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Epigenetic Inactivation of the *RASSF1A* 3p21.3 Tumor Suppressor Gene in Both Clear Cell and Papillary Renal Cell Carcinoma[1]

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ABSTRACT

Renal cell carcinoma (RCC), the most common adult kidney neoplasm, is histopathologically heterogeneous, with most sporadic RCCs (~80%) classified as clear cell (CC) tumors. Chromosome 3p allele loss is the most frequent genetic alteration in RCC but is associated specifically with sporadic and hereditary forms of clear cell RCC (CC-RCC) and is not a feature of non-CC-RCC, such as papillary (chromophilic) RCC. The *VHL* tumor suppressor gene (TSG) maps to chromosome 3p25, and somatic inactivation of the *VHL* gene occurs in up to 70% of CC-RCC tumors and cell lines. However, *VHL* inactivation is not sufficient for CC-RCC tumorigenesis, and inactivation of 3p12–p21 TSG(s) appears to be necessary in CC-RCC irrespective of *VHL* gene inactivation status. Recently, we demonstrated that the candidate 3p21 TSG, *RASSF1A*, is hypermethylated in most small cell lung cancers. We have now investigated the role of *RASSF1A* inactivation in primary RCC tumors. *RASSF1A* promoter methylation was detected in 23% (32 of 138) of primary CC-RCC tumors. In CC-RCC cell lines, *RASSF1A* methylation was associated with silencing of *RASSF1A* expression and restoration of expression after treatment with 5′-azacytidine. The frequency of *RASSF1A* methylation was similar in CC-RCC with and without *VHL* gene inactivation (24% *versus* 21%), and there was no association between epigenetic silencing of the *RASSF1A* and *VHL* TSGs, because 0 of 6 tumors with *VHL* hypermethylation had *RASSF1A* methylation, and *VHL* was not methylated in 26 CC-RCCs with *RASSF1A* methylation. Although 3p allele loss has been reported rarely in papillary RCC, we identified *RASSF1A* methylation in 44% (12 of 27) of papillary RCCs analyzed. Thus: (*a*) inactivation of *RASSF1A* is a frequent event in both CC-RCC and papillary RCC tumors; (*b*) there is no relationship between epigenetic silencing of *RASSF1A* and *VHL* inactivation status in CC-RCC. Fifty-four CC-RCCs analyzed for *RASSF1A* methylation were informative for 3p21 allele loss, and 20% (7 of 35) with 3p21 allele loss demonstrated *RASSF1A* methylation. All informative CC-RCCs with 3p21 allele loss and no *RASSF1A* methylation also demonstrated allele losses at other regions of 3p so that tumorigenesis in these cases may result from: (*a*) haploinsufficiency of *RASSF1A*; (*b*) inactivation of other 3p21 TSGs; or (*c*) inactivation of 3p TSGs from outside of 3p21. *RASSF1A* is the first TSG to be inactivated frequently in both papillary and CC-RCCs. The finding of frequent epigenetic inactivation of *RASSF1A* in papillary RCCs despite previous studies reporting infrequent 3p21 allele loss in this tumor type illustrates how the systematic identification of all major human cancer genes will require detailed analysis of the cancer genome and epigenome.

INTRODUCTION

RCC[3] accounts for ~2% of adult malignancies and is the most common adult kidney neoplasm. Histopathologically, most sporadic RCCs (~80%) are classified as CC-RCCs, and papillary (chromophilic) tumors are the most common form of non-CC-RCC (1). Deletions of the short arm of chromosome 3 are the most widely observed genetic aberration in RCC. Although 3p allele loss is reported in 45–90% of sporadic RCCs, it is associated specifically with hereditary and sporadic CC-RCCs and is infrequent in non-CC-papillary RCC (2–8). Germ-line mutations in the *VHL* TSG are associated with a high risk of CC-RCCs, and somatic inactivation of the *VHL* gene (by loss, mutation, or epigenetic silencing) occurs in up to 70% of CC-RCCs (9–14). However, *VHL* gene mutations are rare in non-CC-RCCs. In contrast, germ-line mutations in the *MET* proto-oncogene cause hereditary papillary (chromophilic) RCCs, and somatic *MET* gene mutations are rare in CC-RCCs (15).

Deletions of 3p are frequent in many adult cancers including lung, breast, ovary, testicular, and head and neck carcinomas (16). *VHL* TSG inactivation does not occur in these tumor types; therefore, several TSGs map to 3p. In addition to the *FHIT* TSG at 3p14, the finding of homozygous deletions in lung and breast cancers has mapped additional TSGs to 3p21 and 3p12. In detailed 3p allele-loss studies of CC-RCCs, we found evidence that loss of 3p12–p21 TSGs is required for tumor development in CC-RCCs with and without *VHL* gene inactivation, and that *VHL* inactivation alone was not sufficient for tumorigenesis (14, 17, 18). Furthermore, chromosome transfer experiments demonstrated that 3p fragments not containing the *VHL* gene (*e.g.*, 3p12–p14 and 3p21–p22) can suppress RCC tumorigenicity (19, 20).

Within 3p12–p21, several distinct regions are proposed to contain TSGs. The *FHIT* gene has been reported to suppress RCC tumorigenicity in nude mouse assays, and reduced expression and hemizygous deletions involving *FHIT* may occur in the majority of CC-RCCs (21–24). However, the role of *FHIT* in RCC tumorigenesis has been questioned (25), and 3p allele loss analysis in CC-RCCs suggests a primary role for 3p21 TSG(s) (14, 18, 26). A critical 120-kb interval for a lung and breast cancer TSG was identified by overlapping homozygous deletions (27, 28). We identified eight genes within this interval, and although mutation analysis of these candidate TSGs in lung tumors and tumor lines revealed only rare inactivating mutations, we and others have demonstrated promoter methylation and absent expression of *RASSF1A* in many lung cancer cell lines and tumors (29–31). In view of the evidence for a 3p21 TSG in CC-RCCs, we have investigated *RASSF1A* as a candidate gene in RCC.

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[3] The abbreviations used are: RCC, renal cell carcinoma; CC-RCC, clear cell RCC; VHL, von Hippel-Lindau; TSG, tumor suppressor gene; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity.

MATERIALS AND METHODS

Patients and Samples

Two hundred eleven sporadic primary RCC tumor and normal (blood or kidney) DNA pairs were analyzed for *RASSF1A* promoter methylation. In addition, we also examined 13 RCC cell lines that had been described previously (11). Tumors were histopathologically classified according to the criteria of Thoenes *et al.* (1). One hundred eighty-one tumors (mostly CC-RCCs) had been analyzed previously for *VHL* gene mutation and methylation status (10, 13, 14). DNA samples from a panel of primary papillary RCCs and corresponding normal tissues were provided by P. Schraml and have been described previously (32).

Molecular Genetic Studies

Bisulfite modification of the DNA was carried out as described previously (30). Briefly, 0.5–1 μg of DNA was denatured by incubation with 0.3 M NaOH for 15 min at 37°C, before sulfonation of unmethylated cytosines by incubation in 3.12 M sodium bisulfite/1 M hydroquinone (pH 5) at [99°C (for 30 s) and 50°C (for 15 min) $\times$ 20 cycles. The sulfonated DNA was recovered using the Wizard DNA clean-up system (Promega) in accordance with the manufacturer's instructions. The DNA was then desulfonated by incubation in 0.3 M NaOH for 10 min at room temperature, before ethanol precipitation and resuspension in water.

DNA sequences specific for the *RASSF1A* promoter region were amplified using primers and conditions described previously (30). Dose-response experiments showed no evidence of significant amplification bias for either methylated or unmethylated DNA (data not shown). Methylated cytosine residues were identified by restriction enzyme digestion. Briefly, 16 μl of the 204-bp PCR product were incubated with 20 units of *Taq*I (Roche) or *Bst*UI (New England BioLabs) for 2 h at 65°C and 60°C, respectively. The possible sizes of the *Taq*I restriction enzyme digestion products are 173, 112, 92, 81, and 31 bp; the sizes of the *Bst*UI digestion products are 172, 121, 89, 83, and 32 bp (30). The restriction enzyme digestion products were then visualized by separation in a 3% agarose gel, and the presence of methylation was verified in a limited number of samples by direct sequencing of the PCR product.

Briefly, PCR products containing bisulfite-resistant cytosines were purified using the Qiaquick gel extraction kit (Qiagen) and ligated into the pGEM-T Easy Vector system (Promega) according to the manufacturers' instructions. Clones were then isolated and sequenced using a dRhodamine sequence cycling kit (Perkin-Elmer) on an ABI 3700 automated sequencer (Applied Biosystem).

RASSF1A Mutation Analysis and Allele Loss Studies

Mutation screening in primary tumor DNA was performed using the PCR-single strand conformation polymorphism method using intronic primers as described previously (30). Aberrantly migrating bands were sequenced on an ABI 377 automated sequencer. Results of detailed 3p LOH analysis on many samples have been reported previously, but alleleotyping at *D3S4604*, which maps close to *RASSF1A*, was performed in a subset of samples. Allelic loss was considered to be present in tumor samples when there was a 50% or greater reduction in signal intensity of an allele in tumor DNA compared with normal DNA.

RASSF1A Expression Analysis

5′-Azacytidine Treatment.

Cell lines KTCL-26, SKRC 39, and SKRC 47 where treated with 5 μM 5′-azacytidine (Sigma Chemical Co.) for 5, 7, and 9 days. The cells where then harvested, and RNA was extracted using the RNeasy Extraction kit (Qiagen). Prior to RT-PCR, the RNA was treated with 10 units of DNase (Sigma Chemical Co.) and incubated at 37°C for 10 min, before re-extraction, as described in the Qiagen RNA clean-up protocol.

RT-PCR of *RASSF1A*. RT-PCR was carried out using the Ready-To-Go RT-PCR bead system (Amersham Pharmacia Biotech) as described in the manufacturer's instructions. Briefly, 0.5 μg of RNA from treated and untreated cell lines was reverse transcribed as described previously (31).

As a control, we also assayed levels of the shorter *RASSF1* isoform C, which has been shown previously to be widely expressed and not susceptible to methylation (31, 33). 123F2E2RT-PCR (5′-ACC TGA CCT TTC TCA AGC TG-3′) was used as the forward primer, with 123F2E31R (5′-CAT CCT TGG GGA GGT AAA AG-3′) used as the reverse primer. Conditions were 95°C for 5 min and a sequence of 95°C (for 30 s), 60°C (for 30 s), and 72°C (for 30 s) 40 cycles, and 72°C for 3 min for 1 cycle.

Cell Culture. RCC cell lines were grown in DMEM supplemented with 10% FCS at 37°C and 5% CO_2.

Statistical Analysis

Comparisons were made by Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Initially, we investigated the frequency of *RASSF1A* promoter methylation in 211 RCCs of known and unknown histopathology. Overall, we identified *RASSF1A* promoter methylation in 59 of 211 (28%) primary tumors. In view of the strong correlations between molecular pathology and histopathological subtypes, we then proceeded to concentrate on 165 RCCs of confirmed histopathology representing the two most common forms of RCC (138 CC-RCCs and 27 papillary tumors).

***RASSF1A* Methylation and Mutation Status in CC-RCC.** Twenty-three % (32 of 138) CC-RCC tumor samples demonstrated *RASSF1A* hypermethylation (Fig. 1). To determine the precise pattern of CpG methylation within the *RASSF1A* CpG island, we directly sequenced 23 clones from 3 CC tumors with *RASSF1A* methylation from nucleotides -110 to $+41$ bp after sodium bisulfite modification (see "Materials and Methods"). Two tumor samples demonstrated methylation of all 16 CpGs within the amplified fragment (Fig. 2*b*), and 1 had methylation at 14 of 16 CpGs. In addition to the promoter fragments demonstrating complete CpG methylation, there were also clones of unmethylated DNA that were attributed to the presence of contaminating normal tissue (tumor samples were not microdissected). For each of the 106 CC-RCCs that did not demonstrate *RASSF1A* methylation, we also analyzed the methylation status of the corresponding blood ($n = 26$) or normal renal tissue ($n = 80$) DNA. In 2 normal samples (both extracted from renal tissue), *RASSF1A* methylation was detected by the *Taq*I restriction digest assay. After excluding a sample switch (by demonstrating the presence of a previously characterized tumor *VHL* mutation in the tumor sample but

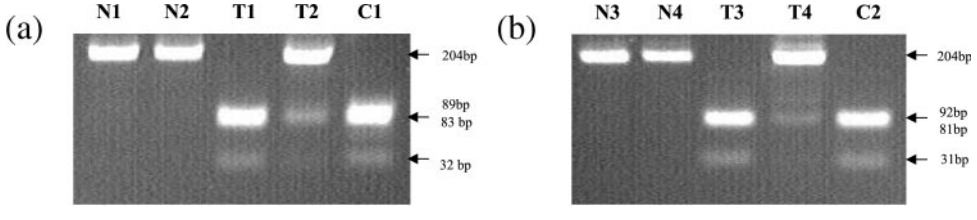

Fig. 1. Restriction enzyme digestion of the *RASSF1A* promoter CpG island of bisulfite-treated normal (*N*), CC-RCC tumor (*T*), and CC-RCC cell line (*C*) samples (generated as described in "Materials and Methods") using *Bst*UI restriction enzyme digestion (*a*), and *Taq*I restriction enzyme digestion (*b*). Samples C1, C2, and positive control [small cell lung cancer cell line DNA methylated at 16 of 16 CpGs in the CpG island (30)] show complete digestion of both alleles, with T2, T3, and T4 showing partial digestion only. Normal samples and T1 were undigested. Sizes (bp) of restriction fragments are shown.

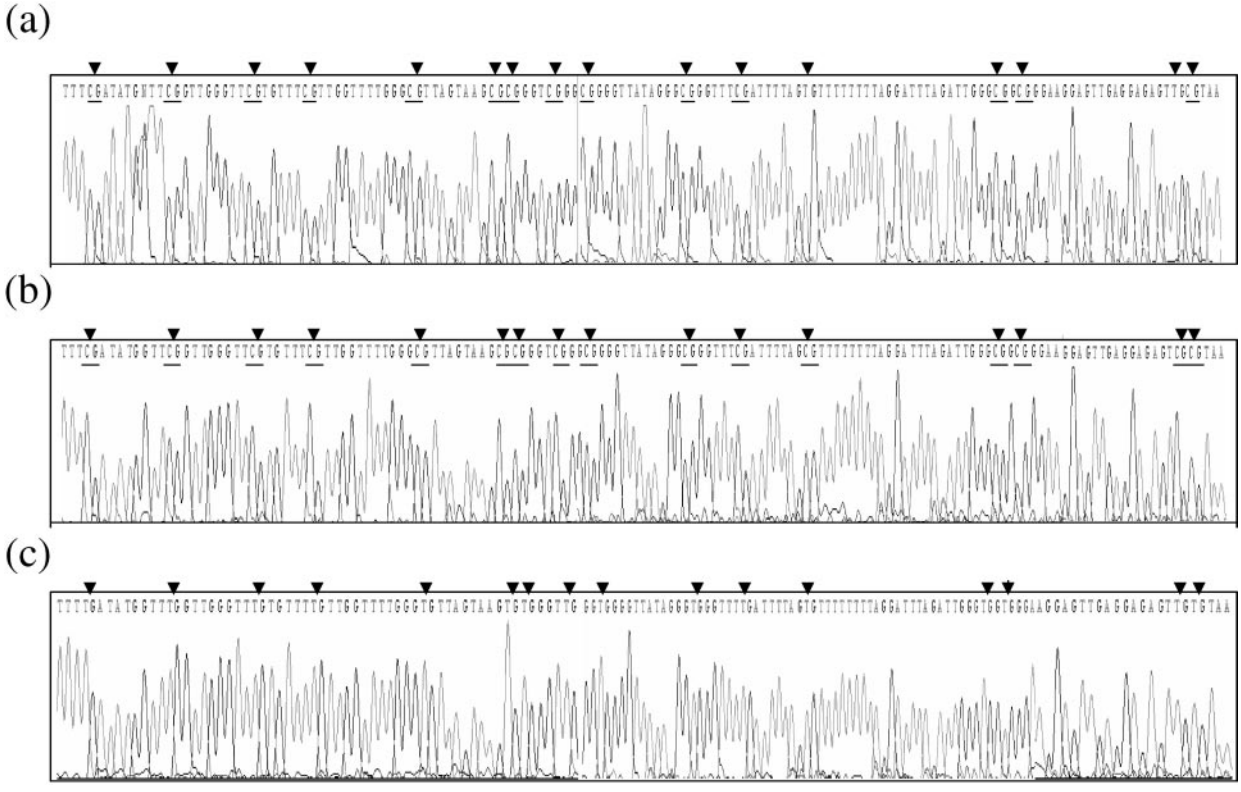

Fig. 2. Bisulfite sequencing of the *RASSF1A* promoter CpG island for papillary tumor DNA exhibiting partial methylation of the CpG island (14 of 16 CpGs methylated; *a*), CC-RCC DNA showing full methylation of the CpG island (16 of 16 CpGs methylated; *b*), and normal kidney DNA exhibiting no methylation (0 of 16 CpGs show methylation). *c*), ▼, CpG; _, CpG exhibiting methylation.

not the normal kidney sample), we proceeded to sequence 16 clones from each of the normal kidney samples from nucleotides −110 to +41 bp after sodium bisulfite modification. Both samples demonstrated methylation at 10 of 16 CpGs, including those constituting one of the *Taq*I recognition sites. This explains the presence of *Taq*I digestion products, although only partial methylation of the fragment had occurred. Sequencing of 2 normal samples not exhibiting restriction digest products revealed minimal methylation of the CpG island in these samples [1 had no CpG methylation (Fig. 2*c*), and 1 had only 2 CpGs methylated].

Eight CC-RCCs with *RASSF1A* methylation were informative for 3p21 allele loss studies. Seven (87%) demonstrated 3p21 allele loss compared with 28 of 46 (61%) informative CC-RCCs without *RASSF1A* methylation ($P = 0.24$). Seven of 35 (20%) informative CC-RCCs with 3p21 allele loss had *RASSF1A* methylation. However, all of the 25 CC-RCCs with 3p21.3 allele loss and no *RASSF1A* methylation that were informative at 3p25 and/or 3p12 showed allele loss in at least one of these additional regions (therefore, there were no informative tumors without *RASSF1A* methylation in which allele loss was limited to 3p21).

Although *RASSF1A* mutations appear to be rare in other tumor types in which epigenetic inactivation is common (29, 30, 32), we proceeded to analyze: (*a*) 1 CC-RCC with *RASSF1A* methylation and no 3p21 LOH; and (*b*) 10 CC-RCCs with 3p21 LOH and no *RASSF1A* methylation for somatic *RASSF1A* mutations. No *RASSF1A* sequence variants were identified in the 11 tumors.

Relationship between *RASSF1A* and *VHL* Gene Mutation and Methylation Status in CC-RCC. The frequency of *RASSF1A* methylation in CC-RCCs without *VHL* gene inactivation was 21% (9 of 43), similar to the 24% (17 of 71) observed in CC-RCC with *VHL* mutations ($P = 0.82$). There was no association between epigenetic inactivation of *RASSF1A* and *VHL* in CC-RCCs; 0 of 6 CC-RCCs with *VHL* methylation demonstrated *RASSF1A* methylation, and 0 of 26 CC-RCCs with *RASSF1A* methylation had *VHL* gene methylation.

***RASSF1A* Methylation in Papillary RCCs.** We detected *RASSF1A* promoter methylation in 12 of 27 (44%) papillary RCCs analyzed. The identification of *RASSF1A* methylation in papillary RCCs was surprising, given the well-recognized rarity of 3p allele loss in this tumor type. Detailed information on the subtype of papillary RCC was available for 19 tumors, and the incidence of *RASSF1A* methylation was similar in type 1 (3 of 10) and type 2 (5 of 9) papillary RCCs. Four papillary RCCs with *RASSF1A* methylation were informative for 3p21 allele loss analysis, and 1 tumor demonstrated allele loss at *D3S4604* close to *RASSF1A*.

Sequencing analysis of methylated clones was carried out to establish the extent of methylation of the positive papillary tumors as described previously. Sequencing of 2 clones of 1 methylated tumor sample showed methylation at 14 of 16 CpGs (Fig. 2*a*).

***RASSF1A* Methylation Is Associated with Transcriptional Silencing.** RT-PCR analysis of two RCC cell lines with *RASSF1A* methylation (SKRC 39 and SKRC 47) demonstrated transcriptional silencing, whereas *RASSF1A* expression was detected in a RCC cell line (KTCL-26) without *RASSF1A* methylation. All three cell lines were then treated with 5′-azacytidine for up to 9 days. 5′-Azacytidine treatment of the *RASSF1A* methylation-negative cell line KTCL-26 did not influence expression of the *RASSF1A* transcript (Fig. 3*a*). In contrast, 5′-azacytidine treatment of the two RCC cell lines with *RASSF1A* methylation resulted in restoration of *RASSF1A* expression after 5 days (Fig. 3, *b* and *c*). The methylation-insensitive *RASSF1C* transcript was present in all samples and was not influenced by treatment with 5′-azacytidine.

***RASSF1A* Methylation and Clinicopathological Information.** To determine whether the presence of *RASSF1A* methylation was associated with significant differences in tumor grade and Tumor-

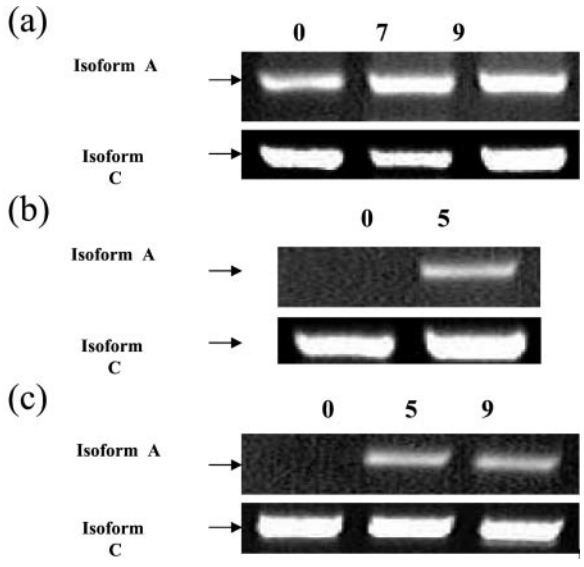

Fig. 3. RT-PCR analysis of *RASSF1A* expression in the following 5-azacytidine-treated cell lines. *a,* KTCL-26 cell line that does not exhibit methylation of the *RASSF1A* promoter CpG island. *b,* SKRC 39 cell line that does exhibit methylation. *c,* SKRC 47 cell line that exhibits methylation. RT-PCR analysis of *RASSF1C* expression is included as a control. Exposure to 5-azacytidine is indicated in days.

Node-Metastasis stage for CC-RCC, we compared these attributes for 77 CC-RCCs for which data were available. However, there were no significant differences ($P > 0.5$) between *RASSF1A* methylated and unmethylated tumors for any of these parameters. In addition, we compared *RASSF1A* methylated and unmethylated papillary RCC samples for age, sex, tumor size, and grade. There were no significant differences for mean age, sex distribution, and tumor grade ($P > 0.4$) between the two groups. Mean tumor diameter was greater for *RASSF1A* methylated tumors (8.21 *versus* 5.94 mm), but this difference was not statistically significant ($t = 1.39$; $P = 0.18$).

DISCUSSION

We have demonstrated that *RASSF1A* promoter methylation is a frequent event in both CC-RCC and papillary RCC. We and others have reported a high frequency of 3p allele loss in CC-RCCs but not papillary RCCs (7, 8, 34–36). The *VHL* TSG maps to 3p25, and somatic *VHL* gene mutation or methylation occurs in up to 70% of CC-RCC tumors and cell lines but not in papillary tumors (10, 11, 13, 14). However, detailed analysis of 3p allele-loss mapping studies have demonstrated that although CC-RCCs harboring somatic *VHL* gene mutations do, in most cases, demonstrate loss of the wild-type *VHL* allele (consistent with a two-hit model of tumorigenesis), 3p allele loss is not limited to 3p25, and all informative tumors analyzed had allele loss at 3p12–p21 (14, 18). In addition, 3p12–p21 allele loss was also present in high frequency in CC-RCCs without evidence of *VHL* inactivation. Thus, 3p12–p21 allele loss (in particular 3p21) appeared to be a critical step in the development of CC-RCC by both VHL-dependent and VHL-independent pathways (14, 18). *RASSF1A* promoter methylation has been reported in most lung cancers and cell lines and in a variable proportion of breast cancers (29–31). In addition, *RASSF1A* suppresses the growth of tumor cell lines in both *in vivo* and *in vitro* studies (29, 31). These findings establish *RASSF1A* as a 3p21 TSG. We demonstrated that *RASSF1A* promoter methylation in RCC is associated with loss of expression in RCC cell lines, and expression is restored after treatment with 5′-azacytidine. These observations are consistent with the hypothesis that epigenetic inactivation of *RASSF1A* is a critical step in the pathogenesis of a significant proportion of CC-RCCs, irrespective of VHL mutation status. The majority of CC-RCCs with *RASSF1A* methylation also demonstrated 3p21 allele loss consistent with a two-hit model of tumorigenesis. However, many CC-RCC tumors with 3p21 allele loss did not show *RASSF1A* methylation. In other tumor types in which *RASSF1A* methylation is frequent (*e.g.,* lung and breast cancers), somatic mutations are rare (29–31). Similarly, we did not find evidence that *RASSF1A* mutations are frequent in CC-RCCs. We cannot exclude the possibility that other 3p21 candidate TSGs may be inactivated in CC-RCCs with 3p21 allele loss and no evidence of *RASSF1A* methylation. However, we note that in none of the CC-RCCs with 3p21 allele loss without *RASSF1A* methylation was 3p allele loss limited to 3p21, and these tumors also showed allele loss at 3p25 and 3p12–p14. Because many CC-RCCs with 3p allele loss show evidence of extensive loss involving most of 3p rather than discrete interstitial deletions, the presence of 3p21 allele loss in a tumor does not necessarily imply that homozygous inactivation of a 3p21 TSG should have necessarily occurred (3p21 loss might be secondary to large chromosomal deletions targeted at another 3p TSG). An interesting hypothesis is that in CC-RCCs in which there is inactivation of a 3p12–p14 TSG, hemizygous loss of 3p12–pter would promote tumorigenesis by inactivating both alleles of a 3p12–p14 TSG (often after inactivation of VHL) and by causing haploinsufficiency of *RASSF1A*. Certainly, TSG haploinsufficiency is emerging as a significant cause of tumorigenesis in some human and murine cancers (37–39).

Although TSG methylation is increasingly recognized as a major contributor to the pathogenesis of many human cancers, the precise etiology of epigenetic changes in cancer are enigmatic. It has been suggested that *cis*-, rather than *trans*-, acting factors are responsible for *VHL* gene methylation in CC-RCCs (40). However, in colorectal cancers, a subgroup of tumors demonstrates a methylator phenotype with a propensity to epigenetic alterations in multiple genes (41, 42). The absence of an association between *VHL* and *RASSF1A* methylation in CC-RCC provides no evidence for the existence of a subgroup of CC-RCCs with a methylator phenotype.

The *RASSF1* gene has several major isoforms because of alternative splicing and promoter usage, but epigenetic silencing of the longer isoform, *RASSF1A*, is specifically associated with cancer. The *RASSF1A* M_r 39,000 predicted peptide contains a RAS association domain, a diacylglycerol binding domain, and a region that is a putative substrate for *ATM* phosphorylation (33). *RASSF1* was shown recently to be an effector of RAS both *in vitro* and *in vivo* studies (33). The finding of *RASSF1A* inactivation in lung cancers was compatible with previous reports implicating mutations or alterations in activity of the RAS signal transduction pathways in the pathogenesis of these tumors. In contrast, RAS mutations are reported to be rare in RCC (43, 44), and the finding of frequent *RASSF1A* inactivation in CC-RCCs will provide new opportunities to develop therapeutic interventions targeted at reversing *RASSF1A* silencing or the downstream consequences of *RASSF1A* inactivation.

The finding of frequent *RASSF1A* methylation in papillary RCCs was unexpected in view of the infrequent reports of 3p allele loss in this tumor type. Furthermore, previous studies have demonstrated that *VHL* and *FHIT* inactivation is specifically associated with CC-RCC and not papillary RCC, whereas *MET* gene mutations are restricted to papillary tumors (15). Thus, *RASSF1A* inactivation provides an important link between the pathways of tumorigenesis in CC-RCCs and papillary RCCs. Nevertheless, the involvement of *RASSF1A* in both tumor types is consistent with the results of chromosome transfer experiments in which a chromosome 3p fragment suppressed tumorigenicity in both CC-RCC and papillary RCC (45). RAS signaling pathways have been implicated in the cellular responses to hepatocyte growth factor/scatter factor (the ligand for *MET*; Refs. 46, 47). Germ-line activating mutations of the *MET* proto-oncogene cause inherited susceptibility to type 1 papillary RCCs (15, 48), but we observed *RASSF1A* methylation in both type 1

(pale cytoplasm, small cell) and a type 2 (eosinophilic cytoplasm, large cell) subtypes of papillary RCCs.

Traditionally, the detection of cytogenetic deletions or allele loss in tumors has been of paramount importance in localizing TSGs. Recently, proposals to identify the molecular pathology of human cancers by complete genomic sequencing have been formulated. The involvement of *RASSF1A* in papillary RCC demonstrates that such an approach will not identify all frequently inactivated TSGs and that sequence analysis of bisulfite-modified DNA may be required to identify TSGs inactivated by epigenetic silencing in regions with infrequent allele loss.

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