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M. Böhm · I. Wieland · C. Stinhöfer
T. Otto · H. Rübben

Detection of loss of heterozygosity in the *APC* tumor suppressor gene in nonpapillary renal cell carcinoma by microdissection and polymerase chain reaction

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Abstract The role of the *APC* (*a*denomatous *p*olyposis *c*oli) tumor suppressor gene in the genesis of nonpapillary renal cell carcinoma is addressed. The frequency of allelic deletion in the *APC* gene was analyzed using microdissection of the tumor specimens and a PCR (polymerase chain reaction)-based assay for the detection of intragenic loss of heterozygosity (LOH). Twelve of 29 carcinomas investigated were informative (41%). In five of these (42%) LOH was detected in the *APC* gene. LOH did not correlate with tumor grade or stage. This high frequency of intragenic LOH suggests an implication of the *APC* gene or a closely linked gene in the genesis of a subset of nonpapillary renal cell carcinoma. The use of a microdissection technique allows the reliable detection of tumor-specific LOH when using a PCR-based assay.

Key words: Kidney cancer · APC · Loss of heterozygosity · Microdissection · Tumor suppressor · Chromosome 5q

Introduction

Inactivation of tumor suppressor genes appears to be a predominant mechanism in the genesis of human cancer. In many sporadic solid tumors, tumor suppressor genes are frequently inactivated by allelic deletion combined with mutation of the remaining allele, or in a smaller percentage by homozygous deletion of both alleles [2, 23, 25]. In nonpapillary renal cell carcinomas, which comprise the majority of renal carcinomas, several tumor suppressor genes may be inactivated during the malignant transformation of renal tissue. However, to date only the *VHL* gene on chromosome 3p25 and the *p53* gene on chromosome 17p have been shown to be involved in renal cell carcinogenesis, and a conclusive "pathway to malignancy" [6] remains to be proposed. It is, therefore, of importance to investigate the relevance of other known tumor suppressor genes in renal cell carcinogenesis.

Cytogenetic [11, 22] and LOH (loss of heterozygosity) [11, 13, 14, 19] analyses indicate that chromosome 5q contains one or more tumor suppressor genes relevant in the genesis of nonpapillary renal cell carcinoma. For locus 5q21-22, available results are equivocal: one group did find an increased LOH frequency in the tumor tissue using several polymorphic markers [13, 14], whereas others did not when using marker D5S346 on chromosome 5q21-q22 [7], marker D5S141 on chromosome 5q21 [4], marker D5S429 [19], or a polymorphism in exon 11 of the *APC* (*a*denomatous *p*olyposis *c*oli) tumor suppressor gene [18].

Allelic deletion can be detected as LOH, and a highly informative polymorphic site has been identified within the *APC* gene [10]. This allows the detection of intragenic allelic deletion in the tumor cells. Intragenic LOH allows a closer assessment of the *APC* gene than the polymorphic markers D5S346, D5S141 and D5S429 mentioned above, which are located outside the *APC* gene.

LOH can be detected by Southern blotting of genomic DNA that has been extracted from a block of tumor tissue and digested with an appropriate restriction enzyme. In recent years the polymerase chain reaction (PCR) has proven to be a fast and effective alternative for the analysis of small DNA regions that requires only the minute amounts of DNA available in many clinically obtained small specimens. However, PCR is very sensitive to contaminating nontumor DNA, which can give positive results and thus obscure the detection of deletions in the tumor DNA. Clinically obtained tumor biopsies are, in contrast to cell lines, usually heterogeneous, consisting of tumor cells, stroma, leukocytes and

M. Böhm (✉) · C. Stinhöfer · T. Otto · H. Rübben
Urologische Klinik und Poliklinik, Universitätsklinikum, D-45122 Essen, Germany

I. Wieland · C. Stinhöfer
Institut für Zellbiologie (Tumorforschung), Universitätsklinikum Essen, D-45122 Essen, Germany

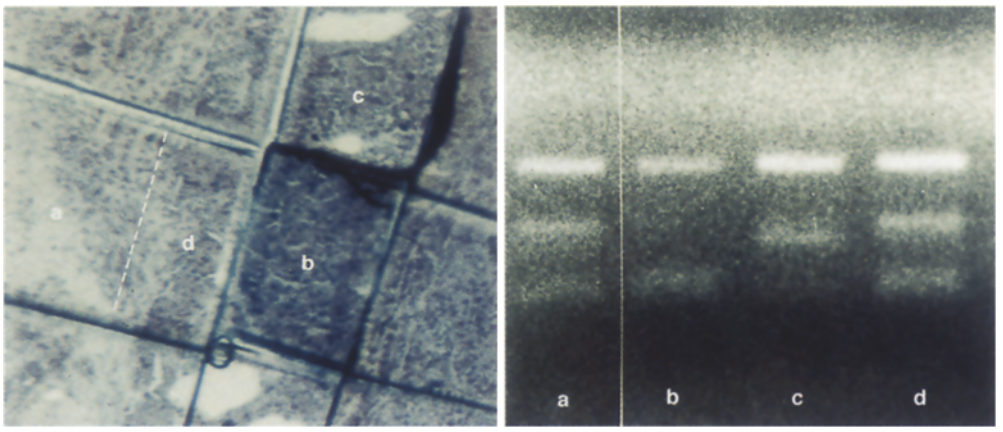

Fig. 1 LOH analysis of exon 11 of the *APC* tumor suppressor gene in renal cell carcinoma of patient 1. *Left panel* Microdissected renal cell carcinoma. The 10-μm cryotome section is placed on a plastic supporter foil, and a rectangular area containing more than 90% tumor cells has been cut out (*b*). Neighboring areas containing about 80% tumor cells (*c*), about 70% tumor cells (*d*), or normal cells only (*a*) are indicated. *Right panel* Agarose gel of *Rsa*I-digested PCR products. *Lane a* normal tissue, *lane b* microdissected tumor cells, *lane c* the section containing about 80% tumor cells and 20% normal cells, *lane d* the section containing about 70% tumor cells and 30% normal cells. The digested fragment is missing in the tumor cells (*lane b*) indicating LOH, i.e. loss of the allele with the *Rsa*I site. The presence of normal cells yields an equivocal result *in lane c*, and obscures the detection of LOH *in lane d*. Band sizes are 132 bp (*upper*), 86 bp (*middle*), and 46 bp (*lower*). The *lower bands* are not evaluable due to interfering primer dimers

surrounding normal tissue. It is, therefore, of prime importance to verify the origin of the DNA prior to introduction into subsequent PCR, in particular when looking for tumor-related LOH or homozygous deletions [2, 24, 28].

In this study, allelic deletion of the *APC* tumor suppressor gene was assessed in nonpapillary renal cell carcinomas using a PCR-based assay in conjunction with a microdissection technique.

Materials and methods

Twenty-nine nonpapillary renal cell carcinomas were removed during routine nephrectomy. Samples of about 1 cm^3 were frozen immediately after resection and stored at −180°C until further analysis [3]. Routine histopathological assessment was done on formalin-fixed, paraffin-embedded specimens according to WHO criteria [15]. Tumors were staged according to the TNM system [21]. After histological verification of each tumor, membrane-mounted cryotome sections were prepared as described [2]. Briefly, 10-μm sections were placed on a plastic supporter membrane (Agfa, Gera or Kopa, Düsseldorf, Germany). Tumor islets and surrounding normal tissue were identified under a stereomicroscope at ×40–100 magnification and excised with a scalpel blade (Fig. 1, left panel). All excised tumor islets contained less than 10% nontumor cells, if not indicated otherwise. Paired islets containing either tumor or normal cells were then lysed as described [2]. Supernatants were used as PCR templates to generate a 132-base-pair PCR product containing a polymorphic *Rsa*I site in exon 11 of the *APC* tumor suppressor gene. After an initial denaturing step at 95°C for 5 min, PCR conditions were 10 cycles for 30 s at 94°C, 1 min at 58°C and 30 s at 72°C, followed by 30 cycles for 30 s at 94°C, 1 min at 54°C and 30 s at 72°C, and a final 10 min at 72°C. The 132-base-pair PCR product is cleaved to an 86- and a 46-base-pair fragment by *Rsa*I digestion if the polymorphic site is present, and it remains uncleaved if the site is absent [8, 24]. *Rsa*I-digested PCR products were electrophoresed on 3% agarose gels and stained with ethidium bromide. At least three tumor islets from different regions of each tumor sample were assessed, and only if the respective allele was undetectable in all of them was the tumour classified as showing LOH.

Results

Of 29 patients with nonpapillary renal cell carcinomas, 17 (59%) were homozygous (i.e. noninformative) at the *Rsa*I site in exon 11 of the *APC* gene (Table 1). The PCR product was cleaved in 13 (76%) of these 17 patients; the remaining four (24%) did not have the restriction site. Twelve patients (41%) were heterozygous, (i.e. informative) for exon 11 of the *APC* gene. In 5 (42%) of these 12 informative cases, LOH was detected in the carcinoma cells, but not in the surrounding normal cells (Fig. 1a, Table 1). LOH was detected if less than about 10% nontumor cells were present in the microdissected tumor islets being introduced into the subsequent PCR reaction (Fig. 1b). If more than about 20% nontumor cells were present in the PCR reaction, the result was equivocal (Fig. 1c) or the detection of LOH was obscured (Fig. 1d). This demonstrates that the verifiable origin of the DNA from tumor tissue is a prerequisite for detection of tumor-specific LOH unequivocally. The results were consistent in all tumors except tumor six, where one of six islets investigated showed LOH, whereas the other five did not. This tumor was considered heterozygous because the majority of tumor islets tested showed no LOH (Table 1).

Two of the five tumors showing LOH in the *APC* gene were in stage T2, two in stage T3 and one in stage T4 (Table 1). There was no significant correlation between tumor stage and LOH in the *APC* gene using a weighted analysis of variance ($P = 0.44$). One of the tumors with LOH was graded G1, three were G2 and

Table 1 Tumor stages and deletion data

Tumor	Age (years)	Sex	Stage	Grade	APC[a]	LOH[b]
1	56	m	T2N0M0	G1	he	LOH
2	59	m	T4N0Mx	G2	he	LOH
3	57	w	T2N0M0	G2	he	LOH
4	61	w	T3N0Mx	G2	he	LOH
5	59	m	T3N2M0	G4	he	LOH
6	59	m	T2N0M0	G1	he	NO[c]
7	53	m	T2N0M0	G1	he	NO
8	51	w	T3N0M0	G2	he	NO
9	69	m	T2N0M0	G2	he	NO
10	54	w	T2N0M0	G2	he	NO
11	55	m	T2N0M0	G2	he	NO
12	74	w	T3N0M0	G2-3	he	NO
13	55	m	T2N0M0	G1	ho+	
14	60	m	T2N0M0	G1	ho+	
15	74	w	T2N0M0	G1	ho+	
16	56	m	T2N0M0	G1	ho+	
17	74	w	T2N0M0	G1	ho+	
18	55	w	T3N0M0	G1	ho+	
19	80	w	T2N0M0	G1-2	ho+	
20	61	m	T2N0M1	G2	ho+	
21	49	m	T3N0Mx	G2	ho+	
22	61	m	T2N0M0	G2	ho+	
23	66	w	T2N0M0	G2	ho+	
24	82	m	T2N0M0	G2	ho+	
25	73	m	T2N0M0	G2	ho+	
26	80	m	T3N0M0	G1	ho−	
27	58	w	T2N0M0	G1-2	ho−	
28	68	w	T4NxM0	G2-3	ho−	
29	77	w	T3N0M0	G3	ho−	

[a] Informativity at exon 11 of the *APC* gene: *he* heterozygous, *ho+* homozygous with *RsaI* site, *ho−* homozygous without *RsaI* site
[b] *LOH* loss of heterozygosity at exon 11 of the *APC* gene, *no* no loss of heterozygosity
[c] Four of five tumor islets tested showed on LOH; one showed LOH

one was G4 (Table 1). A weighted analysis of variance also did not show a significant correlation of LOH with tumor grade ($P = 0.63$).

Discussion

The main findings of this study are that LOH in the *APC* tumor suppressor gene occurs frequently in nonpapillary renal cell carcinomas. This frequency can be detected by PCR-based analysis unequivocally if microdissected material is used.

Tumor-specific LOH in the *APC* gene on chromosome 5q21 occurred in 5 of 12 (42%) informative patients. This is well above the backgound LOH frequency in renal cell cancer of up to 10% reported for most polymorphic sites irrespective of their location in the genome or the function of the related genes [13, 19]. Relevant tumor suppressor genes are characterized by high LOH frequencies. LOH of the *VHL* tumor suppressor gene, for instance, was detected in 16 of 19 (84%) informative non-papillary renal cell carcinomas [17]. The observed frequency of LOH in the *APC* tumor suppressor gene may, therefore, either mean that inactivation of the *APC* gene contributes to the malignant transformation of a subset of nonpapillary renal cell carcinomas, or it may indicate that a tumor suppressor gene relevant for the genesis of nonpapillary renal cell carcinoma is located in the vicinity of the *APC* gene in chromosomal region 5q21.

Renal cell carcinomas are notorious for their genetic heterogeneity. Here, only those tumors that showed LOH in all tumor islets tested were regarded to harbor an allelic deletion. In these five tumors no sign of heterogeneity was detected in the tissue blocks from which the tumor islets were prepared. Samples from other areas of the tumors were not procured for this study, and it remains speculative whether other areas of the tumors, in particular of the T3 and T4 tumors, would also show LOH or not. In tumor 6 one of six islets investigated showed LOH, whereas the other five did not. This may reflect genetic heterogeneity in the *APC* gene in tumor 6. The tumor was, however, considered heterozygous in order not to overestimate the LOH frequency (Table 1).

LOH did not correlate significantly with tumor grade ($P = 0.63$) or stage ($P = 0.44$) in this study. This could mean that in the genesis of some nonpapillary renal cell carcinomas allelic deletion in the *APC* gene is an early step, a phenomenon that has been shown in patients with colorectal carcinoma [16, 20]. It could also mean that allelic deletion in the *APC* gene occurs independent of tumor grade and stage and that the accumulation of a certain number of genetic alterations rather than a specific sequence of steps of genetic alterations renders a normal kidney cell malignant and invasive. However, the number of patients of this study is small and a correlation may thus have been missed for statistical reasons. Others [14, 22] found that LOH on chromosome 5q appears to occur more frequently in grade 2 and 3 clear cell renal tumors than in grade 1 tumors, although this was also not significant. Whether LOH in the *APC* gene bears prognostic relevance in renal cell carcinomas, and if yes whether this is independent of tumor stage and grade, remains to be shown in a larger study.

Twelve of 29 (41%) patients investigated were informative for a polymorphic site in exon 11 in the *APC* gene. This rate of heterozygosity is in the range reported for other patient cohorts (7 of 14 [5], 36 of 71 [9], or 7 of 14 [24]) and suggests that the cohort of this study is not biased.

The frequency of LOH reported here is high compared with other studies on genomic alterations on chromosome 5q in nonpapillary renal cell carcinoma using cytogenetic methods [11, 12, 22] or LOH assays [4, 7, 13, 14, 18, 19]. Cytogenetic analysis implicates chromosome instability at chromosome 5q with a net partial trisomy of chromosome 5q22-qter (reviewed in [11]). Van den Berg et al. [22] reported numerical gain of chromosome 5 in 9 of 79 patients with clear-cell renal cell carcinoma and structural aberrations on chromosome 5q in 18 of 79 patients, the breakpoints being situated in region 5q13-q31. Moch et al. [12] found gains on chromosome 5q in 20% of 46 nonpapillary

renal cell carcinomas using comparative genomic hybridization. Cytogenetic analysis including comparative genomic hybridization has a resolution down to about several megabases. Tumor-specific allelic deletions in relevant tumor suppressor genes, however, can be much smaller [1]. In particular adenocarcinomas may be characterized by smaller deletions than other types of carcinomas, as has been shown for lung carcinomas [26, 27]. Cytogenetic methods may, therefore, not be sensitive enough to detect deletions that affect only a single or few genes.

Chromosomal region 5q21 seems to be an unstable region that is frequently affected and relevant in the tumorigenesis of non-papillary renal cell carcinomas. This manifests on the one hand as allelic deletion in this region as is described here. It may on the other hand lead to malsegregation of chromosome 5 or parts of it during mitosis, thus leading to cytogenetically detectable numerical or partial chromosomal gains and losses. Due to selection during subsequent cell divisions in a growing tumor the cells harboring numerical or partial gains of chromosome 5 may prevail as has been described [11, 22]. Van den Berg et al. [22] not only detected numerical gains of chromosome 5 but also structural abnormalities of chromosome 5q, which occurred twice as frequently. This supports the hypothesis of coincident allelic deletion and cytogenetically detectable abnormalities on chromosome 5 in nonpapillary renal cell carcinoma. The present report, therefore, does not conflict with the respective cytogenetic studies [11, 12, 22].

In this study, the role of the *APC* gene in tumorigenesis was assessed more precisely by using intragenic LOH analysis in exon 11 of the *APC* gene. In contrast, many highly polymorphic microsatellite markers are located in extragenic polymorphic repeat sequences [4, 7, 19] at a more or less well defined distance from the *APC* gene. LOH frequencies can drop sharply at sites distant from the relevant tumor suppressor gene [26]. This may in part explain why these groups did find low LOH frequencies on chromosome 5q21, and why two other groups that used extragenic restriction fragment length polymorphism (RFLP) markers found no LOH [11] or lower LOH frequencies [13, 14] than reported in this study.

The use of microdissection greatly facilitated this study. The simple and inexpensive microdissection method used combines the advantage of a high delineation accuracy with the possibility of assessing the purity of the excised tumor cells *after* the excision. If DNA from more than about 20% nontumor cells is present in the PCR reaction, the detection of LOH is obscured (Fig. 1) due to the nonlinear amplification kinetics of the PCR. This may explain why a group who did not employ a microdissection method did not find increased LOH (2 of 14 tumors investigated) at exon 11 of the *APC* gene [18]. This emphasizes the importance of verifying the origin of the DNA from tumor tissue before introducing it into subsequent PCR, in particular when looking for LOH.

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