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Terminal Deletion of Chromosome 3p Sequences in Nonpapillary Renal Cell Carcinomas: A Breakpoint Cluster between Loci *D3S1285* and *D3S1603*[1]

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ABSTRACT

Deletion of chromosome 3p13–pter sequences is a specific genetic change in nonpapillary renal cell carcinomas (RCC). The *VHL* gene, a putative tumor suppressor gene, has already been cloned from the 3p25–26 chromosomal region. Conflicting cytogenetic and RFLP studies, however, suggest multiple interstitial deletions and additional tumor suppressor genes at chromosome 3p. To investigate the loss of DNA sequences on chromosome 3p in nonpapillary RCCs, we analyzed 41 paired normal and tumor DNAs obtained from short-term cultures of pure tumor cells with 12 polymorphic microsatellite markers covering the 3p11.2–p25 region. Deletion mapping provided evidence for terminal deletion with the most distal breakpoint between *D3S1300* and *D3S1285* loci, which is the site of breakpoint in familial 3;8 translocation predisposing to nonpapillary RCC. All breakpoints, including those occurring in familial translocation 3;6, were clustered in a more than 20-cM-large region between loci *D3S1285* and *D3S1603*. Interestingly, 7 of the 28 cases with 3p deletion showed a recurrent breakpoint between *D3S1603* and *D3S1595*, which cover about 1 cM genetic distance. The results suggest that a tumor suppressor gene, in addition to the *VHL* gene, might be localized somewhere on chromosome 3p distal to the familial 3;8 translocation, or it might be at the breakpoint cluster. Alternatively, the breakpoint serves as a mechanism to lose distal DNA sequences.

INTRODUCTION

Cytogenetic and RFLP analyses left no doubt that alteration of chromosome 3p is associated with the development of nonpapillary RCCs.[3] However, in spite of extensive studies, the exact locus of the tumor suppressor gene(s) remains in question. Comprehensive chromosome studies revealed a terminal deletion of the chromosome 3p segment in nearly 100% of nonpapillary RCCs (1–4). These studies determined the most distal breakpoint at chromosome 3p13–14.1 in sporadic and hereditary nonpapillary RCCs as well. Contrary to these data, interstitial deletions between chromosomal bands 3p14 and 3p23 have been described in other cytogenetic studies (5–7). Interstitial deletions involving chromosomal regions 3p13–14.3, 3p21.3, and 3p21–p24 have also been observed by RFLP analysis of RCCs (8–10). Lubinski *et al.* (11) found interstitial deletions between chromosomal bands 3p21 and 3p12 in RCCs by applying PCR-based microsatellite analysis. Recently, the *VHL* gene has been cloned from the 3p25–26 region, and it is now considered to be the RCC gene (12–13). Reliable cytogenetic and RFLP studies, however, suggest that another gene should be located at the chromosome 3p region, the deletional-mutational inactivation of which is associated with the development of nonpapillary renal cell carcinomas. To localize the RCC gene, we applied the sensitive microsatellite analysis for deletion mapping of chromosome 3p region in nonpapillary RCCs, including those which are associated with constitutional balanced translocations 3;6 and 3;8. To eliminate technical problems, such as contamination with normal cells, and to avoid any doubts in evaluation of the data, DNA extracted from tumor cells growing in short-term culture was used.

MATERIALS AND METHODS

Normal and Tumor DNA. Tumor and normal kidney tissues were obtained from patients at the time of nephrectomy. The most appropriate part of the tumor was separated for cell culture and reference histology, as described earlier (1). One part of this tissue as well as normal parenchymal kidney tissue was frozen in liquid nitrogen and stored at −80°C. The diagnosis of nonpapillary RCC was established by histological and cytogenetic analyses. Tumor cells growing in primary culture were monitored through an inverted microscope for the absence of contamination by normal cells (fibroblast, normal parenchymal cells, and mononuclear cells). Using a combined mechanical-enzymatic cell preparation technique, we have obtained pure tumor cell populations in all cases analyzed (1). DNA was isolated from tumor cells growing in primary cultures or in early passages as well as corresponding normal tissue after proteinase K digestion by phenol-chloroform extraction.

Microsatellite Analysis. To detect allelic loss, we used 12 microsatellite markers (Research Genetics, Inc., Huntsville, AL) covering the chromosome 3p region, which has been suggested for interstitial deletions. One of the microsatellite markers was located at chromosome 3p25, another at 3p21.3, and most of them between 3p14.2 and 3p11.2. The order of loci based on the consensus physical map of chromosome 3p (14), with a minor modification of changing the position of *D3S1511* and *D3S1577*, is shown in Fig. 1. DNA amplification was carried out in 96 polycarbonate plates using an MJ Research PTC100 thermocycler. The reaction was carried out in a 10-μl volume containing 20 ng DNA, 50 μM KCl, 10 μM Tris (pH 7), 1.5 μM $MgCl_2$, 200 μM deoxynucleotide triphosphate, and 2 pmol of each primer, one of them labeled with 2 μCi [γ-^{32}P]ATP. For primers *D3S1284*, *D3S1595*, and *D3S1663*, the concentration of $MgCl_2$ was increased to 3 mM. The samples were covered with mineral oil before being subjected to the following PCR program with a hot start: 10 cycles touchdown (−1°C per cycle) starting with 65°C annealing temperature, and additional 15 cycles composed of 30 s at 94°C, 20 s at 55°C, and 20 s at 72°C. After adding 5 μl stop solution and heating the samples for 4 min to 80°C, 7 μl of the mixture were separated through a 5% denaturing polyacrylamide gel at 80 W for 2 h. The signals were visualized by autoradiography after 1 or 2 days of exposure. LOH was determined by loss of the signal at one of the alleles.

RESULTS

Microsatellite Analysis. The chromosomal analysis has an accuracy of about one chromosomal band, which represents DNA sequences of several megabases. Therefore, we applied the microsatellite analysis to determine the breakpoints more exact at the molecular level. DNA from tumor cells was available in 41 cases, including one tumor each from families with constitutional translocations 3;8 and 3;6. LOH at all informative markers was found in 12 tumors, which corresponds to the cytogenetic data published previously showing a monosomy of chromosome 3. One of the tumors (465) showed loss of chromosome 9 as the only karyotypic change by previous chromosome analysis (1). Corresponding to this finding, no LOH was found at either informative loci on chromosome 3p. The remaining 28 cases showed terminal deletions of different sizes (Fig. 1). Two examples are shown in Fig. 2. The microsatellite markers for loci *D3S1560*, *D3S1289*, and *D3S1300* are mapped to chromosomal regions 3p25, 3p21.3, and 3p14.2, respectively. LOH was detected at these loci in all

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[3] The abbreviations used are: RCC, renal cell carcinoma; VHL, von Hippel-Lindau disease; LOH, loss of heterozygosity.

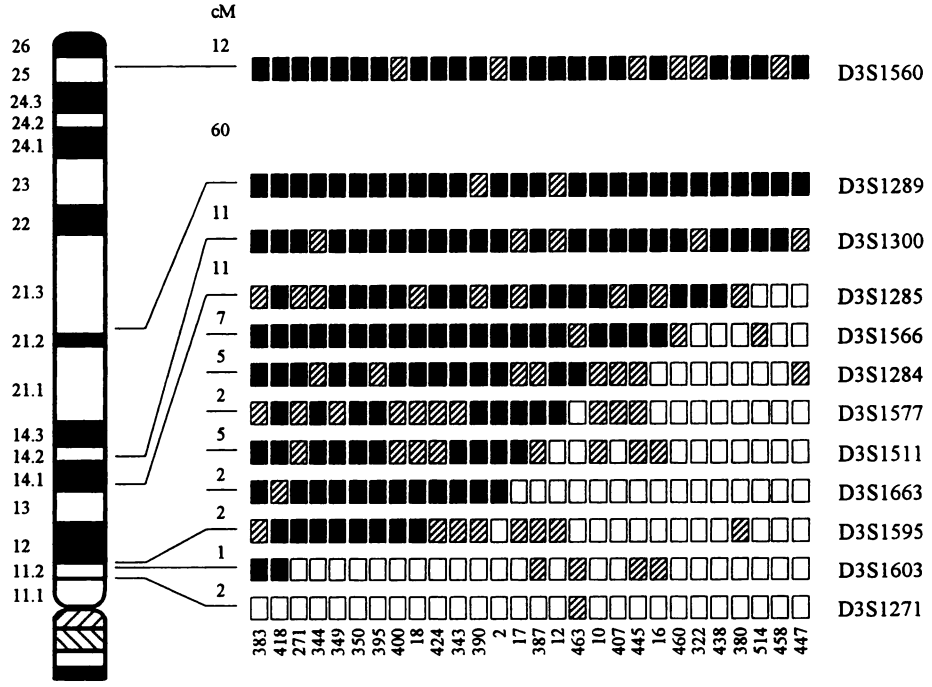

Fig. 1. Result of microsatellite analysis for allelic loss in 28 nonpapillary RCCs. Terminal deletions with different breakpoints were found in all cases. Markers used are indicated on the *right*, whereas their physical location and the genetic distance are shown on the *left*. ■, LOH; ▨, not informative; □, retention of heterozygosity.

Fig. 2. Microsatellite assay of two nonpapillary RCCs. DNAs extracted from pure tumor cells (*T*) and corresponding normal tissues (*N*) were analyzed by PCR using microsatellite markers as indicated. Both tumors 344 and 350 showed terminal deletion distal to the *D3S1603* locus.

informative cases. The three markers cover approximately an 80-cM terminal region of chromosome 3p. The constitutional heterozygosity for the microsatellite marker at *D3S1285* locus (chromosomal band 14.1) was retained in only three cases. One of these tumors (514) was obtained from a patient with the constitutional translocation 3;8. On the other side of the breakpoint cluster, the constitutional heterozygosity at *D3S1271* locus (chromosomal band 3p11.2) was retained in all cases. LOH at the *D3S1603* locus, which is 2 cM distal to the *D3S1271*, was detected in only two cases. The vast majority of the breakpoints are clustered between *D3S1285* and *D3S1603* loci spanning about 20 cM genetic distance. A recurrent breakpoint between *D3S1603* and *D3S1595*, which cover about 1 cM genetic distance, has been found in 7 cases. Unfortunately, the case with constitutional translocation 3;6 (445) was not informative for three of the loci at the breakpoint. However, this study clearly demonstrates that the constitutional breakpoints in both families with predisposition to renal cell carcinomas are located within the breakpoint cluster described here.

DISCUSSION

Most investigators working in this field agree that loss of chromosome 3p sequences is the most characteristic and possibly the first genetic event in the development of nonpapillary RCCs. The role of chromosome 3p alteration was confirmed by an experiment showing that complementation of the entire chromosome 3p segment via microcell fusion can modulate the growth of a RCC cell line (15). A putative tumor suppressor gene, the *VHL* gene, has already been cloned from the chromosome 3p25–p26 region (12). Because somatic mutations of the remaining allele of the *VHL* gene were shown in 50–60% of sporadic nonpapillary RCCs, the *VHL* gene was suggested to be the RCC gene (13). Earlier cytogenetic and RFLP studies as well as our microsatellite analysis showed, however, a constant deletion of more proximal sequences in nearly 100% of nonpapillary RCCs (1–3, 16, 17). Moreover, a recent experimental study using microcell-mediated chromosome transfer demonstrated that introducing DNA sequences from chromosomal regions 3p12–3p14, which do not contain the *VHL* gene sequences, can modulate a phenotype of a RCC cell

line (18). These data suggest that a RCC tumor suppressor gene is located proximal to the *VHL* locus at chromosome 3p.

The exact locus of the RCC gene at chromosome 3p is not yet known. The vast majority of cytogenetic studies described interstitial deletions in RCCs (5–7). These data were confirmed by RFLP analysis of DNAs obtained from RCC tissues (8–10). Based on these studies, more than one tumor suppressor gene at chromosome 3p is now implicated in the genetics of nonpapillary RCCs. A recent study using polymorphic simple sequence repeat markers also described interstitial deletions at different sites between chromosomal bands 3p12–p21 in 9 of the 30 nonpapillary RCCs (11). Thus, even after introducing the very sensitive microsatellite markers for deletion mapping, the trend remains to find multiple RCC suppressor genes on chromosome 3p. Based on our earlier cytogenetic and RFLP analyses, we have some problem following this trend. We have detected the loss of the entire 3p13–pter region of one homologous chromosome in 97% of nonpapillary RCCs by cytogenetic analysis (1, 2). The loss of the chromosome 3p region resulted, in many cases, from a nonhomologous recombination between chromosome 3p and other chromosomes and subsequent loss of a derivative chromosome carrying the 3p13–pter region (19). This is in agreement with loss of one allele of all chromosome 3p markers localized distal from the 3p13–p14.1 boundary. However, our finding is not compatible with the retention of chromosomal sequences at the 3p13–pter region suggested, in many cases, by multiple interstitial deletions.

For positional cloning of the RCC gene, the correct determination of breakpoints as well as the site and size of deleted chromosome 3p sequences are necessary. Chromosomal analysis has an accuracy of about one chromosomal band, which represents DNA sequences of several megabases. Moreover, the exact breakpoint in cases of translocations involving two positive G-bands remains unclear. For example, based on our microsatellite analysis, we had to revise some of the breakpoints determined by earlier chromosome analysis (1, 2), placing it from 3p13 to the other site of the 3p12 chromosomal band, to 3p11.2, or *vice versa*. Small interstitial deletions of some megabases in size are also not detectable in chromosome preparations. Results of RFLP analysis of tumor tissues are often difficult to interpret because of contamination with normal cells. Microsatellite analysis of DNA obtained from pure tumor cells, however, is a sensitive and quick method to determine allelic losses on a clear cut "yes or no" basis. Correspondingly, our study showed a terminal deletion covering at least 80 to 90 cM genetic distance in nearly 100% of nonpapillary RCCs. This deletion comprises a large DNA fragment from the p14.1 band to the terminal region of chromosome 3p, including the *VHL* gene. We did not find interstitial deletions described in previous RFLP studies. Our findings differ in many points from those obtained by Lubinski *et al.* (11). They found retention of constitutional heterozygosity at the locus *D3S1289* in 5 of 12 informative cases, whereas all of the 26 informative tumors in our series showed an LOH at this locus. They observed retention of both alleles in 8 of 14 informative cases for locus *D3S1285*, which was retained in only 3 of 19 tumors in our series. They found interstitial deletion in 9 of 15 tumors, whereas none of the 28 tumors in our series showed such genetic change. Likely, the use of DNA obtained from tumor tissue *versus* tumor cells makes the difference. In our study on sporadic RCCs, the most distal breakpoint occurs at the constitutional breakpoint determined in the familial 3;8 translocation (20). All nonpapillary RCCs, including one from a member of the family with germline 3;6 translocation, had breakpoints between the *D3S1285* and *D3S1603* loci. Thus, the breakpoint cluster comprises more than 20 cM genetic distance. Within this breakpoint region, we found a small recurrent breakpoint cluster of 1 cM genetic distance between *D3S1603* and *D3S1595* loci occurring in 7 of the 28 cases.

As expected from previous cytogenetic studies, microsatellite analysis revealed a breakpoint cluster at chromosomal band 3p12–p13 and a terminal deletion distal to these breakpoints in nonpapillary RCCs. How can we explain these data? One can suggest that the breakpoint directly affects a tumor suppressor gene, or the translocation has a positional effect on a tumor suppressor gene located at the breakpoint cluster on chromosome 3p12–p13. However, the breakpoint cluster covers more than 20 cM genetic distance. Alternatively, the breakpoint is only instrumental to remove the more distal sequences harboring one allele of the RCC suppressor gene, which then might be located somewhere along the 80–90-cM region deleted consequently in nonpapillary RCCs. In this case, applying molecular techniques other than positional cloning is necessary to clone the gene.

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