Intratumoral Heterogeneity of Von Hippel-Lindau Gene Deletions in Renal Cell Carcinoma Detected by Fluorescence in Situ Hybridization

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Abstract

Although chromosome 3p deletions are considered an initial event in clear cell renal cell carcinoma (RCC), the reported prevalence of 3p deletions is highly variable. Because molecular analyses may be influenced by intratumoral heterogeneity, this study was performed to evaluate the genetic heterogeneity of the von Hippel-Lindau (VHL) gene (on 3p25.5) in RCC. Fifty-three clear cell and papillary RCCs were examined by dual-labeling fluorescence in situ hybridization with probes for the VHL gene and chromosome 3 centromere. The results were compared with histopathological phenotype, proliferative activity (Ki-67 labeling index) and 8p/17p deletions (both suggested to be linked to RCC progression). A clear-cut VHL deletion (in more than 40% of cells) was detectable in 69% of clear cell RCCs but was not detectable in nine papillary RCCs. A considerable genetic heterogeneity of VHL deletions was seen in clear cell RCCs including VHL-deleted subpopulations with different chromosome 3 counts within individual tumors as well as populations with and without VHL deletions. 8p22 and 17p13 deletions (each of which were detected in 18% of clear cell RCCs) were both linked to VHL deletions. However, 8p and 17p deletions were not associated with tumor grade, stage, or Ki-67 labeling index. The data indicate that some clear cell RCCs may develop independently of VHL alterations.

Introduction

RCC is the eighth and eleventh most frequent cancer among males and females, respectively. The most common histological subtypes of RCC include clear cell (80%), papillary (about 10%), chromophobe (<5%), and collecting duct (<1%) carcinomas. Previous studies have shown an association between specific patterns of genetic alterations and histological subtypes. Potential diagnostic alterations include polysomy of the chromosomes 3, 7, and 17, considered typical for papillary RCC (1-4), and deletions of the short arm of chromosome 3 (3p).

Cytogenetic studies have shown that 3p deletions are linked to clear cell RCC, occurring in 40-70% of tumors with this histological subtype (5-7). LOH analyses with matched pairs of RCC tumor/normal kidney DNA have detected losses of 3p sequences in about 80% of clear cell RCCs (8-10). A very high prevalence of 3p deletions (98%) has been reported in DNA extracted from tumor cell lines after culture (11). This discrepancy suggests that there may be intratumoral heterogeneity of 3p deletions in RCCs.

Previous molecular studies demonstrated that at least three separate

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3 The abbreviations used are: RCC, renal cell carcinoma; VHL, von Hippel-Lindau; LI, labeling index; FISH, fluorescence in situ hybridization; LOH, loss of heterozygosity.

regions are critical in the development of RCCs, one coincident with the VHL disease gene on 3p25.5 (9, 12-14), one at 3p21-22 (14, 15), and one at 3p13-14 (15-17). The VHL gene on 3p25.5 is the most likely candidate for a clear cell renal tumor suppressor gene (18) because somatic VHL gene mutations were identified in about 50% of RCCs. It seems likely that VHL inactivation occurs even more frequently because hypermethylation of the VHL promoter region, yet another mechanism for gene inactivation, has been observed in an additional 20% of RCCs (19). Although it is assumed that the inactivation of one or more genes on 3p plays a role in RCC initiation (for review, see Ref. 20), it has been suggested that other cytogenetic alterations go along with RCC progression, including deletions of 17p (9) and 8p (5, 9, 11, 21). Deletions of 8p and 17p have been observed in 20-30% of RCCs.

Aiming at a potential diagnostic application of genetic analyses, the extent of genetic heterogeneity that involves potential marker alterations is of importance. To learn more about the patterns of VHL deletions occurring in RCCs and the relationship between VHL deletions and other molecular changes, we analyzed 53 RCCs by FISH using combinations of chromosome (centromere) and locus-specific probes. FISH allows one to visualize specific loci and chromosomes on a cell-by-cell basis and is, therefore, ideally suited to analyze genetic heterogeneity (22).

Materials and Methods

Tumor Material. Frozen tumor samples were available from 53 patients. Forty-two tumors were clear cell RCCs, and 11 were papillary RCCs. The tumors were staged and graded according to the International Union Against Cancer (UICC) and Fuhrman grading system. Twenty-nine tumors were pT2, 23 tumors were pT3, and one tumor was pT4. One tumor was classified as grade 1, 15 were grade 2, 29 were grade 3, and 8 were grade 4. For statistical analysis, stage 3 and 4 tumors were summarized as high-stage tumors. Tumors that were grade 1 or 2 were classified as low-grade tumors; grade 3 and 4 tumors were classified as high-grade tumors.

Immunohistochemistry. Four-μm sections for Ki-67 immunohistochemistry were made from frozen tissue blocks before preparing touch preparations for FISH analysis. Cells with proliferative activity were identified by detecting the Ki-67 antigen. The Ki-67 antigen is expressed in all cells in G1, S, or G2-M phase. The monoclonal antibody MIB1 (1:800; Dianova, Hamburg, Germany) was used for the detection of Ki-67 protein with an Avidin-biotin-complex immunoperoxidase procedure (ABC Elite, Vector Laboratories, Burlingame, CA) without proteinase digestion or postfixation. In this study, antigen retrieval was not performed because fresh frozen tumor material was used. The slides were weakly counterstained with hematoxylin. Nuclei were considered Ki-67 positive if any nuclear staining was seen. Only nuclear staining was considered. Ki-67 LI (percentage of positive cells) were determined by scoring 300 tumor cells in the tumor areas that had the highest density of positive cells.

DNA Probes for FISH. A chromosome 3 centromere probe (p o3.5) was used in combination with a P1 probe mapping to the VHL gene locus (3p25.5, RMC03P052). In addition, combinations of a centromere 17 probe (p17H8) with a 17p13.3 cosmid probe (at the p53 gene locus) and a chromosome 8 centromere probe (pM12) with a 8p22 P1 probe (RMC08P005) were used. All
FISH analyses from three normal kidneys obtained at autopsy served as control samples. The hybridization mixture was denatured for 5 min at 75°C and applied to denatured cells on slides. Hybridization mixture (10 μl) consisted of 10 ng of PI or cosmids probes, 4–10 ng of a biotinylated centromeric probe, and 10 ng of unlabelled, sonicated (200–500 bp) human placental DNA (Sigma) in 50% formamide, 10% dextran sulfate, and 2× SSC (pH 7). Hybridization took place overnight at 37°C. Posthybridization washings and the probe visualization were performed exactly as described previously (23) by using fluorescein-conjugated sheep anti-digoxigenin (Boehringer Mannheim), FITC-conjugated anti-sheep IgG (Sigma), and Texas Red avidin (Vector Laboratories). Nuclei were counterstained with 0.07 μg/ml 4',6-diamino-2-phenylindole in antifade solution.

Scoring of FISH Signals. At least 100 nuclei from each tumor were analyzed. Cells were selected for scoring according to morphological criteria using 4',6-diamino-2-phenylindole staining. Only those cells having a malignant cytological appearance (especially large nuclei) were scored. Small, round lymphocyte-like cells and overlapping or damaged nuclei were disregarded. Slides were analyzed only if two-thirds of the cells were interpretable in representative areas. To avoid misinterpretation due to inefficient hybridization, cells were counted only if at least one bright centromere signal and one cosmide/Pl signal were present. Two cosmide/Pl signals were counted as one if they were situated very close to each other (within <0.5 μm) to avoid misinterpretation due to sister chromatids of cells in S or G2-M phase.

Statistics. Contingency table analysis was used to examine the relationship between tumor grade, stage, histological subtype, and chromosomal alterations. Student's t test was used to evaluate the relationship between chromosomal alterations, tumor grade, stage, and Ki-67 LI.

Results

FISH Analysis

Normal Controls and Threshold Definitions. Imprint preparations from three normal kidneys obtained at autopsy served as controls. The percentage of nuclei with more than two signals per nucleus ranged from 2.5–5% for chromosome 3, 1.6–6.7% for chromosome 8, and 1.6–6% for chromosome 17 in the controls. All samples from normal kidneys showed an equal number of centromere and PI/cosmid signals in ≥90% of cells. Cells with less PI/cosmid signals were attributed to a decreased hybridization efficiency of the PI/cosmid probes compared with the centromere probes.

Conservative cutoff levels for the definition of polysomy, monosomy, and deletion were selected to rule out "false positive" results and also to avoid a classification of tumors on the basis of minor subpopulations. More than two centromere signals per cell (polysomy) in >20% of tumor cells were required to categorize a tumor as polysomic. A monosomy was defined as the presence of >20% of monosomic cells (cells with one centromere signal). The definitions used for deletions of loci were similar to those in previous studies (22–24). As a measure of deletion, the percentage of cells that contained either one centromere signal or fewer cosmide/Pl signals than centromere signals was calculated for each hybridization (defined as the percentage of deleted cells). A tumor was considered to have a deletion if the percentage of deleted cells was ≥40%.

Numerical Chromosomal Aberrations. FISH analyses with centromeric probes were successful in most cases. The slides were interpretable for centromere 3 in 43 tumors (81%), for centromere 8 in 42 tumors (79%), and for centromere 17 in 52 tumors (98%).

VHL Deletions. FISH with a PI probe for VHL (at 3p25.5) and a centromere probe for chromosome 3 was used successfully to define copy number aberrations in 43 tumors (Fig. 2). Nine tumors were excluded from analysis because the hybridization results were considered inadequate for interpretation. 3p deletions according to our definition were not seen in papillary RCCs (n = 9) but were found in 24 of 35 (69%) clear cell RCCs. There was no difference in 3p deletions between tumors of different grades and stages (Fig. 1). The results of our 35 clear cell RCCs are summarized in Fig. 3. The largest deleted tumor cell population was disomic in 15 of 24 (62%) VHL-deleted tumors, polysomic in 5 tumors of 24 (21%); cases 15, 17, 20, 26, and 34), and polysomic in 4 tumors of 24 (17%); cases 12, 24, 29, and 32). There was a considerable heterogeneity of 3p deletion in many tumors including a tumor with polysomic populations with and without deletions (case 15) as well as predominantly disomic tumors having significant fractions of polysomic cells with 3p deletion (cases 13, 14, 16, 22, and 28). Eleven clear cell RCCs showed no 3p deletion (31%; cases 1–11) according to our conservative definition. However, 5 of the clear cell RCC tumors (14%); cases 7–11) had subpopulations of between 20 and 35% of cells with less 3p signals than centromere 3 signals. This may be caused by a decreased hybridization efficiency, but the possibility cannot be excluded that these cells represent true subpopulations with VHL deletions. There were two tumors (cases 1 and 6) that had significant polysomic populations without VHL deletions, which indicates that tumor cells without physical deletion of 3p can exist in clear cell RCCs. Interestingly, there was one tumor (case 15) that had a significant fraction of both deleted and nondeleted polysomic cells.

8p and 17p Deletions. FISH analysis of 8p22 deletions was successful in 41 tumors (Table 1). 8p deletions were observed in 6 of 34 (18%) clear cell RCCs and in 1 of 7 (14%) papillary RCCs. The fraction of 8p22-deleted tumor cells ranged between 61 and 83%.

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<th>Table 1 FISH aberrations and histological tumor subtype</th>
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<td>Monosomy</td>
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<td>Polysomy</td>
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<td>Polysomy</td>
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<td>Chromosome 17</td>
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<tr>
<td>Monosomy</td>
<td>3 (6)</td>
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<td>Polysomy</td>
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<td>Deletion VHL</td>
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There was no association between 8p deletion and tumor grade or stage (Fig. 1). FISH with probes for 17p13 and centromere 17 was successful in 51 tumors. 17p deletions were observed in 7 of 41 (18%) clear cell RCCs and in 1 of 10 (10%) papillary RCCs. The number of cells with 17p deletions ranged from 58 to 88% in these tumors. 17p deletions were not associated with high tumor grade or stage (Fig. 1). All of the clear cell tumors that had a 8p22 (n = 6) and/or 17p deletion (n = 7) also had a 3p deletion. 8p22 deletions and 17p deletions were not observed within the same tumor.

**FISH and Ki-67 LI.** Ki-67 LI could be analyzed in all 54 tumors. The Ki-67 LI was slightly higher in clear cell RCCs (mean, 9.9 ± 9.2%; range, 1.1–48%) than in papillary RCCs (mean 4.3 ± 5.8%; range, 0.8–16%; P = 0.06). Within the group of clear cell RCCs, Ki-67 LI was higher in high stage tumors (n = 21; 12.2 ± 11.6%) than in low stage tumors (n = 21; 7.3 ± 5.0%), although this difference did not reach significance (P = 0.08) in this small set of tumors. There was no significant association between Ki-67 LI and numerical chromosomal alterations or deletions (data not shown).

**Discussion**

In this study FISH was used for the analysis of VHL deletions in RCCs. Previous studies comparing FISH and LOH have shown that these methods are in good agreement (24). It is therefore not surprising that our finding of a VHL deletion in 69% of clear cell RCCs was...
in the range of previous studies in which LOH analyses were performed. It is likely that our result still represents an underestimate because allelic losses due to mitotic recombination cannot be detected by FISH (24). A previous study that showed an allelic loss at 3p in 6 RCCs with cytogenetically normal copies of chromosome 3p (25) suggests that mitotic recombination may be one of the mechanisms leading to VHL loss in clear cell RCCs.

A considerable genetic heterogeneity was seen in our tumors including tumor cells with and without VHL deletion. Nondeleted cells were disomic in most tumors. This raises the question of whether some if not all of these cells were nonneoplastic in nature because a secure distinction between neoplastic and nonneoplastic cells is not possible by fluorescence microscopy. However, the finding of a large fraction of disomic VHL deleted cells in many of these tumors suggests that neoplastic cells were predominantly disomic in these samples. At least one of our tumors (case 15) had both a large subpopulation with VHL deletion and an unequivocal neoplastic subpopulation (being polysomic) without detectable VHL deletion. The possibility of heterogeneous VHL deletions is also supported by the previous findings of van der Hout et al. (25), who demonstrated a 3p deletion in a subset of tumor cells by conventional cytogenetics.

The coincidence of cells with and without VHL deletions in clear cell RCCs is intriguing. Although the combination of mutation and allelic loss is not the only mechanism leading to VHL inactivation (19), it could be expected that in tumors in which VHL deletions are necessary for VHL inactivation, this mechanism would apply in all cells. The simultaneous presence of neoplastic cells with and without VHL deletion in clear cell RCCs is consistent with different scenarios: (a) The observed heterogeneity of VHL deletions is consistent with a role of another tumor suppressor gene on 3p for the initiation and/or
progression of some clear cell RCCs. Cytogenetic and LOH studies have indeed suggested that other loci on 3p may be involved in the biology of sporadic clear cell RCCs (15, 26). It has been shown in the Eker rat model that RCCs can develop via pathways independent of alterations at the VHL locus (27). Interstitial deletions that involve the chromosomal regions 3p13–14.3 and 3p21–24 but not the VHL locus (at 3p25.5) have repeatedly been observed in LOH analyses (9, 14–16). If two different tumor suppressor genes on 3p were involved in the initiation and progression of sporadic clear cell RCCs, one of these 3p genes could be inactivated in all cells and the other one could be inactivated only in a subpopulation of our heterogeneous tumors. In that case, inactivation of the first gene could occur either through the hypermethylatation of its promoter region or through an interstitial deletion proximal to the VHL locus that could not be detected by FISH analysis using the selected set of probes.

(b) It is also possible that subpopulations without detectable VHL deletions by FISH can develop from cells that initially had a 3p deletion through duplication of the remaining allele of 3p. This hypothesis is supported by the findings of Gronwald et al. (28), who failed to detect 3p deletions in some metastases derived from 3p-deleted primary tumors by FISH and by comparative genomic hybridization. These authors hypothesized that the duplication of the remaining allele of 3p in subpopulations of the primary tumor could contribute to metastasis in these tumors by an elevated expression of a putative oncogene on 3p, which would lead to a selective growth advantage. The observations of high-level amplifications at 3p in bladder cancer (3p24; Ref. 29) and breast cancer (3p14; Ref. 30) are additional arguments for the existence of at least one putative oncogene on 3p.

(c) A successive loss of different parts of chromosome 3 that occur during tumor progression is a third possible explanation for subpopulations without detectable VHL deletions (20). Possibly, losses of the remainder of chromosome 3 may occur in tumors that initially had a 3p deletion. Such a mechanism might lead to an additional 3q underrepresentation. The possibility of a biological significance of 3q losses in clear cell RCCs is also supported by our finding of large subpopulations with only one centromere 3 signal in seven of our clear cell RCCs because losses of centromere signals frequently go along with losses of entire chromosomes. We also found relative DNA sequence underrepresentations of 3q in 5 of 11 sporadic clear cell RCCs examined by comparative genomic hybridization (31). However, there are no candidate tumor suppressor genes on 3q that have been implicated in RCC biology yet.

17p and 8p are regions that may harbor other putative tumor suppressor genes with relevance for clear cell RCCs. In this study, deletions of 17p13 and 8p22 were both found in 18% of the tumors. This is consistent with recent allelotyping analyses that showed 8p deletions in 18–33% (11, 21) and 17p deletions in 5–34% of RCCs (9, 10). Both 8p (11) and 17p deletions (10) have recently been suggested as potential prognostic markers because their prevalence was reported to increase with tumor stage. However, in this study we were not able to detect a relationship between 8p and 17p deletions and tumor stage or proliferation rate. Other groups have also failed to find an association between tumor stage and deletions at these loci (21). Taken together, these findings argue against a strong role of genes at these loci for RCC progression because alterations that lead to tumor progression would be expected to accumulate in advanced-stage tumors and to accelerate tumor cell proliferation.

Previous cytogenetic and molecular studies have shown that marked genetic differences exist between histological RCC types (11). Chromosome 3p and 8p (21) deletions were proposed to be specific for clear cell RCCs whereas polosyomies of the chromosomes 3 (4), 7, and 17 were suggested as markers for papillary RCC (1–3). Therefore, it was hypothesized that FISH analysis could facilitate the distinction of RCC subtypes. The absence of VHL deletions in nine papillary RCCs is consistent with previous cytogenetic analyses, which could not detect 3p deletions in papillary RCCs. Accordingly, a VHL deletion that would be detected in a small RCC biopsy or even in a fine-needle aspirate by FISH would indeed argue for a nonpapillary RCC subtype. Interestingly, we found an equally high frequency of polysomy 3, 8, and 17 in both clear cell tumors and papillary tumors. In a previous study, we had found a similar result for polysomy 7 occurring in all of the papillary and 74% of the clear cell RCCs (32). This discrepancy between FISH and cytogenetic data is in agreement with previous studies (33). It was suggested that these discrepancies may be due to a higher sensitivity of FISH in detecting tumor cell subpopulations with chromosome 7 and 17 polysomies. These results show that FISH analysis of polysomy 3, 7, 8, and 17 is not suited to assist diagnosis of RCC subtypes.

In summary, our data show that a considerable heterogeneity of VHL deletion exists within individual clear cell RCCs. This must be taken into account if molecular diagnosis of these tumors is attempted. Although VHL deletion analysis by FISH may have some diagnostic value, our data rule out any relevance of polysomy 3, 8, and 17 or 8p and 17p deletions for distinguishing RCC subtypes.

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References

VHL DELETION IN RCC


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