HIF activation identifies early lesions in VHL kidneys: Evidence for site-specific tumor suppressor function in the nephron

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Summary

Mutations in the von Hippel-Lindau (VHL) gene are associated with hereditary and sporadic clear cell renal carcinoma. VHL acts in a ubiquitin ligase complex regulating hypoxia-inducible factor-1 (HIF-1), but the link between this function and cancer development is unclear. Here we show that in the kidneys of patients with VHL disease, HIF activation is an early event occurring in morphologically normal single cells within the renal tubules. In comparison, dysplastic lesions, cystic lesions, and tumors showed evidence of additional mechanisms that amplify HIF activation. Detection of cells with constitutive HIF activation identified a large number of previously unrecognized foci of VHL inactivation. In proximal tubules these were almost entirely unicellular, whereas multicellular foci were almost exclusively seen in the distal nephron.

Introduction

Tumor suppressor genes isolated by studying kindreds with familial cancer syndromes have given valuable insights into carcinogenesis (Knudson, 2000). One such tumor suppressor gene is *VHL*, mutations in which give rise to von Hippel-Lindau disease (Kondo and Kaelin, 2001; Latif et al., 1993). Affected individuals have a lifetime risk of developing renal cell carcinoma that is in the region of 70%, with the tumors showing somatic loss or inactivation of the second allele. In accordance with Knudson's two-hit hypothesis, sporadic clear cell renal cell carcinomas (CCRCC) show mutation or loss of the *VHL* gene in the great majority of cases (Foster et al., 1994; Gnarra et al., 1994). However, the mechanism(s) underlying this pivotal role in CCRCC remains incompletely understood.

Key insights into VHL function have come from the classical approach of studying the effects of reintroducing genetic material into fully transformed cells. Thus, reintroduction of a wild-type *VHL* gene into transformed tissue culture cells has been shown to influence several aspects of cellular behavior that may be relevant to tumor suppressor function (for review see Kondo and Kaelin, 2001). Specifically, VHL defective renal carcinoma cell lines exhibit failure to degrade hypoxia inducible factor (HIF) α subunits in the presence of oxygen (Maxwell et al., 1999) and failure to assemble a fibronectin matrix (Ohh et al., 1998), and under certain conditions fail to exit from the cell cycle or show increased susceptibility to apoptosis (Pause et al., 1998; Schoenfeld et al., 2000). All these abnormalities in monolayer cultures can be corrected by transfection with a wild-type *VHL* gene.

Though these experiments provide clear evidence of specific VHL functions in a fully transformed cell background, they cannot address important questions about the timing and consequences of tumor suppressor inactivation during progression

SIGNIFICANCE

Since cancer development is a multistep process, the effects of tumor suppressor inactivation must be considered against an accruing background of genetic change that may affect assays of function. The current work exemplifies this for one such property of a tumor suppressor, regulation of HIF by the VHL gene product. The study defines both early and progressive upregulation of the HIF pathway following VHL inactivation, implicating HIF activation in both early and later stages of VHL associated tumorigenesis. Furthermore, identification and analysis of very early lesions showed that they promote angiogenesis, and that effects of VHL inactivation on proliferation are site specific within the nephron. This provides a focus for understanding VHL tumor suppressor function in kidney cells.

from a normal cellular phenotype to overt carcinoma. In other types of cancer, DNA studies have demonstrated inactivation of tumor suppressors such as APC at an early stage in carcinogenesis, but such studies have necessarily centered on cells that have already become morphologically abnormal (Kinzler and Vogelstein, 1996; Shih et al., 2001). Other strategies for analyzing early events in cancer development have focused on induced gene expression or gene inactivation in animal models (Hakem and Mak, 2001; Hanahan and Folkman, 1996; Wu and Pandolfi, 2001). Overall, there is a paucity of markers that permit analysis of events in human cancers from the first changes in morphologically normal cells (Porter, 2001). In an effort to address this problem, we sought to study activation of the HIF system during the development of VHL-associated CCRCC.

VHL acts as the recognition component of a multiprotein E3 ubiquitin ligase complex, capturing HIF-α chains which have undergone oxygen-regulated hydroxylation of specific prolyl residues and resulting in their ubiquitylation and destruction (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). In renal carcinoma cells lacking functional VHL, HIF-α subunits accumulate even in the presence of oxygen and form a transcriptionally active complex that directs the expression of a constitutive hypoxia-like pattern of gene expression (Maxwell et al., 1999). Since HIF targets include angiogenic growth factors, and successful angiogenesis is a key step in tumor development beyond a size of approximately 1 mm^3 (Hanahan and Folkman, 1996), it seems likely that HIF activation contributes to later stages of VHL-associated tumorigenesis. To date, however, it has not been established whether activation of the HIF transcriptional cascade is an early consequence of VHL loss-of-function in normal mammalian cells, or whether this only happens after other events occur during the evolution of renal carcinoma.

We reasoned that evaluating the HIF system in kidneys from patients with VHL disease would address important issues concerning the timing and consequences of dysregulation of this pathway. Tumor-associated nephrectomy specimens from VHL patients were therefore analyzed for evidence of activation of the HIF system. Stabilization of HIF-α subunits was studied using isoform-specific antibodies for HIF-1α and HIF-2α. HIF transcriptional activity was assessed by examining expression of selected target genes involved in different cellular processes: carbonic anhydrase 9 (*CA9*) (Ivanov et al., 1998; Wykoff et al., 2000), *VEGF* (Iyer et al., 1997), and *GLUT-1* (Ebert et al., 1995).

Results

HIF activation in early VHL-associated lesions

To detect activation of the HIF pathway, we first immunolabeled sections for the HIF target carbonic anhydrase IX (CAIX) using the monoclonal antibody M75 (Pastorekova et al., 1992), which has been extensively validated for detection of this protein. Expression of the *CA9* gene is regulated by the VHL/HIF system via a HIF responsive element in the promoter, and its protein product, CAIX, constitutes an attractive marker for HIF activation because the inducible response is of particularly high amplitude and the protein is very stable (Ivanov et al., 1998; Wykoff et al., 2000).

Eleven nephrectomy specimens from 10 patients with VHL disease were labeled with CAIX antibody. The material in each case included overt CCRCC and apparently normal renal parenchyma. In some cases there were also cysts and dysplastic tubules. As expected from previous studies of sporadic tumors (Liao et al., 1997; Wykoff et al., 2000), overt CCRCC labeled intensely for CAIX (Figure 1A). A high level of CAIX was also expressed by the epithelial cells lining the benign cystic lesions characteristic of VHL disease (Motzer et al., 1996) (Figure 1B). In addition, focal sites of CAIX expression scattered throughout the larger mass of CAIX-negative renal parenchyma were observed (Figures 1C–1H). Some of these regions exhibited distorted or cystic tubular architecture, and in some there was evidence of non-monolayer growth since the cells were not all in direct contact with the basement membrane (Figure 1H). Intriguingly, however, most foci of CAIX-positive epithelial cells were identified in apparently normal tubules (Figures 1C–1F). In each patient, CAIX-positive cells were most often seen as single tubular cells (Figures 1C–1E), although multicellular foci were also observed (Figures 1F–1H). In the material examined, we observed 545 foci of CAIX expression. We examined adjacent sections stained with hematoxylin and eosin for morphological abnormalities (for example, cystic change or distortion of the tubular structure), which would be differentiated from normal renal parenchyma by routine histological evaluation. Only 21 such regions were readily discernible, each of which corresponded to a multicellular focus of CAIX labeling.

In contrast to our finding of numerous foci labeling for CAIX in this material, 20 nephrectomy specimens from patients with sporadic CCRCC, or sporadic renal tumors of other histological types, did not contain any such foci of CAIX-positive epithelial cells in the kidney around the tumor (data not shown).

To confirm that the foci of CAIX expression represented activation of the HIF pathway, we examined expression of two other HIF-responsive genes encoding the glucose transporter GLUT-1 (Ebert et al., 1995) and the angiogenic growth factor VEGF (Iyer et al., 1997). Immunolabeling for GLUT-1 revealed high levels in overt CCRCC, and also in collections of cells in the nontumorous renal parenchyma that resembled the CAIX expressing foci. As expected for a gene whose expression is also regulated by other pathways (Ebert et al., 1995; Hiraki et al., 1988), GLUT-1 expression was somewhat less restricted than CAIX, being present in erythrocytes, in a small subset of glomerular cells, and occasionally in collecting ducts, where CAIX labeling was virtually never observed (see below). However, examination of serial sections confirmed that the boundaries of the CAIX-positive early lesions corresponded exactly to those of the GLUT-1 expressing foci, as would be anticipated if these foci represent cells manifesting mutational activation of the HIF/VHL pathway (Figure 2A). As a third marker of HIF activation, VEGF expression was analyzed. In contrast to GLUT-1 and CAIX, which are transmembrane proteins, VEGF is secreted, and was therefore studied using in situ mRNA hybridization. As reported previously in normal kidney, VEGF expression was less restricted than CAIX, being prominent in glomerular epithelial cells (Brown et al., 1992). However, discrete foci of abnormally high VEGF expression were clearly evident in some tubules, and in adjacent sections these were precisely concordant with foci of CAIX expression (Figure 2B). These lesions therefore coexpress three independent HIF target genes that are involved in different cellular processes.

To investigate the VHL status of these lesions, we first labeled sections with a monoclonal antibody to VHL, IG33. In the normal renal parenchyma, this antibody labeled collecting, distal and proximal tubules, with stronger signal (+++) in the distal

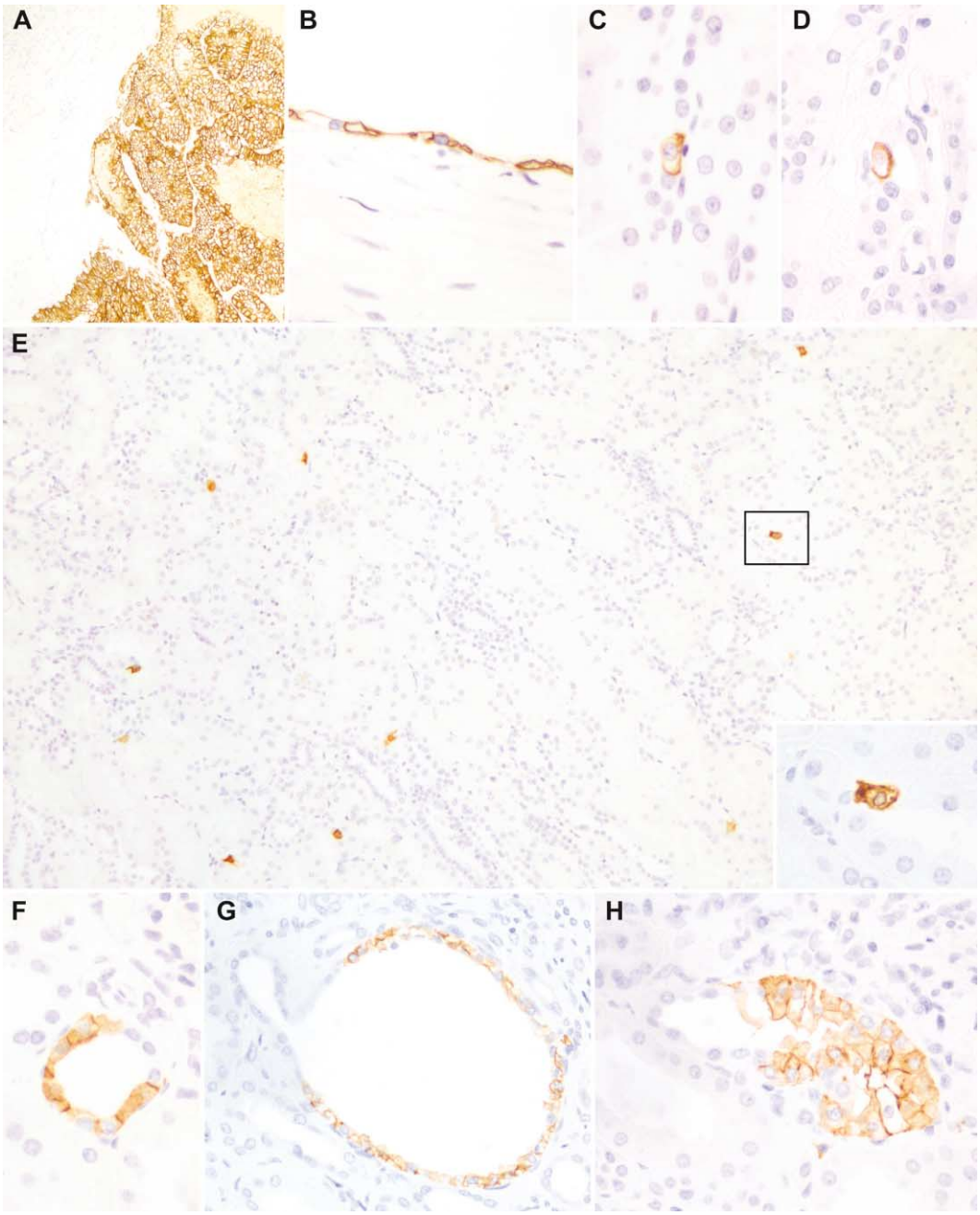

Figure 1. CAIX immunoreactivity in kidneys of VHL patients

A: A region of overt CCRCC showing labeling of all cells (final magnification ×50).
B: The wall of a cyst, showing labeling of flattened epithelial cells lining the cyst (×360).
C and D: Morphologically normal regions showing CAIX expression in single epithelial cells (×360).
E: Low magnification view including a number of isolated tubular cells expressing CAIX (×110). Inset shows one of these single cellular foci (×360).
F: A tubular profile in which the majority of the lumen is lined by cells expressing CAIX (×360).
G: Distorted tubular profile showing an extensive region of CAIX labeling (×240).
H: A tubular profile containing a region of dysplasia which expresses CAIX (×300).

tubules. Comparison of the IG33 staining with the CAIX staining in serial sections revealed that there was a striking diminution, or loss, of VHL immunoreactivity which corresponded precisely with regions of CAIX positivity (Figure 2A). In particular, this pattern of overlap was evident in numerous foci where the CAIX-positive cells constituted only part of the tubular profile, eliminating any need for cross-comparison of different tubular profiles. Since IG33 recognizes the N-terminal portion of VHL that is unnecessary for tumor suppressor function (Iliopoulos et al., 1998; Schoenfeld et al., 1998), the sharply reduced (but not absent) staining probably represents persisting expression of functionally inactive mutant protein that retains this epitope.

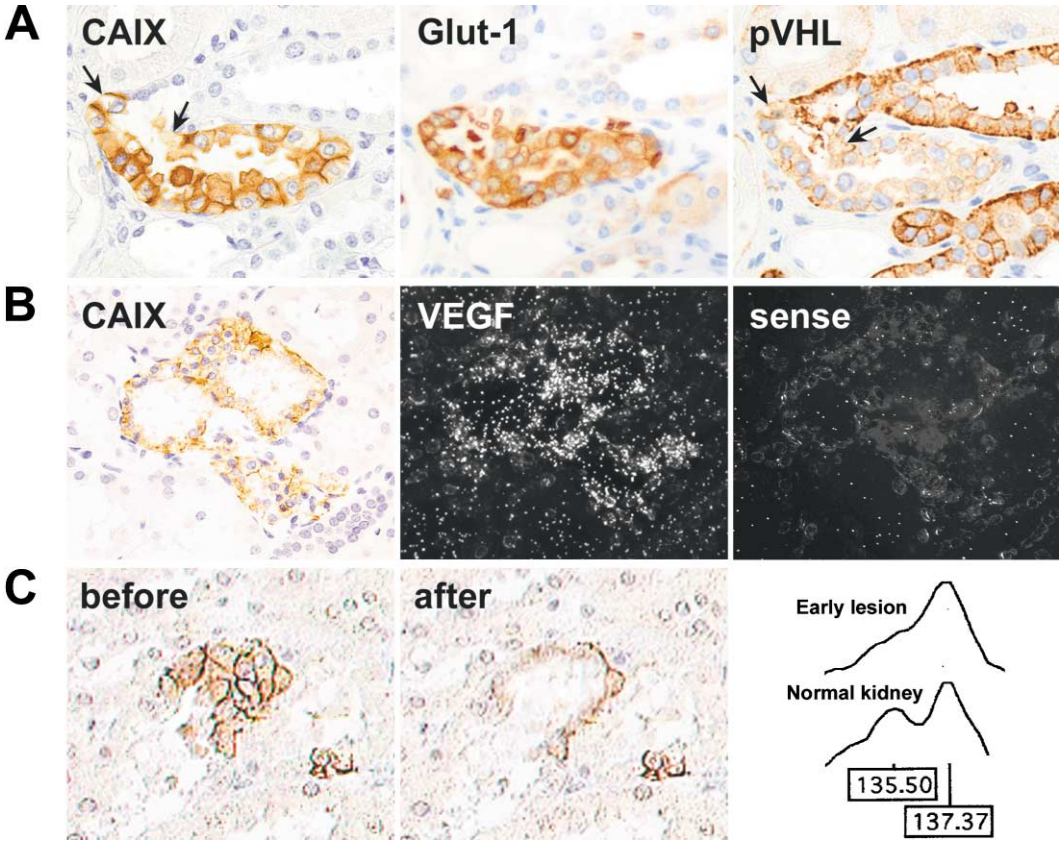

Figure 2. Expression of HIF target genes and analysis of VHL status in foci of HIF activation

Sections were labeled with antibodies or hybridized with riboprobes to *VEGF* as indicated.
A: A single tubular profile showing cells that express CAIX and cells that do not. The region labeled for CAIX shows a high level of expression of GLUT-1. In this portion of the distal tubular profile, a marked reduction in labeling for VHL is seen (from +++ to +), with the boundary corresponding precisely to the boundary of the CAIX immunolabeled region. Arrows indicate this boundary. Note that labeling for VHL is more intense (+++) in the distal tubular profiles than in the proximal tubules (top of field, +), a small vessel, and interstitial cells (−).
B: Three tubular profiles are seen in which the epithelial cells are immunoreactive for CAIX. In situ hybridization of an adjacent section shows a high level of VEGF mRNA in these cells (dark field view). Hybridization to a sense probe as a control indicates that the signal is specific.
C: Laser capture microdissection and PCR allelotyping. A tubular profile containing an early focus was identified by CAIX immunolabeling (left panel). This focus was captured using laser microdissection (center panel). The right panel shows the allelotype at D3S1038 of this early lesion (upper trace) and from a group of cells which were negative for CAIX and were captured from the same section. The wild-type allele is lost in the early lesion.
(Final magnification: **A:** 300×; **B:** 210×; **C:** 300×).

In further experiments, we used an antibody that recognizes the C terminus of VHL, A1.2 (Schoenfeld et al., 1998), and also undertook DNA analysis of the VHL locus. When we labeled control kidneys and VHL kidneys with A1.2, we observed signal in a tightly restricted portion of the nephron, being essentially confined to the collecting tubule, collecting duct, and a subset of distal tubular profiles, suggesting that labeling with this antibody is less sensitive than with IG33. Consequently, A1.2 immunolabeling was only informative concerning VHL expression in a subset of lesions. In this subset, CAIX-expressing cells that occupied part of the tubular perimeter showed complete loss of A1.2 immunoreactivity when compared to their neighbors (data not shown).

In another approach, we used laser capture dissection to isolate small multicellular foci and analyzed markers linked to the *VHL* gene by PCR. Amplification was successful in a total of seven samples from four different nephrectomy specimens; in each case, 3p25 allele loss (at D3S1317 or D3S1038) was demonstrated in the region of the *VHL* gene (Figure 2C). In two of these patients (three foci) it was possible to establish which marker allele was linked to the mutant *VHL* allele, and in each instance, the marker allele linked to the wild-type *VHL* allele was lost in the foci. Taken together, the immunolabeling and DNA analysis demonstrate that the CAIX-positive foci harbor a defect in the normal *VHL* allele.

The regulatory HIF-α subunits exist as two main isoforms that are controlled by VHL-mediated proteolysis, HIF-1α and HIF-2α (Maxwell et al., 1999). To analyze the characteristics of HIF activation in the different lesions, we next labeled sections using specific antibodies for each isoform. In the normal renal parenchyma of VHL patients, clearly demarcated foci of HIF-1α staining were observed, both in proximal and in distal tubular cells. Analysis of serial sections showed that these foci of HIF-1α expression were precisely concordant with the CAIX-positive early lesions (Figure 3A). Comparison of different foci on the same section revealed that while HIF-1α labeling was only of

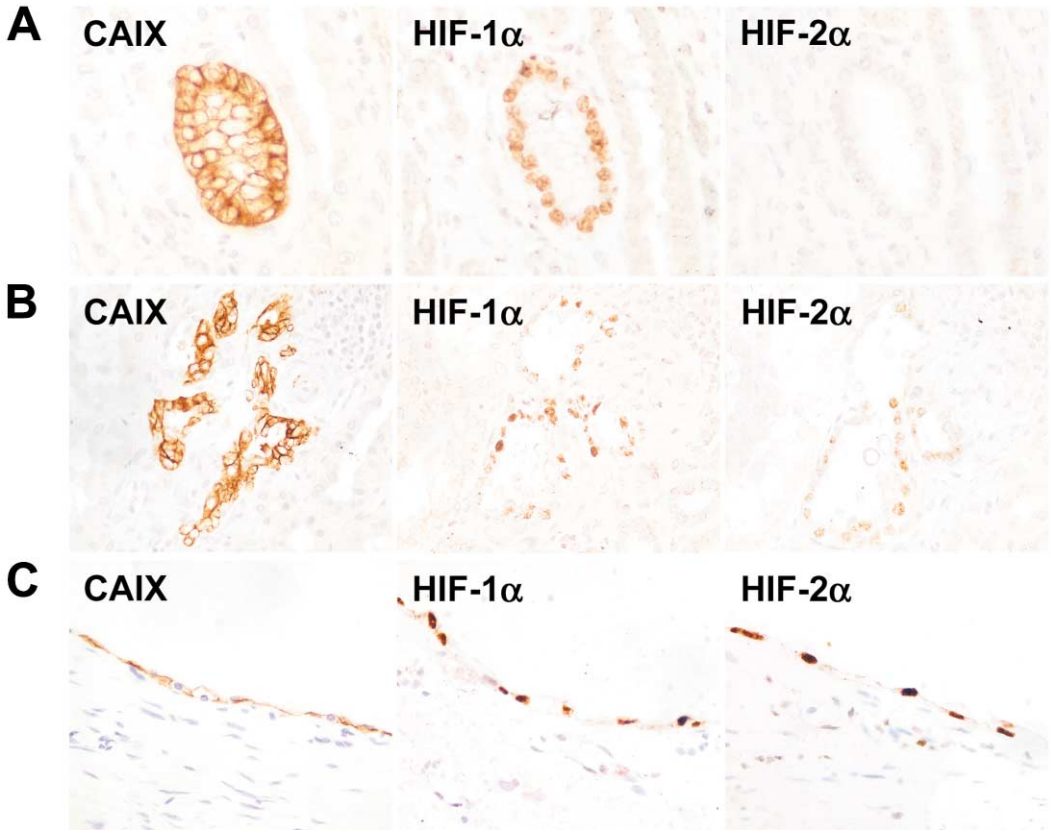

Figure 3. Labeling for individual HIF-α subunits in foci of HIF activation

Adjacent sections or defined sections from a series were labeled with antibodies as indicated.
A: A tubule in which epithelial cells express CAIX. The tubule shows clear but weak nuclear labeling (+) for HIF-1α. HIF-2α was virtually undetectable.
B: A distorted tubular structure. Immunoreactive cells for CAIX show nuclear labeling for both HIF-1α and HIF-2α (+).
C: A cyst lined by cells expressing high levels of CAIX. These cells show moderate staining for both HIF-1α and HIF-2α (++).
(Final magnification: **A**: 300×; **B** and **C**: 180×).

weak intensity (+) in the earliest lesions, it was further upregulated (++) in more advanced lesions, with labeling being consistently more intense in the epithelium lining cystic lesions, and in overt CCRCC (+++), than in the earlier foci (Figures 3A–3C, and data not shown). In contrast to the findings for HIF-1α, we detected virtually no HIF-2α immunoreactivity in the CAIX-positive foci that were morphologically normal or near normal (Figure 3A). HIF-2α was detected at weak intensity (+) in the small subset of foci where the tubular and/or cellular morphology was abnormal (Figure 3B), with more intense signal in cysts (++, Figure 3C) and in regions of overt CCRCC (+++) (data not shown).

Cellular morphology, site of origin, and size distribution of lesions

Immunohistochemical identification of cells manifesting mutational dysregulation of the HIF/VHL pathway enabled further studies of the site, size, morphology, and staining patterns of these very early lesions. Examination of immunolabeled sections indicated that essentially all single cell profiles and the large majority of multicellular foci were not readily morphologically distinguishable from surrounding tubular cells. To analyze the morphological features of the multicellular foci in more detail, we selected 29 multicellular CAIX-positive foci which occupied part of a tubular profile, and examined cellular morphology in the adjacent sections stained with hematoxylin and eosin. In only 6 of these foci could we discern any alteration in nuclear morphology, nuclear to cytoplasmic ratio, or tubular architecture. However, in the majority of foci (19/29), the cytoplasm of CAIX-positive cells in early lesions exhibited a "clear cell" appearance (Figure 4A). That we observed this feature reminiscent of overt CCRCC (Motzer et al., 1996) in many of the early lesions suggests that, like HIF activation, it occurs at a very early stage in tumor development.

Morphological examination of the early CAIX-positive foci revealed that they arose from more than one segment of the nephron. No foci were evident in the thin limbs of the loop of Henle. Where a single CAIX-positive cell was seen, this was almost always in the proximal tubule. In contrast, when foci containing more than one cell were examined, they involved distal tubules in almost all cases, although occasional multicellular foci showed major morphological alterations such that the tubular segment could not be determined with certainty. To confirm the location of the multicellular foci within the nephron, serial sections were labeled for Tamm-Horsfall protein (uromodulin), which is specifically expressed in the thick ascending limb

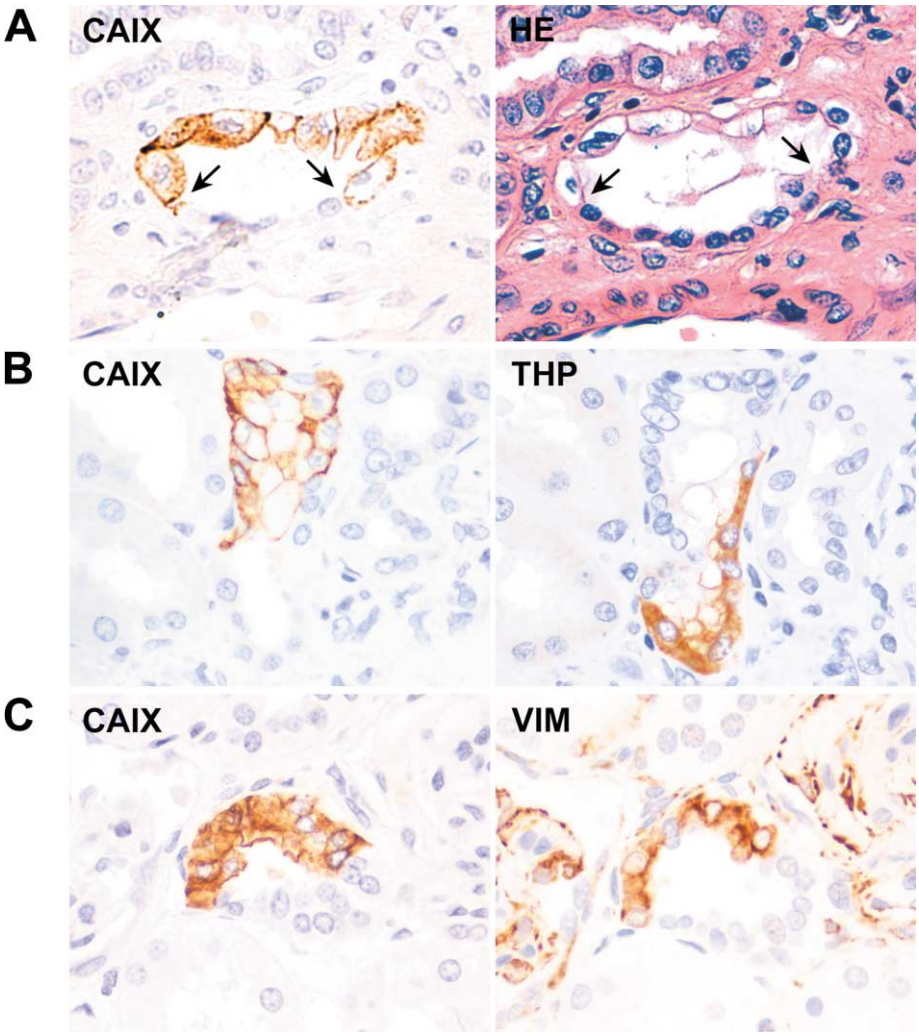

Figure 4. Multicellular foci of CAIX immunoreactivity involve distal tubules and show features of clear cell RCC

Serial sections (**A** and **C**) or defined sections from the series (**B**) were labeled for CAIX and other antibodies or examined morphologically after staining with hematoxylin and eosin.

A: Within a tubular profile, the cells labeling for CAIX show a "clear cell" appearance.

B: A tubular profile that includes a focus of CAIX-positive cells is seen to label with antibody to Tamm-Horsfall protein (THP), showing that this is a distal tubule. Note that the group of cells within the profile which are labeled for CAIX do not label for Tamm-Horsfall protein.

C: A distal tubule closely related to two glomeruli is partly lined by CAIX-positive cells. These cells are seen to express vimentin, which is not expressed by normal tubular epithelial cells, but is seen in the glomerular mesangial cells.

(Final magnification: **A**: 420×; **B** and **C**: 450×).

of Henle's loop and the early distal convoluted tubule (Sikri et al., 1981). This showed that multicellular foci occurred in tubular profiles which labeled strongly for Tamm-Horsfall protein (Figure 4B). However, we found that in these tubular profiles the cells that expressed CAIX showed loss of Tamm-Horsfall protein compared to their neighbors (Figure 4B). This demonstrates that loss of VHL function alters expression of a gene involved in normal distal tubular cell function. Conversely, when we examined the expression of vimentin, which is not expressed by normal tubular epithelial cells, but is expressed by CCRCC (Waldherr and Schwechheimer, 1985), we found that this was expressed in the CAIX-positive foci (Figure 4C).

To further analyze the size distribution of lesions arising in different parts of the nephron, we conducted a morphometric analysis of sections derived from all eleven nephrectomy specimens. Altogether, 524 lesions could be classified as arising from proximal or distal tubules based on the appearance of adjacent cells in the tubular profile. We argued that any sustained net proliferative advantage should lead to a geometric increase in the number of cells in the lesion. Table 1 shows that in each kidney, the size distribution of lesions arising in the distal nephron was compatible with such an effect, whereas the size distribution for proximal tubules was strikingly different, being almost always confined to single cell profiles over a substantial number of identified lesions.

In order to gain insight into specific processes relevant to tumor development, we next assessed vascularity in the region of CAIX-positive foci and the proliferative index of the CAIX-positive cells. We used an antibody to CD31 to label endothelial cells and used CAIX labeling of adjacent sections to identify CAIX-positive foci. We observed a substantial increase in the extent of vascularization around tubules containing CAIX-positive cells (Figure 5A). These vessels also expressed laminin and were strongly immunoreactive for CD34 (data not shown), thus confirming that they were blood vessels and not lymphatics (Breiteneder-Geleff et al., 1999; Ezaki et al., 1990; Paal et al., 1998). Quantitative analysis in two patients showed that CD31 labeling was directly contiguous with over 80% of the perimeter of distal tubules containing CAIX-positive cells, irrespective of whether they displayed a normal or altered architecture (Figures 5A and 5B and data not shown). In contrast, CD31 labeling was only adjacent to approximately 25% of the basement membrane of distal tubules that did not contain CAIX-positive cells (Figure 5B). Equivalent results were obtained for CD34 (data not shown). This marked increase in vascularization around CAIX-positive foci shows that they are promoting effective angiogenesis.

Table 1. Distribution of focus size is different in proximal and distal tubules

	Area (mm^2)	Proximal tubule (number of cells)				Distal tubule (number of cells)						
		1	2–4	5–16	17–64	1	2–4	5–16	17–64	*PT*	*DT*	*p*
1	160	16	2	0	0	3	3	2	0	1.17	2.87	0.0083
2	232	33	0	0	0	0	3	5	3	1.00	14.27	<0.00001
3	225	11	3	1	0	1	2	4	3	1.67	20.90	0.0006
4	321	37	5	2	0	6	9	6	3	1.43	7.87	<0.00001
5	436	18	1	0	0	5	4	4	0	1.05	3.69	0.0004
6	134	10	3	0	0	25	12	15	4	1.31	4.77	0.0114
7	238	10	0	0	0	0	1	0	0	1.00	3.00	0.0044
8	28	10	0	0	0	0	2	4	0	1.00	6.50	0.0002
9	320	12	0	0	0	10	6	1	2	1.00	5.05	0.0067
10	80	86	6	0	0	6	16	12	5	1.06	6.61	<0.00001
11	64	47	1	0	0	9	8	5	1	1.02	3.78	<0.00001

Morphometric assessment of the CAIX-labeled foci was undertaken for each of the eleven nephrectomy specimens. The number of cells comprising each focus was counted and the site of origin in the nephron assessed on the basis of tubular morphology. Each row contains data from one nephrectomy specimen. Foci in distal tubules were generally multicellular. In contrast, those in proximal tubules almost exclusively contained a single CAIX-positive cell in the plane of the section. *PT* and *DT* give the mean number of cells per focus in the proximal tubule and distal tubule, respectively, for each nephrectomy specimen. The difference in the number of cells in distal and proximal tubular foci was statistically significant in each nephrectomy specimen (Wilcoxon rank sum test).

To directly examine the rate of proliferation within the multicellular foci of VHL inactivation in tubules that were morphologically near normal, we performed labeling experiments for the proliferation-associated marker Ki67. In the two nephrectomy specimens with the largest number of multicellular lesions, we assessed the Ki67 labeling index both in the multicellular CAIX-positive foci and in distal tubules which were CAIX-negative. In one case, 5/210 (2.38%) CAIX-positive cells labeled for Ki67, compared to 28/4964 (0.56%) distal tubular cells which were negative for CAIX, giving a 4.25 fold increase. In the second case, 4/232 (1.72%) CAIX-positive cells were positive for Ki67 compared to 11/5196 (0.21%) distal tubular cells which were negative for CAIX, giving an 8.19-fold increase in Ki67 labeling. In each case the difference in Ki67 labeling was highly statistically significant ($p = 0.000123$, 0.00164, respectively, using Poisson approximation). We also assessed the rate of apoptosis using TUNEL labeling. Although some apoptotic cells were observed in the regions of overt CCRCC, we did not find a single TUNEL-positive cell in the early multicellular lesions, and TUNEL labeling was virtually absent in the CAIX-negative distal tubules.

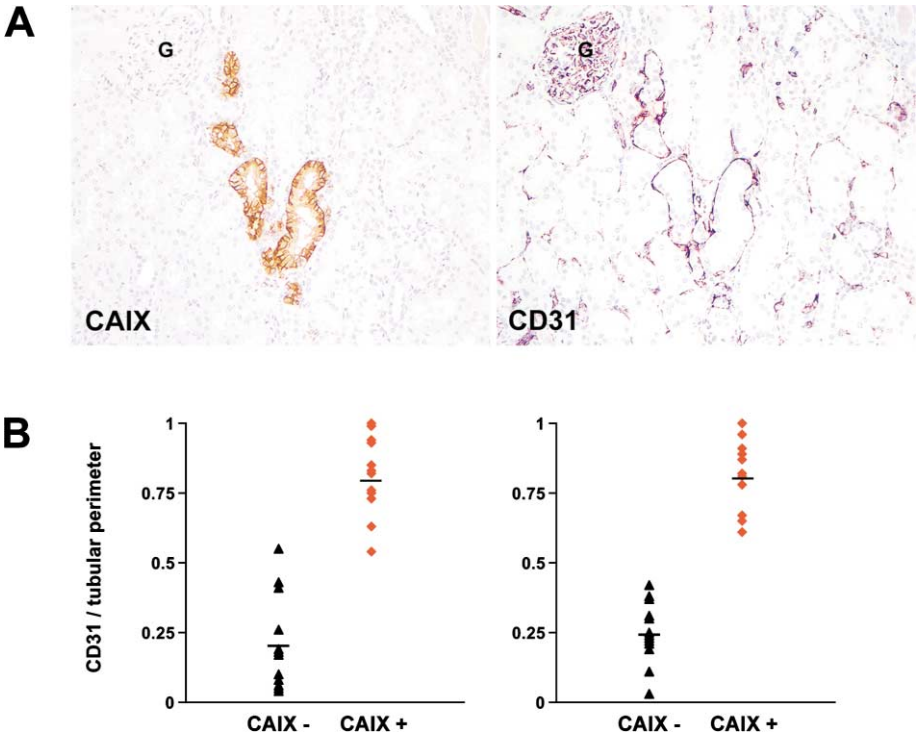

Figure 5. Evidence that early lesions are angiogenic

A: Serial sections were labeled with antibodies to CAIX and CD31. Several tubular profiles are seen to be lined by epithelial cells containing CAIX. These tubular profiles are almost completely surrounded by CD31 immunolabeling, in marked contrast to the other tubular profiles. A glomerulus, G, is visible in the upper left part of the field (Final magnification: ×90).

B: Morphometric analysis of CD31 immunolabeling in two nephrectomy specimens. The proportion of the perimeter of selected tubular profiles that was directly adjacent to CD31 immunolabeling was calculated. Tubular profiles that contained CAIX-positive cells (red symbols; right column in each panel) were more extensively surrounded by CD31 immunolabeling than tubular profiles in the same section that were negative for CAIX (black symbols, left column). The Wilcoxon rank sum test indicated a statistically significant difference in both specimens ($p < 0.00001$).

Discussion

In this work we have demonstrated that the HIF pathway is activated at a very early stage in the evolution of neoplastic kidney lesions in VHL disease. HIF upregulation in single cellular profiles and concordant patterns of staining for HIF-1α and HIF target gene products in foci of VHL loss of function indicate that activation of the HIF transcriptional response is either an early or an immediate consequence of VHL inactivation. Activation of HIF and target genes such as *CA9* therefore provides a robust system for the positive identification of early foci of VHL inactivation, and an unusual opportunity to observe the steps that lead from apparently normal tubular epithelial cells to CCRCC.

In the current study, this has provided several insights into VHL-associated renal disease. First, it enabled the characteristics of HIF pathway activation to be examined. For HIF-1α protein, progressively higher levels of immunoreactivity were observed in morphologically normal foci, dysplastic foci, cystic lesions, and CCRCC. With HIF-2α, the protein was not detected in the earliest foci, but staining of increasing intensity was present in more advanced lesions. Although the difference in detection of HIF-1α and HIF-2α in the earliest foci could relate to differences in the sensitivity of detection, studies of hypoxic rat kidney tissue indicate that HIF-1α is the isoform normally activated by hypoxia in renal tubules, whereas HIF-2α is normally confined to nontubular cell populations (C. Rosenberger and S.J.M., unpublished data). Thus, it is likely that VHL inactivation leads to early or immediate upregulation of the normal tubular cell isoform, HIF-1α, with further events serving to amplify the response during progression to CCRCC. The influence of different non-VHL oncogenic pathways on HIF activation has been demonstrated in VHL competent tissue culture cells (for reviews see Maxwell et al., 2001; Semenza, 2000). Our observations indicate that multiple pathways may also influence the HIF system in VHL defective native neoplastic lesions.

Second, the ability to identify very early phakomata within otherwise normal tubular profiles enabled observations on the characteristics of these lesions. Mechanisms of tumor suppression have been divided into gatekeeping and caretaking functions. Loss of a gatekeeping function leads directly to a net proliferative advantage, whereas loss of a caretaker function allows enhanced generation or survival of genetic mutations that ultimately favor the emergence of clones with such an advantage (Kinzler and Vogelstein, 1997). Though the tumor suppressor mechanisms of VHL remain unclear, the gene product has been classified as a gatekeeper (Kondo and Kaelin, 2001). In keeping with this, reexpression in transformed renal carcinoma cells has a major effect on growth as model tumors (Iliopoulos et al., 1995).

Surprisingly, when all identified foci in the kidneys from VHL patients were considered, we found a very low frequency of multicellular compared to single cell lesions, implying that any advantage in terms of an overall increase in survival/proliferation rate over cell death is small in comparison with the rate of mutational inactivation of VHL. When we examined the proliferation rate in the multicellular foci of VHL inactivation, the labeling index was 4- to 8-fold higher than we observed in control tubular profiles. This suggests that the early proliferative advantage conveyed by VHL inactivation may be relatively modest compared to other mutations in tumor suppressor genes with gatekeeper functions. For example, *APC* inactivation in the earliest detectable lesions in colonic epithelium is associated with Ki67 labeling in virtually every cell (Shih et al., 2001). Furthermore, when the size distribution of lesions was compared for foci that could clearly be assigned as having a proximal versus distal origin, a striking difference was observed. Distal tubular lesions manifest a range of size profiles with an approximate geometric size distribution, consistent with a net survival/proliferative advantage. In contrast, this was not observed for lesions sited in proximal tubules, which were essentially confined to single cells. This indicates that the tumor suppressor mechanisms associated with VHL are likely to be cell specific within the nephron. In this respect, the distal tubule has some interesting attributes. First, cell turnover in this region under normal circumstances is several fold greater than in the proximal tubule (Nouwen et al., 1994). Second, in the normal kidney, expression of the mitogens epidermal growth factor and transforming growth factor-α is found in this region, but not in the proximal tubule (Hise et al., 1996; Nouwen et al., 1994; Salido et al., 1989). Interestingly, TGF-α expression is influenced by VHL status in fully transformed RCC cells, which show growth dependence on the TGF-α/EGFR pathway (de Paulsen et al., 2001).

Since our studies are not sequential, they do not formally "track" the origin of CCRCC. Indeed, the size distribution suggests that the great majority of the smallest foci of VHL loss do not progress further. Nevertheless, that VHL loss appears to lead to a proliferative advantage that is selective for the distal tubule suggests that the associated tumors arise from this portion of the nephron and not from the proximal tubule. Supporting this interpretation, in a recent preliminary report, mice with cell-specific inactivation of VHL in the proximal tubule did not develop cysts or CCRCC (Haase et al., 2001). Other evidence on the origin of CCRCC has been based on structural and marker protein expression studies of the cells in overt CCRCC, and there have been proponents of a proximal tubular origin, a primitive stem cell origin, and a distal tubular origin (Motzer et al., 1996). Absence of Tamm-Horsfall protein has previously been cited as a major piece of evidence against origin from the distal tubule (Wallace and Nairn, 1972). However, our analysis of early foci within distal tubules showed a striking loss of Tamm-Horsfall protein expression, indicating that this interpretation is misleading.

An important challenge will be to elucidate the link between VHL loss-of-function and proliferation in distal tubular epithelial cells and whether HIF activation is involved in this process. In contrast to the current uncertainty of the link between VHL inactivation and proliferation, the route to increased vascularity can be reasonably attributed to HIF activation, which was recently shown to be sufficient to promote increased formation of competent blood vessels in normal skin (Elson et al., 2001). What is intriguing is the activation of angiogenesis in these very early lesions, in contrast to the usual position in solid tumors where angiogenesis is a relatively late event in tumor evolution (Hanahan and Folkman, 1996). One implication of this is that these early lesions are likely to be unusually well oxygenated compared to both normal tissues and other evolving solid tumors.

Finally, in addition to insights into tumorigenesis, identification of these premalignant lesions could be useful clinically. First, a finding of CAIX-positive foci in the surrounding renal parenchyma in surgical material from patients with sporadic CCRCC might identify a small subset with a greatly increased

risk of developing further tumors who would benefit from specific surveillance. Second, it may be possible to use the high level expression of CAIX on the cell surface to target premalignant foci in patients with VHL germline mutations.

Experimental procedures

Clinical material

Eleven nephrectomy specimens from ten VHL subjects were studied. Nephrectomies were performed at ages ranging from 26 to 53. One patient had the second kidney removed two years after the first procedure. Each kidney specimen included multiple cysts, as well as single or multiple solid tumor lesions of the clear cell type. Specimens were formalin fixed and embedded in paraffin wax.

Immunohistochemistry

5 μm sections mounted on Snowcoat X-tra slides (Surgipath) were dewaxed in xylene and rehydrated using graded ethanol washes. For antigen retrieval, sections were immersed in preheated Dako target retrieval solution (cat. # S1699) (HIF; Glut-1; VHL; Vimentin; Ki67; CD31; CD34) or 10 mM Na citrate buffer (pH 6.0) (Tamm-Horsfall protein) and treated for 90 s in a pressure cooker, or incubated for 5 min. at room temperature with Proteinase K (Dako, cat. # S3020) (laminin). Antigen retrieval was not necessary for CAIX or A1.2 labeling. Primary antibodies were: mouse monoclonal anti-human HIF-1α H1α67 (Novus biologicals, cat. # 100-123), 1/1000; rabbit polyclonal anti-mouse HIF-2α PM8 antiserum (Talks et al., 2000), 1/3000; mouse monoclonal anti-human CAIX M75 (Pastorekova et al., 1992), 1/50; rabbit polyclonal anti-human GLUT-1 (Dako, cat. # A3536), 1/200; mouse monoclonal anti-human VHL IG33 (NeoMarkers, cat. # MS-690-P0), 3 μg/ml; mouse monoclonal anti-bovine vimentin (Dako, cat. # M7020), 1/200; mouse monoclonal anti-human Tamm-Horsfall protein (Cedarlane Lab., cat. # CL1032A), 1/400; rabbit polyclonal anti-human Ki67 (Dako, cat. # A0047), 1/100; mouse monoclonal anti-human CD31 (Dako cat. # M0823), 1/40; mouse monoclonal anti-human VHL, clone A1.2 (Schoenfeld et al., 1998), 1/400; mouse monoclonal anti-human CD34 (Dako cat. # M7165), 1/50; rabbit polyclonal anti-rat laminin (Dako cat. # Z0097), 1/25. Negative controls were performed for all experiments and included irrelevant primary immunoglobulins of the same isotype or species. Antigen/antibody complexes were revealed by means of the Catalyzed Signal Amplification system (Dako, cat. # K1498, K1499, K1500) (HIF) or Envision system (Dako, cat. # K4006, K4010) (all other antigens) according to the manufacturer's instructions. Sections were counterstained with hematoxylin for 30 s, dehydrated in graded ethanol washes, and mounted in DPX (Lamb). Labeling intensity for VHL and HIFα subunits was assessed in a semiquantitative fashion, with grading from − (absent) to +++ (strongly positive), as indicated in Figures 2 and 3.

TUNEL staining

TUNEL staining was performed using an in situ cell death detection kit (Roche cat. # 1684817) according to the manufacturer's instructions.

In situ hybridization

The VEGF riboprobe consisted of nucleotides −16 to +217 of the human $VEGF_{121}$ isoform. This probe also recognizes VEGF isoforms 165, 189, and 206. Sense and antisense RNA probes were transcribed from linearized template (MAXIscript in vitro transcription kit [Ambion]) with [^{33}P]-UTP (Amersham). In situ hybridization was performed as described previously (Clark et al., 1996). Briefly, riboprobes were hybridized at 30,000 cpm/μl for 18 hr at 55°C. Slides were then washed, treated with RNase A, coated with emulsion, and exposed for 21 days at 4°C.

Laser capture microdissection

Sections were mounted on uncoated slides and incubated at 37°C overnight. Immunohistochemistry for CAIX was performed essentially as described above. After a brief hematoxylin counterstain, sections were thoroughly dehydrated and left to stand in xylene for 10 min. Slides were removed from xylene and air dried. Microdissection was performed using the PixCell II LCM System (Arcturus Engineering Inc.) with a beam diameter of 7.5 μm for single cells or small clusters of cells. Cells were collected onto HighSensitivity caps (Arcturus Engineering Inc.).

Molecular genetic analysis

DNA was extracted from microdissected cells using the Qiaquick DNA Mini kit (Qiagen). PCR amplifications of microsatellite markers D3S1038 and D3S1317 (see http://www.ncbi.nlm.nih.gov/entrez/ for primer sequences) were performed in 10 μl reactions with 2 μl DNA, 1× *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.5 U Thermoprime plus *Taq* Polymerase (ABgene), 200 μM each dNTP and 2.0 pmol each primer. The forward primer was labeled with a fluorescent dye (6-Fam for D3S1317 and Hex for D3S1038). After initial denaturation at 95°C for 5 min, PCR was conducted for 35 cycles of 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by a final extension for 5 min at 72°C. PCR products were analyzed on an ABI 377 DNA analyzer. PCR product sizes were determined by reference to an internal standard, and using Genescan v3.1.2 and Genotyper v2.5.2 software (Applied Biosystems).

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