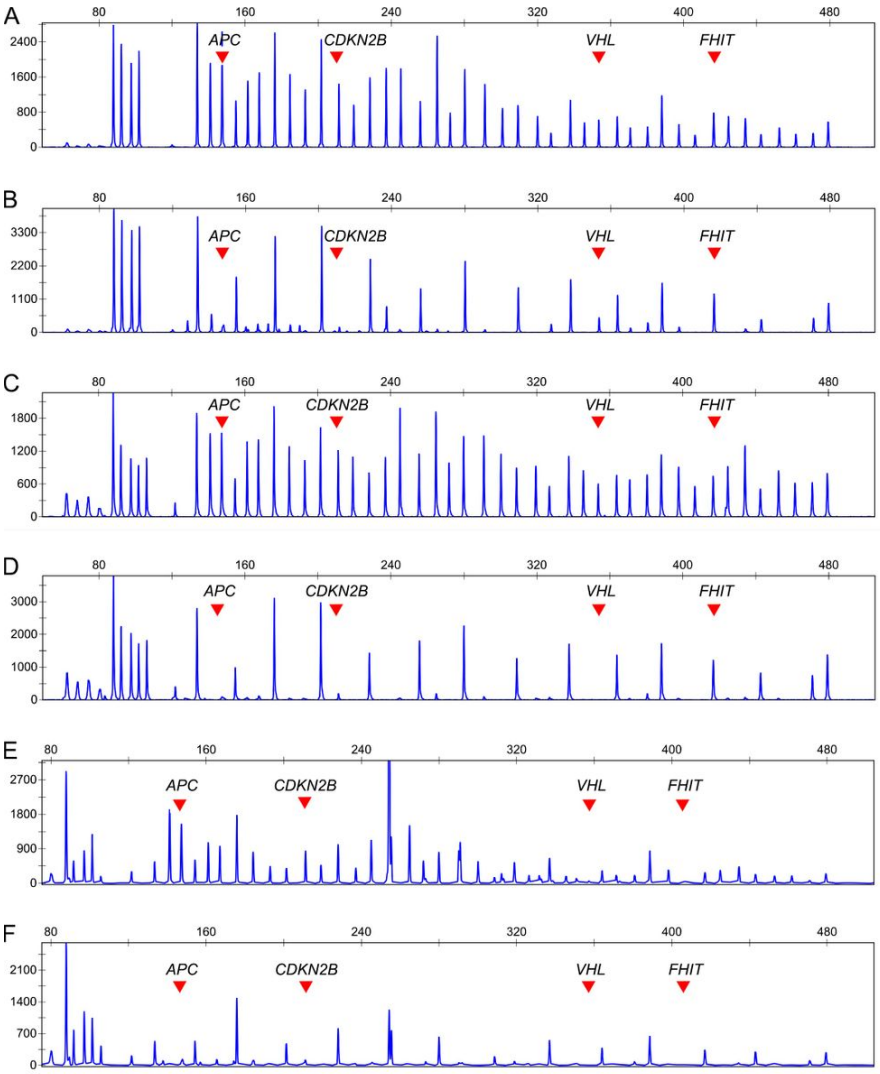

Figure 2

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

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