Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9700–9704, October 1994
Genetics

Silencing of the *VHL* tumor-suppressor gene by DNA methylation in renal carcinoma

JAMES G. HERMAN*†‡, FARIDA LATIF†§, YONGKAI WENG†¶, MICHAEL I. LERMAN§, BERTON ZBAR§, SUE LIU¶, DVORIT SAMID‖, DAH-SHUHN R. DUAN¶, JAMES R. GNARRA¶, W. MARSTON LINEHAN¶, AND STEPHEN B. BAYLIN*,**

*Oncology Center and **Department of Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD 21231; §Laboratory of Immunobiology, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21702-1201; and ¶Urologic Oncology Section, Surgery Branch, and ‖Clinical Pharmacology Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892

Communicated by Hamilton O. Smith, July 7, 1994

ABSTRACT **Mutational inactivation and allelic loss of the von Hippel–Lindau (*VHL*) gene appear to be causal events for the majority of spontaneous clear-cell renal carcinomas. We now show that hypermethylation of a normally unmethylated CpG island in the 5′ region provides another potentially important mechanism for inactivation of the *VHL* gene in a significant portion of these cancers. This hypermethylation was found in 5 of 26 (19%) tumors examined. Four of these had lost one copy of *VHL* while one retained two heavily methylated alleles. Four of the tumors with *VHL* hypermethylation had no detectable mutations, whereas one had a missense mutation in addition to hypermethylation of the single retained allele. As would be predicted for the consequence of methylation in this 5′ CpG island, none of the 5 tumors expressed the *VHL* gene. In contrast, normal kidney and all tumors examined with inactivating *VHL* gene mutations but no CpG island methylation had expression. In a renal cell culture line, treatment with 5-aza-2′-deoxycytidine resulted in reexpression of the *VHL* gene. These findings suggest that aberrant methylation of CpG islands may participate in the tumor-suppressor gene inactivations which initiate or cause progression of common human cancers.**

Alterations of the pattern and regulation of DNA cytosine methylation have been recognized as consistent molecular changes in human tumors, although the functional consequences have remained difficult to establish (1, 2). An overall increase in DNA methyltransferase activity, which catalyzes cytosine methylation, is found in many tumors (3–5) and also in the premalignant stages of tumor progression (4, 5). Paradoxically, initial studies found a decrease in overall DNA methylation in tumors (6, 7). However, more recent data have shown regions of hypermethylation (8–11) which involve normally unmethylated CpG-rich DNA, termed "CpG islands" (12). These islands are often found in and around the 5′ regulatory areas of genes, and methylation would either render or mark the gene as transcriptionally inactive (12). In normal cells, CpG islands on autosomal chromosomes remain unmethylated. However, many islands on the transcriptionally inactive X chromosome (13) or in selected genes silenced by parental imprinting (14–16) are extensively methylated. The active participation of this methylation in silencing imprinted genes has recently been shown (17).

It has been hypothesized that aberrant methylation of CpG islands of tumor suppressor genes might provide a mechanism for inactivation during tumor progression (18), but evidence for this with well-defined tumor-suppressor genes has been limited. The best evidence for this model exists with the retinoblastoma (*RB*) gene, where abnormal methylation of the CpG island was found in 1 of 21 retinoblastomas (19) and 5 of 32 unilateral spontaneous retinoblastomas (20). No mutations of the *RB* gene were detected in the latter study, though the size of the *RB* gene prevented complete exclusion of mutations. *In vitro* methylation of sites 5′ to these *RB* methylation loci does produce inactivation of the *RB* promoter (21). However, expression data for tumors with aberrantly methylated *RB* genes has not been reported.

In the present study, we have examined the possibility that methylation changes in the CpG island of the von Hippel–Lindau (*VHL*) gene may account for loss of expression of this newly defined tumor-suppressor gene in renal carcinomas. This gene was recently identified as containing the germ-cell mutation on chromosome 3p that is associated with VHL disease (22). In a recent report, 57% of clear-cell renal carcinomas are shown to have mutations of the *VHL* gene and 97% had loss of one *VHL* allele (23). A similar incidence of mutation (56%) was found in clear-cell tumors from patients in Japan (24). In a study of some of these same tumors, we now show that abnormal CpG island methylation is associated with inactivation of the *VHL* gene in a significant number of clear-cell renal carcinomas where this gene is not mutated in the coding region.

MATERIALS AND METHODS

Southern Method. Five micrograms of DNA was digested with excess restriction enzyme (100 units of *Not* I, 50 units of *Xba* I, or 75 units of *Sma* I) overnight under appropriate conditions specified by the supplier (New England Biolabs). Restriction digests were ethanol precipitated and analyzed by electrophoresis in 2% agarose gels as described (8), and images were obtained with a PhosphorImager (Molecular Dynamics).

RNase Protection Assay. PCR primers 1 (upstream; ref. 23) and 2 (downstream; 5′-TTT-GGT-TAA-CCA-GAA-GCC-CAT-CGT-3′) were used to generate a 349-nt fragment with the g7 cDNA (22) as a template. The fragment was subcloned into pCRII plasmid with the TA subcloning kit (Invitrogen). Inserts were sequenced to assess orientation and to eliminate any clones with mutations due to subcloning. Antisense probes for RNase protection were generated by linearizing the plasmid with *Xho* I and using SP6 polymerase and an *in vitro* transcription kit (Ambion, Austin, TX) in the presence of [α-^{32}P]CTP. Probes were purified by gel electrophoresis and assays performed with the RPAII kit (Ambion). An internal 18S rRNA control (Ambion) was included in each reaction mixture.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: VHL, von Hippel–Lindau.

†J.G.H., F.L., and Y.W. contributed equally to the data.

‡To whom reprint requests should be addressed.

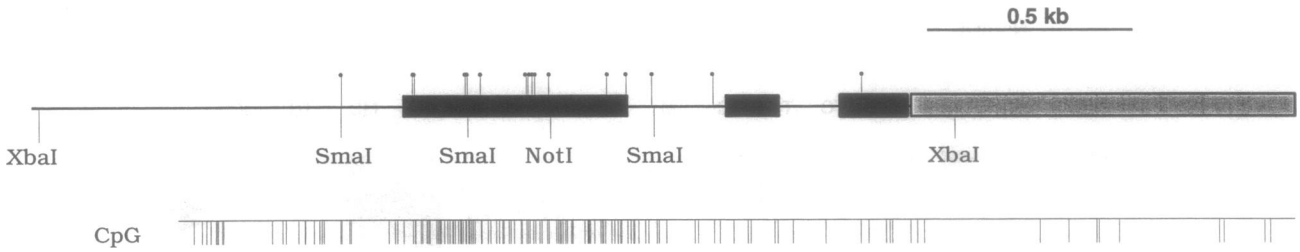

FIG. 1. Representation of the *VHL* gene with exons 1–3 in black and the 3′ untranslated region in gray. Restriction sites for *Xba* I, *Sma* I, and *Not* I are labeled, and the 16 *Hpa* II sites are shown by dots. Below, the distribution of CpG dinucleotides reveals the dense clustering of these sites spanning exon 1 and constituting a CpG island.

RESULTS

Methylation of the *VHL* Gene. We examined 26 spontaneous clear-cell renal carcinoma cell lines for methylation abnormalities of the CpG island in the first cloned exon of the *VHL* gene (Fig. 1). This region fulfills the criteria for an island, having a G+C content of 70% and having a CpG/GpC ratio of >1 (25). As shown by the frequency of the normally underrepresented CpG dinucleotide in Fig. 1, this island spans the first cloned exon. Numerous restriction enzymes—including *Not* I, *Sma* I, and *Hpa* II, which are methylation sensitive and known to be highly associated with CpG islands—have recognition sites in this region (Fig. 1). These enzymes will not cleave if there is methylation of cytosines in CpG dinucleotides included in their recognition sequences.

Using Southern analysis, we found that 21 (81%) of 26 tumor cell lines retained in *VHL* the unmethylated pattern typical for a CpG island and identical to the pattern seen in normal kidney. In these, as well as in normal kidney, the *Not* I site (examples in Fig. 2) was unmethylated. In contrast, we found 5 (19%) of 26 tumor cell lines in which the CpG island of *VHL* was methylated. In these, 4 had virtually all cells, and 1 had 50% of cells, methylated at the *Not* I site (Fig. 2). To assess the extent of CpG island methylation, additional methylation-sensitive enzymes were used. These same five renal carcinoma cell lines had methylation of two or all three of the *Sma* I sites (Fig. 3) in the *VHL* CpG island. Normal kidney and the 21 other renal carcinoma cell lines were unmethylated at all *Sma* I sites (examples in Fig. 3). These abnormally methylated renal cell lines also were methylated at most or all of the numerous *Hpa* II sites, in contrast to DNA from normal kidney and other renal cell lines (examples in Fig. 4).

Methylation Changes Precede Culture and Metastasis. Changes in DNA methylation can occur during cell culture, and cultured immortalized cells universally have areas of CpG island methylation (9). To verify that the changes we found were not produced by cell culture, we examined DNA obtained directly from tumor tissue from which the cell cultures with abnormal *VHL* methylation were started. All five tumors prior to culture had the same abnormal high molecular weight *Not* I fragment identified in cell lines (Fig. 2, lanes T). However, retention of bands reflecting unmethylated sites was seen in each fresh specimen. While these normal bands could reflect the same cellular heterogeneity seen in one of five culture lines (Fig. 2), they more likely represent contamination of tumor with non-neoplastic cells. In previous studies, fresh renal carcinomas have been found to contain up to 60% lymphocytes and other normal cells (26).

Further evidence for the clonal nature of the *VHL* methylation abnormalities was found by investigating both primary tumors and metastatic lesions from the same patients. For such studies, fresh tumor or cell cultures were available from both the primary tumor and metastatic lymph nodes in six patients. In each case, including two of the patients with abnormal *VHL* methylation (UOK 102 and 121), the methylation status was identical in both primary and metastatic lesions (Figs. 2 and 3). Thus, abnormal methylation appears to be an event which precedes metastatic progression.

Specificity for Clear-Cell Renal Carcinoma. We also examined the papillary histologic subtype of renal cell carcinoma for the presence of abnormal methylation of the *VHL* gene. Loss of the short arm of chromosome 3 (3p) is rare in this type, in sharp contrast to the clear-cell histology where consistent loss of 3p has been found (27–32). In addition, mutations have not been found in the *VHL* gene in this subtype (23). In 6 papillary cell lines and 5 uncultured papillary renal cell tumors, the normal unmethylated pattern of *VHL* was found (data not shown), further suggesting that

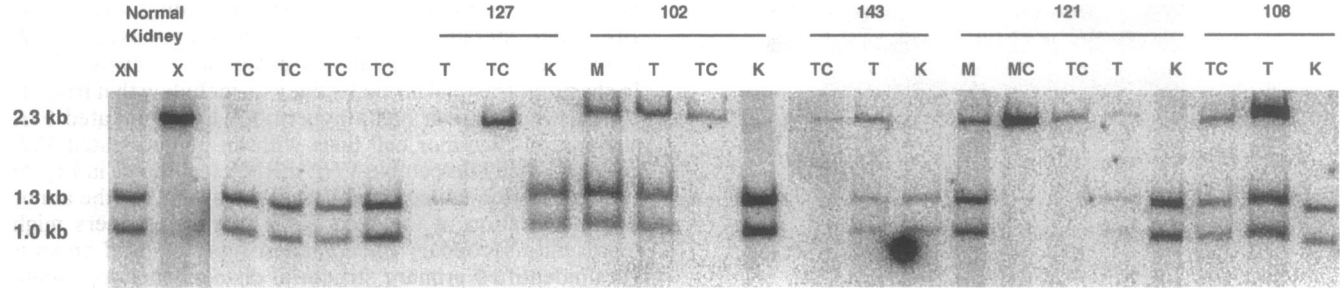

FIG. 2. Methylation state of *Not* I recognition site as assessed by Southern hybridization with the *VHL* gene G7 probe (22). Lanes 1 and 2 contained DNA from normal kidney, cleaved with *Xba* I and *Not* I (XN) or *Xba* I alone (X). All other lanes contained double digests with *Xba* I and *Not* I. Lanes labeled TC alone are examples of 21 tumors from which DNA underwent complete cleavage of the 2.3-kb fragment, yielding bands of 1.3 and 1.0 kb. Labels above sets of lanes indicate patient number with DNA from primary tumor (T), primary tumor cell culture (TC), lymph node metastasis (M), lymph node metastasis cell culture (MC), and adjacent normal kidney (K). All normal kidney DNA samples from patients with tumor DNA methylation were unmethylated. DNA from tumors 127, 102, 143, and 121 was completely methylated at the *Not* I site, whereas DNA from tumor 108 was partially methylated, indicating some cellular heterogeneity within the culture (lanes marked TC). Primary tumor prior to cell culture (lanes T) also had methylation of the *Not* I site. However, these tumors contained some DNA which was unmethylated at this sequence reflecting either tumor cell heterogeneity or, more likely, contamination with normal tissue (24). Tumor metastases (M) shared the methylation pattern of the primary tumors (102 and 121).

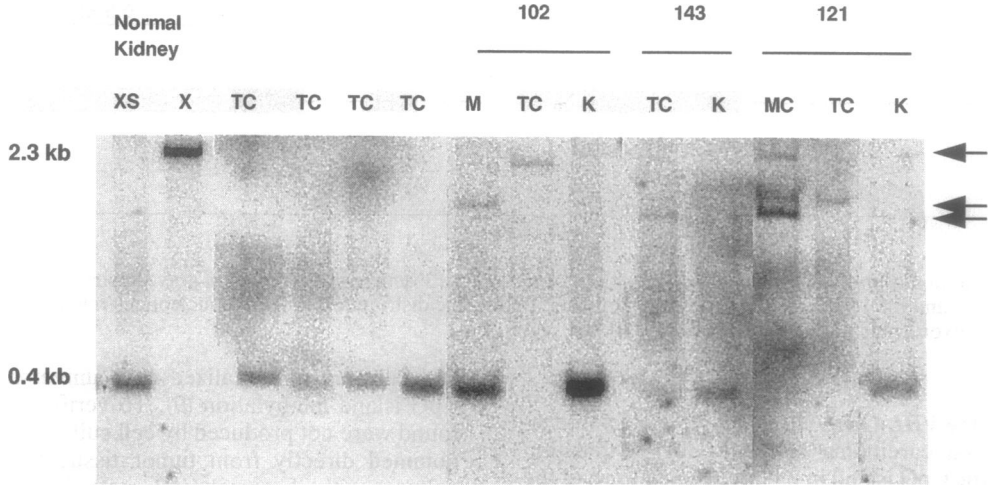

FIG. 3. Methylation state of the *Sma* I sites in the *VHL* gene in selected renal tumors. All samples were digested with *Xba* I and *Sma* I except normal kidney DNA, which was digested with *Xba* I alone (X). Labels are the same as in Fig. 2. Arrows mark abnormal methylation, with the full-length 2.3-kb fragment representing methylation of all *Sma* I sites, and intermediate fragments reflecting methylation of some but not all of the *Sma* I sites. The small, 0.4-kb band is found when *Sma* I sites 2 and 3 are unmethylated.

inactivation of *VHL* is not present in this cell type. In addition, other tumors which frequently delete 3p but do not have mutations of *VHL* (23) retained the normal unmethylated pattern of the *VHL* CpG island (11 lung cancer cell lines and 6 pheochromocytomas; data not shown). Our data further support the specificity of abnormalities of *VHL* in general, and hypermethylation, in particular, for clear-cell renal carcinoma.

Mutations and Loss of Heterozygosity. We next compared the methylation status of the *VHL* gene with the previously documented presence of *VHL* mutations and the allelic status of the gene in the tumors studied above. Eighteen of the 21 tumors with normal *VHL* methylation had *VHL* mutations in the sequenced coding region (23). In contrast, 4 of the 5 tumors having hypermethylation of *VHL* had no detectable mutations in the sequenced coding region (tumors UOK108, 121, 143, and 127, ref. 23). Three of these 4 (UOK108, 121, 143) had lost one *VHL* allele and retained a single heavily methylated allele, while the fourth tumor (UOK127) had two abnormally methylated wild-type *VHL* alleles. The fifth abnormally methylated tumor (UOK102; ref. 23) had a missense point mutation in a single allele which was also heavily methylated. While this mutation, substitution of arginine for proline at codon 209, produces a change in charge, it is more conservative than most of the other 78 mutations found in sporadic clear-cell tumors (23, 24), which typically produce major alterations in the predicted protein. It was also unique to this tumor and had not been found among 95 germ-line mutations in familial VHL disease (33). Thus, 4 of the 7 tumors without detectable mutations had abnormal methylation of the *VHL* CpG island.

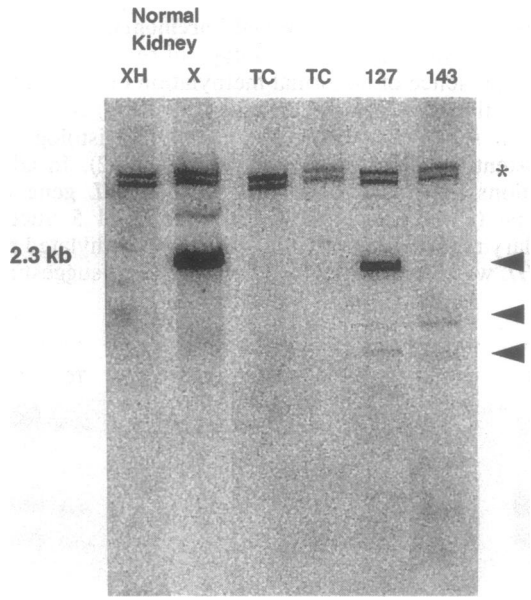

FIG. 4. Methylation state of the *Hpa* II sites in the *VHL* gene in selected renal tumors. All samples were digested with *Xba* I and *Hpa* II, except normal kidney DNA, which was digested with *Xba* I alone (X). The asterisk marks two DNA fragments recognized by the G7 probe which are outside the CpG island and these allow assessment of DNA loading. DNA from normal kidney and tumors marked TC did not demonstrate recognizable fragments, indicating extensively fragmented DNA. Arrows mark large fragments resulting from methylation of most or all (2.3-kb fragment) of the 16 *Hpa* II sites in selected tumors.

Methylation Correlates with Loss of *VHL* Expression. As mentioned previously, CpG island methylation plays an active role in maintaining silencing of parentally imprinted genes (14–17) and also in silencing genes on the inactivated X chromosome (13). We thus examined the expression status of the *VHL* gene at the RNA level in cell lines with unmethylated and abnormally methylated *VHL* genes. All five samples with hypermethylation of *VHL*, including tumor UOK 102, which contained a point mutation, did not express *VHL* transcripts as assessed by RNase protection analysis (Fig. 5). In sharp contrast, all normal kidneys—including that from the patient whose tumor had a hypermethylated, mutated *VHL* allele—and 23 tumor cell lines with an unmethylated *VHL* gene had easily detectable *VHL* mRNA (examples in Fig. 5).

One question concerning these data is whether the abnormal methylation of the *VHL* gene in renal cancers might represent a secondary phenomenon superimposed on an as yet unidentified primary structural change, such as a mutation in the promoter region. In this case, the lack of *VHL* transcripts could be mediated by the primary event and not the CpG island methylation. Similar questions have been raised about the role of CpG island methylation in silencing of genes on the inactive X chromosome and in silencing of autosomal imprinted genes. In the latter instances, demethylation of these genes indicates that the methylation is, at the least, required for maintenance of the silenced transcription (17). We have addressed this issue in our study by treating the cell line UOK 121 (containing a hypermethylated and silent

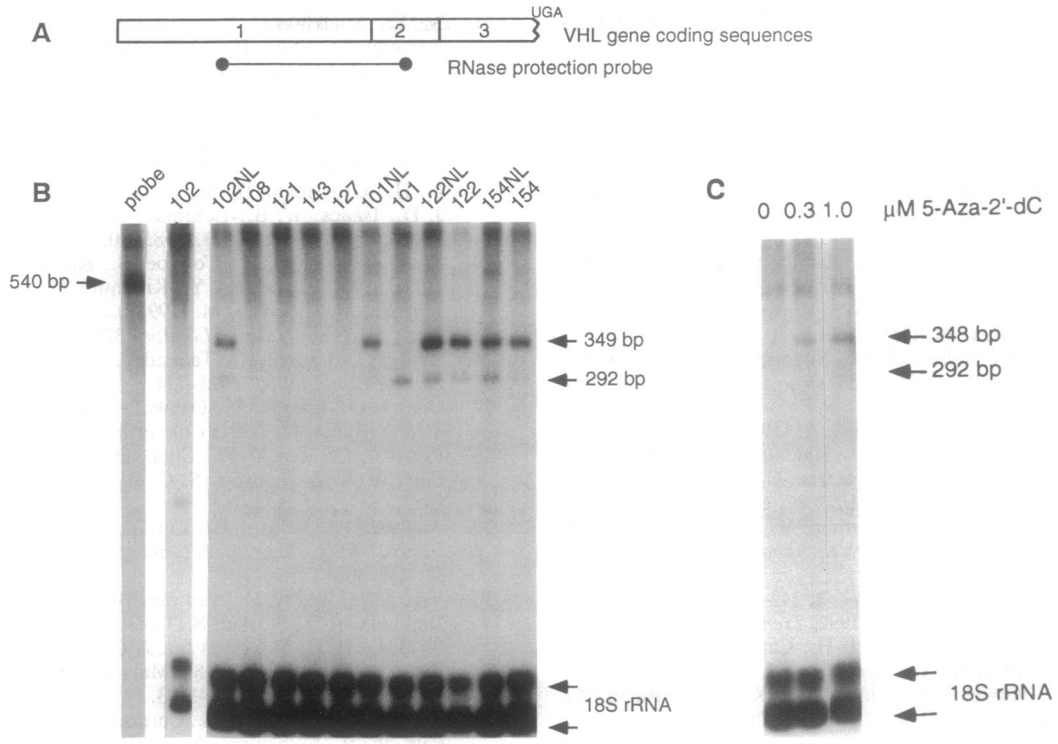

FIG. 5. (*A*) The probe used for RNase protection covers 292 nt of the first exon and 57 nt of the second exon of the *VHL* transcript. A 349-nt protected fragment would be expected from the exon 1–2–3 form of the transcript, and a 292-nt fragment would be expected from the exon 1–3 form of the transcript. (*B*) VHL gene expression in renal carcinoma cell lines and in cells cultured from proximal renal tubule cells from the same patient (NL). Note that the exon 2 splice acceptor site is mutated in the UOK 101 tumor (23) and only the exon 1–3 transcript was seen. An internal control probe for 18S rRNA was included in each reaction mixture. The larger size (540 nt) of the undigested probe reflects vector sequences. (*C*) Reexpression of the *VHL* gene. Tumor cell line UOK 121 (121 in *B*) was treated with 0.3 and 1 μM 5-aza-2′-deoxycytidine for 5 days and *VHL* expression was analyzed by RNase protection. Both doses resulted in expression of the *VHL* transcript.

VHL gene) with the "demethylating" agent 5-aza-2′-deoxycytidine. As seen in Fig. 5*C*, such treatment results in reexpression of the *VHL* transcript, indicating that the promoter remains otherwise intact. In this cell line, methylation appeared to be essential for the absence of *VHL* gene expression.

DISCUSSION

The above findings provide evidence that methylation of the CpG island of the *VHL* tumor-suppressor gene is associated with transcriptional inactivation in a subset of clear-cell renal carcinomas, most of which do not contain a mutated *VHL* gene. This finding, in 19% of the tumors we studied, when combined with the mutational inactivation found in 18 (69%) of the 26 tumors examined, documents inactivation of *VHL* in 88% of these spontaneous renal carcinomas. These data and those in a recent study (23) define inactivation of the *VHL* gene as the likely initiating event in clear-cell renal carcinoma. In addition, we have recently correlated germ-line mutations which completely inactivate the VHL protein with the development of renal cell carcinoma. Other germ-line mutations which are predicted to cause only partial inactivation of the VHL protein were less likely to produce renal cell carcinoma and more likely to cause an inherited form of pheochromocytoma (33). Our data are consistent with this model, in that the DNA methylation events we have observed for the *VHL* gene are associated with complete inactivation of expression of *VHL*.

Inactivation of tumor-suppressor genes is increasingly recognized as a fundamental mechanism of tumor initiation and/or progression. Deletions and point mutations are common mechanisms of gene inactivation of one allele for other known tumor-suppressor genes (34). In most cases, the second allele is deleted by mechanisms involving loss of either a portion of a chromosome or a whole chromosome. Our results demonstrate that DNA methylation may represent another mechanism of tumor-suppressor gene inactivation, which can function identically to inactivating mutations in association with loss of a second allele. In addition, the results in one tumor indicate that abnormal CpG island methylation can inactivate the *VHL* gene even when both wild-type alleles are retained. Previous studies of tumor-associated aberrant methylation of a CpG island on chromosome 17p suggest that this abnormality may precede allelic loss and could be associated with the structural predisposition to this event (35, 36). Our findings for the *VHL* gene represent the best characterized example of the direct relationship between CpG island methylation and lack of expression of a tumor-suppressor gene in a common form of human cancer. This, coupled with previous findings that tumors and immortalized cells often contain hypermethylated CpG islands (8–11), suggests that aberrant methylation may prove to be a relatively common mechanism for silencing tumor-suppressor genes in other forms of human neoplasia.

We wish to thank Dr. Don Sens of the Medical University of South Carolina for providing normal renal proximal tubular cell cultures, Drs. Fray Marshall and James Brooks for additional papillary cell tumors, and multiple colleagues in our laboratory programs for review of the data. For J.G.H. and S.B.B., work was supported by National Institutes of Health Grant CA 43318 and a gift from the Clayton Foundation. D.-S.R.D. was supported by the William K. Warren Foundation.

1. Baylin, S. B., Makos, M., Wu, J., Yen, R.-W., de Bustros, A., Vertino, P. & Nelkin, B. D. (1991) *Cancer Cells* 3, 383–390.
2. Spruck, C. H., III, Rideout, W. M., III, & Jones, P. A. (1993) *DNA Methylation: Molecular Biology and Biological Signifi-*

cance, eds. Jost, J. P. & Saluz, H. P. (Birkhäuser, Basel), pp. 487–509.
3. Kautanien, T. L. & Jones, P. A. (1986) *J. Biol. Chem.* **261,** 1594–1598.
4. El-Diery, W. S., Nelkin, B. D., Celano, P., Yen, R. W. C., Falco, J. R., Hamilton, S. R. & Baylin, S. B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3470–3474.
5. Issa, J.-P. J., Vertino, P. M., Wu, J., Sazawal, S., Celano, P., Nelkin, B. D., Hamilton, S. R. & Baylin, S. B. (1993) *J. Natl. Cancer Inst.* **85,** 1235–1240.
6. Goelz, S. E., Vogelstein, B., Hamilton, S. R. & Feinberg, A. P. (1985) *Science* **228,** 187–190.
7. Feinberg, A. P., Gehrke, C. W., Kuo, K. C. & Ehrlich, M. (1988) *Cancer Res.* **48,** 1159–1161.
8. de Bustros, A., Nelkin, B. D., Silverman, A., Ehrlich, G., Poiesz, B. & Baylin, S. B. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5693–5697.
9. Antequera, F., Boyes, J. & Bird, A. (1990) *Cell* **62,** 503–514.
10. Jones, P. A., Wolkowicz, M. J., Rideout, W. M., III, Gonzales, F. A., Marziasz, C. M., Coetzee, G. A. & Tapscott, S. J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6117–6121.
11. Makos, M., Nelkin, B. D., Lerman, M. I., Latif, F., Zbar, B. & Baylin, S. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1929–1933.
12. Bird, A. P. (1987) *Trends Genet.* **3,** 342–348.
13. Riggs, A. D. & Pfeifer, G. P. (1992) *Trends Genet.* **8,** 169–174.
14. Ferguson-Smith, A. C., Sasaki, H., Cattanach, B. M. & Surani, M. A. (1993) *Nature (London)* **362,** 751–755.
15. Stoger, R., Kubicka, P., Liu, C.-G., Kafri, T., Razin, A., Cedar, H. & Barlow, D. P. (1993) *Cell* **73,** 61–71.
16. Bartolomei, M. S., Webber, A. L., Brunkow, M. E. & Tilghman, S. M. (1993) *Genes Dev.* **7,** 1663–1673.
17. Li, E., Beard, C. & Jaenisch, R. (1993) *Nature (London)* **366,** 362–365.
18. Nelkin, B. D., Przepiorka, D., Burke, P. J., Thomas, E. D. & Baylin, S. B. (1991) *Blood* **77,** 2431–2434.
19. Gregor, V., Passarge, E., Hopping, W., Messmer, E. & Horsthemke, B. (1988) *Hum. Genet.* **83,** 155–158.
20. Sakai, T., Toguchida, J., Ohtani, N., Yandell, D. W., Rapaport, J. M. & Dryja, T. P. (1991) *Am. J. Hum. Genet.* **48,** 880–888.
21. Ohtani-Fujita, N., Fujita, T., Aoike, A., Osifchin, N. E., Robbins, P. D. & Sakai, T. (1993) *Oncogene* **8,** 1063–1067.
22. Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F. M., Orcutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L., Schmidt, L., Zhou, F., Li, H., Wei, M. H., Chen, F., Glenn, G., Choyke, P., McClellan, M. W., Weng, Y., Duan, D. S. R. *et al.* (1993) *Science* **260,** 1317–1320.
23. Gnarra, J., Tory, K., Weng, Y., Smidt, L., Wei, M. H., Li, H., Latif, F., Liu, S., Chen, F., Duh, F.-M., Lobensky, I., Duan, D. R., Florence, C., Poza Hi, R., Walther, M. M., Bander, N. H., Grossman, H. B., Brauch, H., Pomer, S., Brooks, J. D., Isaacs, W. B., Lehrman, M. I., Zbar, B. & Linehan, W. M. (1994) *Nat. Genet.* **7,** 85–90.
24. Shuin, T., Kondo, K., Torigoe, S., Kishida, T., Kubota, Y., Hosaka, M., Nagashima, Y., Kitamura, H., Latif, F., Zbar, B., Lerman, M. I. & Yao, M. (1994) *Cancer Res.* **54,** 2852–2855.
25. Bird, A. (1986) *Nature (London)* **321,** 209–213.
26. Zbar, B., Brauch, H., Talmadge, C. & Linehan, M. (1987) *Nature (London)* **327,** 721–724.
27. Ogawa, O., Kakehi, Y., Ogawa, K., Koshiba, M., Sugiyama, T. & Yoshida, O. (1991) *Cancer Res.* **51,** 949–953.
28. Presti, J. C., Rao, P. H., Chen, Q., Reuter, V. E., Li, F. P., Fair, W. R. & Jhanwar, S. C. (1991) *Cancer Res.* **51,** 1544–1552.
29. Anglard, P., Trahan, E., Liu, S., Latif, F., Merino, M. J., Lerman, M. I., Zbar, B. & Linehan, W. M. (1992) *Cancer Res.* **52,** 348–356.
30. Van der Hout, A. H., van den Berg, E., van der Vlies, P., Dujkhuizen, T., Storkel, S., Oosterhuis, J. W., de Jong, B. & Buys, C. H. C. M. (1993) *Int. J. Cancer* **53,** 353–357.
31. Brooks, J. D., Bova, G. S., Marshall, F. F. & Isaacs, W. B. (1993) *J. Urol.* **150,** 1278–1283.
32. Presti, J. C., Reuter, V. E., Cordon-Cardo, C., Mazumdar, M., Fair, W. R. & Jhanwar, S. C. (1993) *Cancer Res.* **53,** 5780–5783.
33. Chen, F., Kishida, T., Yao, M., Hustad, T., Glavac, D., Dean, M., Gnarra, J., Orcutt, M. L., Duh, F.-M., Glenn, G., Green, J., Hsia, Y. E., Lamiell, J., Li, H., Wei, M. H., Smidt, L., Tory, K., Kuzmin, I., Stackhouse, T., Latif, F., Linehan, W. M., Lehrman, M. & Zbar, B. (1994) *Hum. Mutat.*, in press.
34. Weinberg, R. (1991) *Science* **254,** 1138–1145.
35. Makos, M., Nelkin, B. D., Chazin, V. R., Cavenee, W. K., Brodeur, G. M. & Baylin, S. B. (1993) *Cancer Res.* **53,** 2715–2718.
36. Makos, M., Nelkin, B. D., Reiter, R. E., Gnarra, J. R., Brooks, J., Isaacs, W., Linehan, M. & Baylin, S. B. (1993) *Cancer Res.* **53,** 2719–2722.