The Genetic Locus NRC-1 within Chromosome 3p12 Mediates Tumor Suppression in Renal Cell Carcinoma Independently of Histological Type, Tumor Microenvironment, and VHL Mutation

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ABSTRACT

Human chromosome 3p cytogenetic abnormalities and loss of heterozygosity have been observed at high frequency in the nonpapillary form of sporadic renal cell carcinoma (RCC). The von Hippel-Lindau (VHL) gene has been identified as a tumor suppressor gene for RCC at 3p25, and functional studies as well as molecular genetic and cytogenetic analyses have suggested as many as three separate regions of 3p that could harbor tumor suppressor genes for sporadic RCC. We have previously functionally defined a novel genetic locus nonpapillary renal carcinoma-1 (NRC-1) within chromosome 3p12, distinct from the VHL gene, that mediates tumor suppression and rapid cell death of RCC cells in vivo. We now report the suppression of tumorigenicity of RCC cells in vivo after the transfer of a defined centric 3p fragment into different histological types of RCC. Results document the functional involvement of NRC-1 in not only different cell types of RCC (i.e., clear cell, mixed granular cell/clear cell, and sarcomatoid types) but also in papillary RCC, a less frequent histological type of RCC for which chromosome 3p LOH and genetic aberrations have only rarely been observed. We also report that the tumor suppression observed in functional genetic screens was independent of the microenvironment of the tumor, further supporting a role for NRC-1 as a more general mediator of in vivo growth control. Furthermore, this report demonstrates the first functional evidence for a VHL-independent pathway to tumorigenesis in the kidney via the genetic locus NRC-1.

INTRODUCTION

Genetic abnormalities in RCC\(^3\) have indicated a consistent involvement of chromosome 3 loci in the genesis of the disease. High frequency LOH as well as cytogenetic aberrations involving the short arm of chromosome 3 have been observed in the vast majority of sporadic RCCs (1, 2). The familial form of RCC involves defined translocation events with breakpoints in 3p (3). These types of analyses in combination with functional genetic approaches have suggested as many as three separate regions within 3p that could harbor tumor suppressor genes for RCC.

The most distal region of 3p (3p25) contains the VHL gene. Inheritance of the VHL gene predisposes to the development of a number of different tumor types including spinal hemangioblastomas, retinal angiomas, pheochromocytomas, as well as renal and pancreatic cysts (4). The VHL gene is mutated in 57% of sporadic RCC, suggesting an important role for VHL in the sporadic form of RCC as well (5). Introduction of the VHL cDNA into RCC cell lines has functionally indicated that VHL is a tumor suppressor gene for RCC (6).

Genetic alterations in lung cancer, as well as renal cell, ovarian, uterine cervical, and testicular carcinoma, implicate a 3p region more proximal than 3p25 to contain a putative tumor suppressor gene. High frequency LOH has been reported in SCLC (100%), RCC (95–100%), and ovarian carcinoma (57%) in the 3p21.1–3 region (1, 2, 7–10). Functional genetic screens developed in our laboratory were used to identify regions of 3p with tumor suppressor functional activity. For these experiments, a defined fragment of 3p was identified in microcell hybrids generated in the A9 fibrosarcoma background which suppressed tumor formation in athymic nude mice (11). The region of functional tumor suppressor activity was first limited to a 2-Mb region within 3p21-p22 (11) and later refined by Daly (12) and others (13) within 3p21.3 by detection of homozygous deletion in SCLC.

In addition to the 3p21.3 region, high frequency LOH in the region 3p13–14.2 has also been documented (14, 15). Furthermore, the breakpoint region in familial RCC lies within 3p14. Recently, the FHIT gene was isolated from the breakpoint region in familial RCC and includes the FRA-3B region, which is the most common fragile site in the human genome (16). Multiple deletions and alternative transcripts have been observed in a number of different tumor types.

Evidence for a tumor suppressor gene in the 3p region proximal to FHIT has been shown by our previous functional studies in which we transferred an intact human chromosome 3 and subsequently a centric fragment of 3p (encompassing the 3p14–q11 region by cytogenetic analysis) into a nonpapillary RCC line SN12C.19 (17). In all experiments, the 3p centric fragment mediated a dramatic tumor suppression and rapid induction of tumor cell death after s.c. injection of microcell hybrids in athymic nude mice (17). Physical mapping of suppressed and unsuppressed fragment-containing microcell hybrids limited the region containing the tumor suppressor locus NRC-1 to within 3p12 and distinct from the VHL gene (18). The NRC-1 critical region directly overlaps a 5–7-Mb homozygous deletion region observed in the SCLC line U2020 (19). Thus, either the most proximal region of 3p contains multiple genes, each involved in different tumor types, or there is a more general tumor suppressor gene in this interval.

One of the critical questions to be addressed regarding the putative 3p tumor suppressor genes, then, is whether there is a cell type specificity to their involvement in the kidney and more broadly within the diverse histological tumors for which 3p LOH has been documented. The criteria for classification of renal cell tumors is based largely on histopathological parameters and does not seem to correlate directly with clinical outcome. Renal cell tumors are classified by histological observation as either papillary or nonpapillary (including tumors of solid, alveolar, tubular, or cystic) origin (20). Cell types of RCC include clear cell, granular cell, mixed (clear and granular) cell, pleomorphic, and spindle type (20). Most reports of LOH and cytogenetic alterations within 3p in sporadic RCC have involved the more common nonpapillary form. Few reports have indicated an association between 3p cytogenetic aberrations or high frequency LOH and the papillary form, indicating a possible cell type specificity for the 3p genes in RCC. Additional support for cell type specificity in different
histological RCCs has been shown in that an inherited form of papillary RCC has been characterized that involves a t(X;1) translocation as well as an t(X;17) translocation, unlike the nonpapillary familial form, which has been characterized by 3p translocation events (21). Kovacs (22), in fact, has proposed a new classification system for renal tumors based on cytogenetics and LOH indicating that 3p cytogenetic aberrations are consistent only with the nonpapillary form and not the papillary. The implications of this system would be toward the use of LOH for diagnosis of RCC and classification of tumors by histological type.

Thus, much evidence supports a cell type specificity to the RCC tumor suppressor genes on 3p. However, more recent reports document 3p LOH in papillary RCC (two of seven; Ref. 23). One allelotype study indicated that although no 3p LOH was observed in papillary RCC, that the remainder of genome wide losses on chromosomes 6q, 8p, 9p, 9q, and 14q were found in both papillary and nonpapillary RCCs (24). These data suggest that, other than chromosome 3 involvement, the remainder of high frequency losses suggest a common pathway to tumorigenesis in papillary and nonpapillary RCC. However, only three markers on 3p were tested in this allelotype study, none of which mapped into the most proximal 3p region. Although no high frequency cytogenetic alteration within 3p has been observed in papillary RCC, Hughson et al. (25) reported a papillary RCC with two normal copies of 3 that showed significant 3p LOH by RFLP analysis. Their data indicated that one copy of chromosome 3 was lost, followed by a nondisjunction event that placed two copies of the remaining chromosome in the tumor. Thus, two apparently normal copies of 3 were observed cytogenetically, with LOH observed in 3p and 3q. These data suggest that chromosome 3 loss/nondisjunction events could be a mechanism involved in the genesis of papillary RCC. It therefore remains to be definitively determined whether chromosome 3p loci play a role in all types of kidney cancer.

We now present extensive data to document that the chromosome 3p12 locus NRC-1 functionally suppresses tumors in RCC regardless of the histological type of RCC or the microenvironment of the tumor. Furthermore, because NRC-1 maps within the 3p12 homozgyous deletion region in SCLC, these functional studies predict the potential involvement of NRC-1 in different histological cancers as well. Results from this study, furthermore, provide evidence that NRC-1 suppresses tumor formation of RCC in the presence of a VHL mutation and thus provide the first functional evidence for a VHL-independent pathway to renal tumorigenesis via NRC-1.

MATERIALS AND METHODS

Cell Lines. The A498 cell line was isolated from the primary kidney tumor of a 52-year-old female (26). The KRC-7 line was established in our laboratory from a primary kidney tumor and is a nonpapillary RCC of sarcomatoid origin.4 The SN12C.19 line is a subcloned line of SN12C, derived from a mixed clear cell, granular cell nonpapillary RCC (27). The HA(3)IIaa cell line contains the introduced 3p centric fragment in the A9 mouse fibrosarcoma cell background (17). Cell lines were maintained in DMEM (high glucose)/F12 medium containing single neo-tagged human chromosomes has been described previously (28). Briefly, micronucleation of donor HA(3)IIaa was accomplished by 48-h mitotic arrest using 0.06 μg/ml Colcemid. Enucleation of micronucleate populations was accomplished using 10 μg/ml cytochalasin B and centrifugation (27,000 × g for 70 min). Microcells were filtered through 5- and 3-μm nuclease filters to eliminate whole cells and enucleated whole cells and to select for the smallest of the microcells, corresponding to the transfer of single chromosomes.

Cytogenetic Analysis. G-11 analysis of hybrid lines followed the protocol of Bobrow (29). G-banding analysis was performed as described (30).

Microsatellite Analysis. Microsatellite polymorphism analysis followed the method of Lott et al. (18). Microsatellite PCR was performed using primers synthesized by Research Genetics (Huntsville, AL). Before amplification, the forward primer was end labeled with 32P by T4 polynucleotide kinase (Promega, Madison, WI). PCR amplification was performed in a 25-μl reaction volume containing: 0.63 μM concentration of forward and reverse primers, 100 ng of template DNA, 0.2 mM deoxynucleotide triphosphates, 1.5 mM MgCl2, 0.63 unit AmpliTaq polymerase (Perkin-Elmer, Foster City, CA), and HEPES buffer (10 mM HEPES, 50 mM KCl, pH 8.3). After initial denaturation and addition of AmpliTaq, reaction products were subjected to 23 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 2 min. Samples were then denatured and loaded on a 6% acrylamide gel with 33% formamide and 6 μL urea. Electrophoresis was performed at 60 W for −2–3 h. Subsequently, gels were vacuum dried and exposed to autoradiography film overnight at room temperature.

In Vivo Assays for Tumor Suppression. Cells (5 × 106) of hybrid or parental origin were injected s.c. into athymic nude mice (5–7 weeks of age) as described previously (17). Cell viabilities were performed using trypan blue exclusion immediately before and after injections. For renal subcapsular injection, the protocol of Fidler et al. (31) was followed. Athymic nude mice were anesthetized with methoxyflurane. After a left subcostal incision, made to allow access to the left kidney, a tuberculin syringe with 30-gauge needle was inserted from the lower pole to just below the renal capsule on the superior pole of the kidney. Then 0.05 ml, containing 1 × 106 cells, was slowly injected, resulting in a subcapsular bleb. The incision was closed with a single layer of wound clips.

SSCP Analysis and DNA Sequence Analysis. SSCP and DNA sequencing were performed as described previously (18).

RESULTS

NRC-1 Mediates Tumor Suppression in Papillary as well as Nonpapillary RCC. We previously reported tumor suppression and rapid cell death of RCC in vivo after introduction of the 3p centric fragment into a nonpapillary RCC line SN12C.19 (17). To test the role of NRC-1 in papillary RCC, we transferred the 3p centric fragment from HA(3)IIaa (an A9 microcell hybrid containing the pSV2neo-tagged subchromosomal region 3p12–q11; Fig. 1) into a papillary RCC cell line A498 (Fig. 2a; Ref. 26). The A498 cell line was obtained from the American Tissue Culture Association, where it had

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Fig. 2. Microcell fusion experiments into the A498 RCC cell line. 

a, G-banded metaphase spread of A498(3i) series microcell hybrid; 
b, microsatellite analysis of A498 indicating homozygosity for four of four markers in the 3p12 region; 
c, tumor volumes of A498 hybrids in the second experiment after injection into athymic nude mice; 
d, wet weights from the second experiment in which A498(3i) series hybrids were injected into nude mice (graph indicates mean kidney wet weights; bars, 95% confidence intervals; 
e, mutation screening for VHL in A498 (arrow, mutation).
been classified as being of papillary origin. Confirmation of papillary histology was obtained by histological examination of A498 tumors formed after injection into athymic nude mice. Results indicated that histologically, tumor nodules manifested papillary and solid areas. The papillary component comprised the majority of the histological patterns and showed fronds and rosettes formed of central fibrovascular core lined by cuboidal tumor cells. Tumor cells were cytomorphologically similar in both the solid and the papillary patterns. This cell line was also examined cytogenetically and found to contain two normal copies of chromosome 3; however, microsatellite screening of A498 indicated homozygosity for four of four markers tested in the 3p12 interval containing NRC-1 (Fig. 2b). Given the high degree of heterozygosity for the microsatellite markers tested (>75–85%), results suggest that A498 may be hemizygous for this region of 3p. Twelve microcell hybrid clones were generated in the A498 background after transfer of the 3p centric fragment via microcell fusion using HA(3)IIaa as the donor line. All clones were extensively characterized by G-banding for the presence of any donor mouse chromosomes and by classical cytogenetics. Results indicated the presence of the centric fragment at high frequency (89–97%) in all hybrid clones examined.

Fig. 3. Microcell fusion experiments into the KRC-7 RCC cell line. a. LOH analysis of tumor cell line/normal tissue in KRC-7; b. G-banded metaphase spread indicating the presence of the 3p centric fragment in the KRC-7 sarcomatoid hybrid KRC-7(3i)B; c. microsatellite analysis using markers in the distal region of the introduced fragment indicating retention of the region in the transferred fragment in wo of three hybrids; d. in vivo summary of injections of KRC-7 series hybrids in athymic nude mice; e. SSCP analysis (arrow, shift); f. DNA sequencing of the VHL gene (arrow, mutation) in KRC-7.

Five microcell hybrid clones [A498(3i) series] as well as parental A498 controls were injected s.c. at 5 × 10^6 cells in the right flank of each of three athymic nude mice. Tumor volumes were measured biweekly for 2 months, after which time the tumors were excised and wet weights were determined. Four of the five hybrid clones demonstrated a dramatic tumor suppression ranging from 2- to 5-fold relative to parental controls (data not shown). Clone A498(3i)14 formed tumors of equivalent or slightly smaller size than parental controls, whereas A498(3i)3 formed tumors larger than A498. This experiment was repeated with an additional microcell hybrid clone for a total of six microcell hybrids containing the introduced 3p fragment. Tumor volumes were calculated again biweekly (Fig. 2c), and final tumor wet weights were determined (Fig. 2d). At the end of 47 days after injection, the parental A498 line formed tumors of 1417 mm^3, which required that the experiment be terminated. In this experiment, tumor volumes for A498(3i)2, A498(3i)19, and A498(3i)7 all ranged from 138–444 mm^3, again resulting in a greater than 3–10-fold suppression of tumor formation (P = 0.0495 for each hybrid clone). A(3i)10 was also suppressed as in the first experiment in the range of 617 mm^3. In the second experiment, A498(3i)14 and A498(3i)3 hybrids were intermediate in tumor formation (P = 0.827 and P = 0.513, respectively) relative to parental controls with no obvious deletions observed by microsatellite analysis. Thus, in two separate experiments, comparison between wet weights and tumor volumes indicated a dramatic suppression of tumorigenicity. As in previous experiments, complete suppression of tumorigenicity mediated by the chromosome 3p fragment was not observed, with outgrowth of tumors at the end of the experiment. Explants derived from tumors formed at the end of the experiment were cultured in vitro and examined by G-banding for the presence of the 3p fragment. Results indicated a loss of the introduced fragment in tumor explants in the range of 32–98% in hybrid clones, with the least suppressed clone A498(3i)14 retaining the fragment in only 2% of the population. These results indicate the involvement of the tumor suppressor locus NRC-1 in not only nonpapillary RCC but
also the papillary form of the disease as well, which confirm and extend our previous findings that the 3p12–q11 fragment of <20 Mb, inclusive of the centromeric region, mediates tumor suppression in two different RCC cell lines.

**NRC-1 Mediates Tumor Suppression in Different Cell Types of Nonpapillary RCC.** Initial studies which defined the tumor suppressor locus NRC-1 were performed by the transfer of the 3p centric fragment region into a nonpapillary RCC cell line SN12C.19, which is of mixed granular cell, clear cell origin. To study the involvement of the NRC-1 locus in different cell types of nonpapillary RCC, we established over 20 new cell lines of different histological origin.\(^4\)

KRC-7 represents a rare sarcomatoid variant of RCC, the karyotype of which contains two normal copies of chromosome 3 and two copies of a chromosome 3 with a cytogenetic deletion of 3p12–3pter. LOH studies were performed for the 3p12 interval in the KRC-7 cell line versus adjacent normal tissue from the tumor originating KRC-7 (Fig. 3a). Results indicated LOH for four of four markers tested. Thus, although KRC-7 contains multiple copies of chromosome 3, the 3p12 region is hemizygous, indicative that nondisjunction/loss may have occurred. Microcell hybrids were then constructed by transfer of the 3p12–q11 region from Ha(3)Ia into KRC-7. Unlike fusions in SN12C.19, which generated 30–40 hybrids/fusion, three microcell fusions were attempted in KRC-7, and only three hybrids in the KRC-7 background were obtained from one experiment. In all hybrids, however, the centric fragment was represented at high frequency (85–90%; Fig. 3b). Microsatellite analysis conducted on the KRC (7) series hybrids indicated that two of three hybrids retained distal marker D3S1577 in the NRC-1 critical region (Fig. 3c). One hybrid, KRC7(3i)B, retained the introduced 3p fragment at high frequency by cytogenetic analysis; however, a deletion of D3S1577 (additional markers examined were uninformative) was found upon microsatellite screening. KRC-7 hybrids were injected at 5 × 10^6 cells s.c. into athymic nude mice (five mice per hybrid line tested). Tumors were visible in the KRC-7 parental line injected at 6 weeks after injection. Wet weights of the KRC-7 line averaged 0.1 g 9 months after injection. Two of the three KRC-7 hybrids [KRC-7(3i)B and KRC-7(3i)C] failed to form any tumors during the 9-month study (Fig. 3d). The third clone [KRC-7(3i)A] was also completely suppressed for tumorigenicity in three of five mice injected. The remaining two mice, however, formed tumors larger than parental controls (Fig. 3d). Thus, although the parental line was not as aggressive as SN12C.19 or A498 in forming tumors in vivo, detection of 2–10-fold differences in hybrid tumor wet weights were possible and representative of previous experiments. Furthermore, because KRC-7(3i)B contained a deletion of 3p12 sequences as well as failed to form tumors in vivo, this microcell hybrid deletion clone may prove valuable to further limit the NRC-1 critical region. KRC-7 is the only RCC line under study for which complete suppression of tumorigenicity was observed; because it is a newly established line, it may be more representative of the original tumor than the other lines under investigation that have been in culture for prolonged periods and perhaps acquired additional genetic aberrations. The combined results of the A498 and the KRC-7 study (Table 1) indicate that the NRC-1 locus mediates tumor suppression independently of histological type and cell type of RCC and perhaps represents a more general tumor suppressor gene in the 3p12 region involved in diverse histological tumors.

**Orthoptic Injection of Microcell Hybrids Containing the 3p Centric Fragment Indicates That Tumor Suppression via NRC-1 Is Independent of the Microenvironment of the Tumor Cells.** To determine whether the microenvironment of the tumor would affect hybrid growth rates in vivo, the same series of microcell hybrids previously reported by s.c. injection in the SN12C.19 background (17) were also injected orthotopically into the kidney of athymic nude mice. SN12C.19 contains multiple copies of chromosome 3, as well as an unbalanced t(3;8) translocation,\(^3\) resulting in a derivative chromosome composed of most of the 3p arm of chromosome 3 (3pter–3p14.3 or 3p14.2) fused to the centromere and long arm of chromosome 8. For this experiment, two hybrids containing the introduced centric fragment and which were suppressed in s.c. injections were used [SN19(3i)YY and SN19(3i)FF]. A G-banded metaphase of SN19(3i)YY is shown in Fig. 4a. One hybrid containing two copies of an intact chromosome 3 [SN19(3i)WW] was also injected; for controls, one 3p centric fragment-containing hybrid [SN19(3i)KK] that had a longer latency period for tumor formation, but eventually formed large tumors in s.c. injections, was also used. Microsatellite screening of hybrid clones indicated that all retained the distal markers in the NRC-1 critical region (Fig. 4b). Parental controls and an irrelevant chromosome 2 microcell hybrid, SN19(2)L, were also injected. Each line was injected into the kidneys of 10 athymic nude mice. Kidneys were excised 60 days after injection and weighed. Results (Fig. 4c) directly correlated with data obtained from s.c. injections. The average wet weight of parental SN12C.19 injected kidneys was 2.2 g; injections with a control chromosome 2 microcell hybrid showed a slight decrease in growth (1.8 g) that was not statistically significant as compared with parental controls (P = 0.221) using a Mann-Whitney statistical analysis. However, orthotopic growth of the two hybrids, SN19(3i)FF (0.7 g, P = 0.007) and SN19(3i)YY (0.8 g, P = 0.007) was suppressed significantly. SN19(3i)WW, which contained two copies of the introduced chromosome 3, was the most suppressed for tumor formation with an average kidney wet weight of 0.6 g (P = 0.002). Hybrid SN19(3i)KK, which formed intermediate tumors between suppressed and parental cells in s.c. injections, again demonstrated an intermediate level of tumor suppression (1.6 g, P = 0.125) which was not significantly different from parental controls. Thus, tumor suppression via NRC-1 is independent of the tissue-specific controls in the kidney and supports the role of NRC-1 in different histological and cell types of RCC.

**Tumor Suppression via 3p12 Locus NRC-1 Is Independent of VHL Mutation.** We next tested whether a requirement for tumor suppression via NRC-1 is a wild-type VHL gene. Given that both NRC-1 and VHL are tumor suppressor genes for RCC, then if NRC-1 was in fact upstream of VHL in the same pathway, tumor suppression

<table>
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<th>RCC histological type</th>
<th>Donor chromosome introduced</th>
<th>Tumor suppressor phenotype</th>
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\(^{5}\) S. Pathak, personal communication.
might be abrogated in the presence of an inactivating mutation in VHL. If, on the contrary, NRC-1 is either downstream of VHL or in an independent pathway, then NRC-1 would be expected to mediate tumor suppression regardless of the absence of VHL.

To test the requirement of VHL for tumor suppression via NRC-1, we examined the status of VHL in the RCC cell lines under study. SN12C.19 is wild type for VHL by SSCP analysis (Fig. 4d). However, A498 and KRC-7 contain mutations in VHL. SSCP analysis of KRC-7 indicated a slight shift in one band relative to the normal tissue control (Fig. 3e). Sequence analysis indicated a C-G transition mutation in exon 2 at amino acid 186 in KRC-7 (Fig. 3e). A498 was previously used as recipient for transfection of wild-type VHL cDNA in experiments to document in vitro growth control via VHL in RCC (6). The cell line contains a previously reported 4-base deletion at nucleotides 639–642, resulting in a frameshift and lack of normal expression of the VHL protein (6). Sequencing of the VHL gene in the A498 line in our laboratory confirmed these data (Fig. 3e). Results clearly indicate that hybrids in the A498 and KRC-7 background (with VHL mutation), when injected s.c. into athymic nude mice, were suppressed for tumor formation as were hybrids in the SN12C.19 background (without VHL mutation), both in s.c. and orthotopic injections. We conclude that the NRC-1-mediated pathway to tumorigenesis is functional.

Fig. 4. Microcell fusion experiments into the SN12C.19 cell line. a, G-banded metaphase of SN12C.19 series hybrid SN19(3i)YY (arrow, presence of the 3p centric fragment); b, microsatellite analysis of SN12C.19 hybrids indicating the presence of the introducted 3p12 region in hybrid clones; c, wet weights of tumors after injection of SN12C.19 hybrids orthotopically into the kidney of athymic nude mice (graph denotes mean kidney wet weights; bars, 95% confidence intervals); d, SSCP analysis of SN12C.19 for the VHL gene. Control DNA was obtained from normal male lymphocytes.
ally independent of VHL, either downstream from VHL in the same pathway or in a completely independent pathway from VHL. Complementation of the pathway to tumorigenesis in the kidney, therefore, can be accomplished via NRC-1, even in a background of VHL mutation.

**DISCUSSION**

The critical events underlying the genesis of sporadic RCCs are not well understood. Clearly, the VHL gene is mutated at high frequency in sporadic RCCs, and replacement of VHL into RCC cell lines suggests a function as a tumor suppressor gene. However, inheritance of the VHL gene results in a diversity of tumor types and not just RCC, and the generation of these different tumor types is not easily explained by a strict genotype/phenotype relationship. Given this wide spectrum of primarily benign tumors that develop in VHL-affected individuals, one must consider the possible interactions of other genes that might influence the progression of VHL-associated malignancies. Binding of the VHL/Elongin B,C complex with Hs-CUL-2, a member of the Cdc53 protein family, has been shown (32). The Cdc53 gene in yeast has been implicated in the targeted degradation of cell cycle proteins and acts as a putative “gatekeeper” gene that monitors the balance between controlled cell division and cell death. Null mutation of the Ce-cul1 homologue in Caenorhabditis elegans produced increased cell numbers in many cell lineages examined (33). Studies on VHL-associated RCCs have documented, by both cytogenetic and LOH analyses, that losses occur commonly along the whole 3p arm. A likely mechanism for malignant conversion in VHL-associated tumorigenesis involves not only inheritance of a gatekeeper gene such as the VHL gene, which may predispose to the hyperplastic state, but also the loss of additional genes, such as NRC-1, which may be critical for malignant progression in RCC.

The results of this study indicate that the genetic locus NRC-1, syntetic to VHL on chromosome 3p, can function independently of VHL to suppress tumors in sporadic RCC. The exact role that NRC-1 plays in the genesis of kidney cancer will await the identification of the tumor suppressor gene in this region. In our previous functional studies in which the 3p centric fragment was introduced into the RCC cell line SN12C.19, cell death was observed in the smallest tumors at week 1 after injection of hybrid cells in nude mice, and there was no evidence of angiogenesis in tumors at later stages based on morphology. At the periphery of the dead core of the tumor, there was an interface of cells actively undergoing apoptosis.6 The formal possibility exists that NRC-1 may play a critical role in the regulation of angiogenesis, downstream of VHL, and that loss of angiogenesis may result in induction of apoptosis as seen in our functional model. Thus, we can begin by addressing the role of NRC-1 in a programmed cell death pathway in the kidney and the possible interrelatedness of a programmed cell death pathway and the VHL pathway associated with angiogenesis regulation and entrance into G0. Mutations of the VHL gene have been shown to result in up-regulation of vascular endothelial growth factor and angiogenesis (34). Hanahan and Folkman (35), in a transgenic model for pancreatic islet cell tumors (one of two tumor types in VHL that progress to malignancy, RCC is the other), have shown that apoptosis in islet cell tumors in mice is highly dependent on persistent angiogenesis. When transgenic mice were treated with angiogenesis inhibitors, tumor growth was impaired and vessel density was reduced. The S-phase cell fraction remained high, whereas the apoptotic incidence increased significantly in the small tumors arising in mice treated with angiogenesis inhibitors. These results imply that the vasculature of the tumors may be a paracrine regulator of apoptosis and that inadequate vascularization can also cause tumor cell apoptosis.

In this report, we have also documented that introduction of NRC-1 into histologically diverse RCC tumor lines results in tumor suppression and that this tumor suppression is independent of the microenvironment of the tumor. These data suggest that the tumor suppression mediated by NRC-1 is independent of potential differential gene expression in the different cell types of RCC. These data also call into question the histological classification of RCC tumors based on presence or absence of 3p LOH. LOH is only as accurate as the markers available. Previous allelotyping studies excluded 3p12 loci in the region of NRC-1 in their analysis. As documented in this report, the 3p12 region is important in different histological types of RCC and potentially other malignancies involving 3p aberrations. Only when the syntenic loci on 3p are isolated, however, can the formal definition of the selectivity and generality of these important tumor suppressor genes be elucidated.

**ACKNOWLEDGMENTS**

We are grateful to Dr. I. J. Fidler and Mike Wilson for conducting orthotopic injections and James Luca for technical assistance.

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The Genetic Locus \textit{NRC-1} within Chromosome 3p12 Mediates Tumor Suppression in Renal Cell Carcinoma Independently of Histological Type, Tumor Microenvironment, and \textit{VHL} Mutation

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