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ARTICLE

Microsatellite Analysis of Chromosome 3p Region in Sporadic Renal Cell Carcinomas

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The etiology and progression of renal carcinomas (RCC) is still poorly understood. RCC have been classified into several pathological entities. The most frequent type, clear cell carcinoma, accounts for about 80% of sporadic RCC and shows several chromosome abnormalities documented both by conventional cytogenetics, loss of eterozygosity (LOH) and replication error (RER) studies. In 10 clear cell type sporadic RCC we evaluated LOH and RER using a set of 10 microsatellite markers covering the chromosome 3p region, which has been suggested for interstitial deletions. Electrophoresis was performed by automated sequencer ABI Prism 377 and data were analyzed with Genescan and Genotyper 2.5 softwares. We revealed allelic loss in 48,7% of informative microsatellites and a single case of RER. We found the highest LOH frequency in 3p25-26 region where maps Von Hippel-Lindau (VHL) oncosuppressor gene. In addition, DNA hypermethylation, an alternative mechanism of VHL gene silencing, was evaluated by methylation-specific PCR. However hypermethylation status was not detected in any of our tumor samples. (Pathology Oncology Research Vol 8, No 4, 241–244)

Keywords: microsatellite instability, renal cell carcinoma, LOH, RER

Introduction

Renal cell carcinoma (RCC) is the most frequent malignant disease accounting for about 3% of all adult malignancies, and its etiology is poorly understood.[1] Multiple genetic changes that lead to loss of growth control may contribute to development of RCC.[2] Cytogenetic and molecular studies have suggested frequent genetic alterations on chromosome 3p, including microsatellite instability, characterized by the presence of random contractions or expansions in length of simple sequence repeat, in both ereditary and sporadic form of RCC.[3-10] RER (Replication Error) is caused by a defect of DNA mismatch repair function as a result of mutations in mismatch repair genes. LOH (Loss of heterozygosity) is an important molecular mechanism that results in the loss of one of the two alleles at a specific locus. LOH of polymorphic DNA markers, in tumors compared with normal tissue, is a sign of somatic deletion and has been used as a clonal marker of neoplasia.[11] High frequencies of loss of heterozygosity of the short arm of chromosome 3 have been detected in various RCC types and this strongly suggests that more than one tumor-suppressor gene is located at this chromosomal region.[4-6] The VHL gene, a tumor suppressor gene mapped on 3p25-26 is associated with a large fraction (>70%) of sporadic RCC as well as the hereditary form.[12-14] The aim of this study was to investigate the frequency and the presence of microsatellite instability in RCC clear-cell type by comparing DNA tumor fingerprints with constitutional DNA.

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Materials and methods

Tumor collection and DNA extraction

Ten non-papillary sporadic RCC were examined in this study. Tumor and normal kidney tissues were obtained from patients at the time of nephrectomy. The diagnosis of clear-cell RCC was established by histological analyses. DNA was isolated from tumor cells after proteinase K digestion by phenol-chloroform extraction. 100ng were used as a template in 25 μl reactions.

Microsatellite analysis

To determine LOH/RER at the short arm of chromosome 3, we analyzed all samples in 10 loci: D3S1560, D3S1038, D3S1297, D3S1597, D3S2387, D3S1076, D3S1067, D3S1300, D3S2757 and D3S4260. The order of loci is according to the consensus physical map of chromosome 3p ***(www.gdb.org).*** We choose these 10 markers to represent three regions, 3p25-pter, 3p21 and 3p14 of potential interest in RCC clear-type carcinogenesis.[1,4,5] The Polymerase Chain Reaction was carried out in 25 µl containing 50-100 ng genomic DNA; amplification was performed in 50 mmol/L KCl, 10 mmol /L Tris-HCl (pH 8.3), 5 mmol/L $MgCl_2$, 200 µmol/L dNTPs, and 0.5 µmol/L for each fluorescent primer set. AmpliTaq DNA polymerase (1 U Ampli Taq Gold Applied Biosystems Foster City KA USA) was added for each 25 µl reaction. PCR was performed by the 9700 Thermal Cycler (Applied Biosystems, Foster City KA, USA); cycling parameters for the reactions were optimized for each exon. Primers used for amplification, repeat type, size range, and dye label are reported in ***table 1.*** One µl of the fluorescent PCR was added to 6 µl of formamide and 1 µl of ROX 350 size standard, loaded on 6% polyacrylamide 8-M urea gel in a 377 Automated Sequencer (Applied Biosystems). The data were collected automatically and analyzed by GeneScan software; finally, the Genotyper software was used for the allele scoring and assessement of LOH.[11]

Methylation analysis

To detect VHL gene hypermethylation, a multiplex PCR was developed by using primers that flank the ***NotI*** site (F CGG AGG GCG GAG AAC TGG; R GAG GGC TCG CGC GAG TTC) and primers from exon 2 that act as a control for failure of PCR (F CAC CGG TGT GGC TCT TTA ACA A; R ACA TCA GGC AAA AAT TGA GAA CTG G).[13] Two micrograms of DNA were digested with ***NotI*** in at least two independent experiments. 100 nanograms of digested DNA were used in a multiplex 30 µl PCR reaction. Fragment were amplified in a 9700 Thermal Cycler (Applied Biosystems, Foster City KA, USA) for 30 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C. The reactions contained 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U Ampli Taq Gold (Applied Biosystems Foster City KA USA) and 1µM primers (final concentrations). In presence of VHL hypermethylation a 208-bp fragment is observed only; the second fragment (262bp) amplifies if ***NotI*** digestion fails because of methylation of cytosines in the ***NotI*** recognition site.

Table 1. **Primers used in the study**

Microsatellite	*Locus*	*Primers*	*Dye label*	*Repeat type*	*Size*
D3S1038	3p26.1 3p25.2	TCCACTAAGAGGCTTCCTAG* AAAGGGGTTCAGGAAACCTG	FAM	Di	115 bp
D3S1560	3p25-26 3pter-3p24.2	GCATCTACAGGGGGTGTCT* AGGCTGATTTTCAGCACAA	NED	Di	242 bp
D3S1297	3pter-3p25	CATAATTTGCTGCTTTGGAT* TGCACATTAAAGGAACAGGT	HEX	Di	219 bp
D3S2387	3pter-3qter 3pter-3p24.2	AAAGCTAGAAGGAGCTGGCT* GGGTGACAGAGTGAGATGTG	NED	Te	191 bp
D3S2757	3p14.2	TCACCTGTGTTTGGTTTGGA* TGCACATTAAAGGAACAGGT	FAM	Di	318 bp
D3S1597	3pter-3p24.2	GCAAATCGTTCATTGCT* AGTACAAATACACACAAATGTCTT	HEX	Di	171 bp
D3S1067	3p21.1-3p14.3	TCATCTATCTCCCAACTGTTGAG* GAGCACTACCTGTTTAAGATAGG	HEX	Di	95 bp
D3S4260	3p14.2	CTGCAAAGAGGAAGGAAGGG* TGTGAACTGTCAATCCATCCA	FAM	Te	214 bp
D3S1076	3p21.2-3p21.1	ATTCCCTGAATATGATCCCAACTG* ACCTAAGAGCAATCACTTAACTTAG	FAM	Di	95 bp
D3S1300	3p21.1-3p14.2	GCCAATTCCCCAGATG* AGCTCACATTCTAGTCAGCCT	FAM	Di	217 bp

Table 2. **Clinical data of RCC patients**

Patient	*Sex*	*Tumor Histology*	*Tumor grade*	*Tumor stage*	*Tumor size (cm)*	*Microsatellite instability (%)*
R4	M	Clear-cell	G2	pT1	3,6x3,2x3	1/10 (10)
R5	F	Clear-cell	G2	PT3a	12x10x8	3/10 (30)
R9	M	Clear-cell	G3	pT2	9x8x8	5/10 (50)
R13	M	Clear-cell	G2	pT1	3,2x3x3	3/10 (30)
R16	M	Clear-cell	G2	pT1	4x3x2,8	7/10 (70)
R19	F	Clear-cell	G1	PT1	2,5x2x2	6/10 (60)
R57	M	Clear-cell	G3	pT2	6,5x5x4,6	1/10 (10)
R73	M	Clear-cell	G3	pT1b	8x8x7	3/10 (30)
R81	F	Clear-cell	G2	PT1	3,7x4x4	0/10 (0)
R83	F	Clear-cell	G2	PT1	4x4x3	9/10 (90)

Results

The presence of microsatellite instability at the marker loci D3S1560, D3S1038, D3S1297, D3S1597, D3S2387, D3S1076, D3S1067, D3S1300, D3S2757 and D3S4260 was investigated in 10 patients with sporadic RCC clear-cell type *(Table 2)*. The analysis was performed by using semiautomated procedures based on multicolor fluorescently labeled microsatellite markers and an automated sequencer for allele detection.[11] In this study we revealed allelic loss in 48,7% (38/78) of informative microsatellites (78 microsatellites out of 100 studied resulted informative). We found the highest LOH frequency (55.8%) in 3p25-26 region where the Von Hippel-Lindau oncosuppressor gene maps.[12] In 9 of 10 patients with RCC, LOH was observed in more than one marker locus. In one patient, LOH was detected in 9 loci out of 10 studied. In 6 (60%) of 10 patients, instability at microsatellite D3S1038 was found and in 5 patients (50%) it was observed at microsatellites D3S1560, D3S1297 and D3S1067. These results are reported in

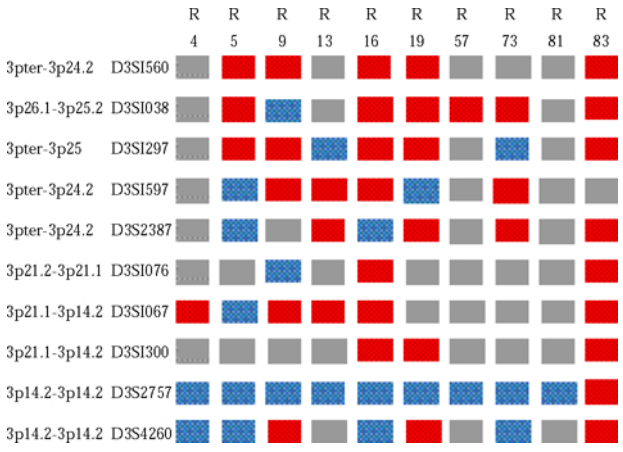

Figure 1. *Results of microsatellite analysis in 10 tumors with clear cell type RCC. In red LOH; in purple, not informative; in gray absence of heterozygosity.*

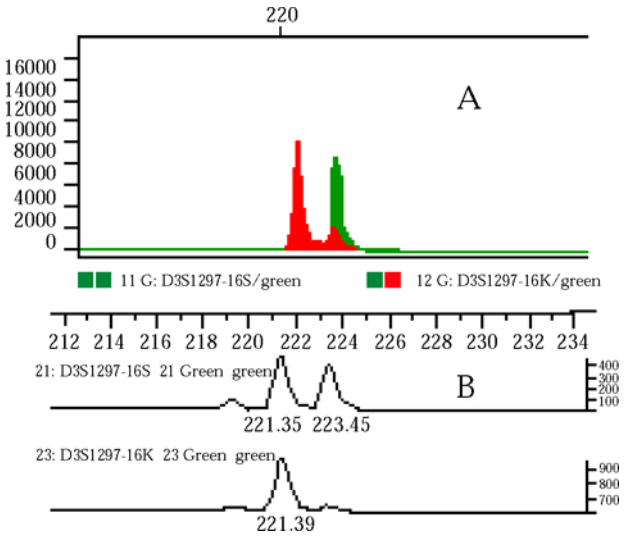

Figure 2. *It is reported one example of alterations of microsatellite sequence: LOH at D3S129/RCC obtained with automated sequencer.* ***A.*** *Genscan software;* ***B.*** *Genotyper software.*

Figure 1. Figure 2 shows one example of alterations of the microsatellite sequence at D3S1297 which shows the presence of LOH a in tumor sample. In one patient we detect RER-like a strong production of extra peaks, extending several repeats units from normal alleles and being stronger than the constitutional alleles (***Figure 3***). A tumor is RER+ if two or more markers out of five were positive;[11] in our samples five out of ten markers were positive for RER. DNA hypermethylation was not observed in any of our tumor samples.

Discussion

Analysis of microsatellite instability is a promising technique to investigate somatic changes that occur during tumor progression.[2,3,10] Evaluation of LOH and/or RER is

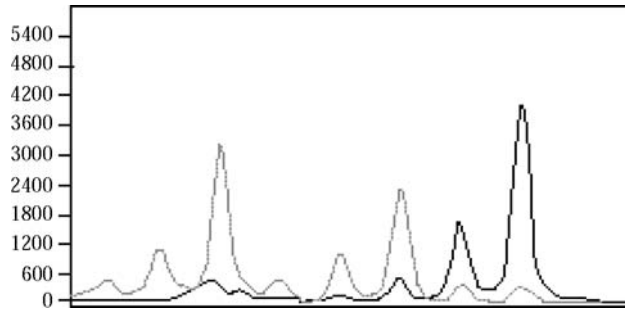

Figure 3. *Example of RER+ detected in five loci out of ten analysed in R9 sample (grey) like a strong production of extra peaks, extending several repeats units from normal alleles (black) and being stronger than the constitutional alleles.*

also a useful method for early diagnosis of tumors, and in some cancer types correlation between microsatellite instability and diagnosis has also been reported.[5,8,9] In particular LOH at chromosome 3p occurs in a large proportion (98%) of non-papillary RCC of sporadic origin,[7] so detection of LOH at this level represent a crucial point in the differential diagnosis of renal cell tumors.[15] Our data revealed a high frequency of LOH in the 3p region (48,7%) with the highest LOH frequency in the 3p25-26 region where the Von Hippel-Lindau oncosuppressor gene maps, and a very low frequency of RER, in keeping with previously studies.[12-14,16-19] Because VHL is a tumor suppressor gene, two mutational events are required for its inactivation as suggested by the "two hit" model of tumorigenesis.[13] We have investigated DNA hypermethylation as alternative mechanism of VHL gene silencing in sporadic RCC. Nevertheless the hypermethylation status was not observed in any of our tumor samples. As reported previously,[18] DNA hypermethylation is a rare condition in renal carcinoma cell lines of the clear-cell type.[16-17] Screening for intragenic mutations may provide evidence of other intragenic mechanisms involved in VHL inactivation. Probably due to the very small numbers of patients analyzed, no association was found between LOH and stage of disease, tumor size and histological grade. Analysis of a larger cohort of patients may allowed us to correlate microsatellite instability with clinical characteriastic.

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