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ORIGINAL ARTICLE

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Loss of heterozygosity and methylation of p16 in renal cell carcinoma

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Abstract To investigate the possible role of genomic aberrations of chromosome 9p21 in the tumorigenesis of human renal cell carcinoma (RCC), 40 sporadic RCCs were studied using PCR analyses. The tumours were predominantly low stage and low grade. Loss of heterozygosity (LOH) was observed in nine of 39 informative cases, but no homozygous deletion was noticed. Hypermethylation of the promoter region of p16 occurred in eight of the 40 RCCs. No correlation was found between hypermethylation of the p16 gene and LOH on 9p21. A similar level of LOH and methylation was observed in the 40 RCCS regardless of histology, grade and stage. These results suggest that inactivation of p16 and the possibility of other unknown tumour suppresser genes located on other chromosomes could be involved in the pathogenesis of RCC.

Keywords Loss of heterozygosity · Methylation · p16 gene · Renal cell carcinoma

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Introduction

Loss of chromosome 3p occurs frequently in RCCs and represents the most common genetic event described in this cancer [20]. Because sporadic adult cancers are known to arise from multiple genetic events, it is likely that other suppresser loci important in renal cell carcinogenesis remain to be discovered.

Allelotype and deletion mapping studies have identified the loss of chromosomes 5q, 6q, 10q, 13q and 17p in approximately 15–30% of sporadic RCCs [6, 20]. Recently, p16, a candidate tumour suppresser gene (TSG), was mapped to 9p21 [14]. Intensive study of the gene and its product has revealed frequent alterations that inactivate the function of this gene through at least three mechanisms: mutation, homozygous deletion, and methylation of the promoter region [16]. p16 is an inhibitor of the cyclin D/CDK4 and cyclin D/CDK6 kinase complexes. The inactivation of p16 may allow efficient phosphorylation of pRb by increasing kinase activity of these cyclin D/CDK complexes and promote cells into S phase by releasing E2F transcription factor. Microsatellite analysis with markers close to the p16 gene revealed that small homozygous deletions, unlike mutations, represent a major mechanism of p16 inactivation. Homozygous deletions of p16 have been found in primary bladder carcinoma, glioma, mesothelioma, T-cell acute lymphoblastic leukaemia, melanoma, prostate carcinoma, sarcoma, ovarian and renal cell carcinomas [2, 4, 10, 13, 17, 21, 22]. However, homozygous deletions or intragenic mutations were detected infrequently in primary cancers with loss of heterozygosity (LOH) at 9p21 [3, 19]. This controversy suggests that: (1) a second TSG resides at this locus, or (2) this gene is inactivated by some alternative means in most tumour types. In recent studies, methylation of the 5′CpG island of p16 was identified and precisely associated with complete transcriptional block and inactivation in head and neck, lung, brain, breast, colon, oesophageal and bladder cancers [5 ,8, 12, 18, 19, 23].

To study the LOH and methylation status of the p16 gene and its correlation with clinical and pathological data, we screened 40 sporadic RCCs using PCR-based techniques.

Materials and methods

Paired, normal tumour samples were collected from 40 patients with primary, non-familiar RCC who underwent radical nephrectomy at San Carlos Hospital between 1993–1998. After collection, they were frozen immediately. Representative sections from cancer samples were stained with haematoxylin and eosin to establish tumour type according to the World Health Organisation classification and the grade of differentiation according to the Fuhrman classification [7]. All normal tissue was confirmed to be free of tumour.

DNA extraction

DNA from tissues was extracted using the phenol/chloroform method. Only tumour samples containing $< 30\%$ normal cells were analysed.

LOH analysis

A total of 50 ng genomic DNA from normal and tumour samples was amplified using PCR. Primers were obtained from Progenetic (Table 1). DNA amplification was done following Kim's conditions [15]. Control reactions were done in parallel with each PCR test. LOH was defined as a $> 50\%$ reduction of the intensity by visual inspection in either of the two alleles as compared with those in normal control panels.

Methylation analysis

A total of 1 μg tumor DNA was denatured at alkaline pH and high temperature in 50 μl reactions and treated with 1 ml 3 M sodium bisulphite and 12.5 μl 40 mM hydroquinone. Samples were incubated at 50°C for 16 h. Sodium bisulphite converts unmethylated cytosines to uracils and leaves methylated cytosines unchanged. Uracil is replicated as thymine during PCR, allowing for methylation analysis by designing primers that preferentially anneal to sequences containing either methylated (CpG) or unmethylated (TpG) sites. Bisulphite-converted DNA was purified using the Wizard's resin filter method (Promega). The samples were incubated at 37°C for 15 min with 0.3 NaOH in 50 μl reactions and again purified on DNA QIAamp columns (Quiagen).

All PCR products were separated on 8% polyacrylamide-TBE 0.5% gel using a leader (PhiX 174/Hae III Markers, Promega) as a size marker. Gels were fixed with acetic acid-ethanol and visualised with 0.1% silver staining.

Statistical analysis

To ssses methylation and LOH at p16, we analysed normal and cancerous tissue from 40 patients with RCC. The statistical significance was determined by a χ^2, Fisher's exact test or Student's *t*-test. Relative risks and their confidence intervals were calculated with the use of the estimated regression coefficients and their standard errors. A *P*-value < 0.05 was considered significant. Analysis was performed using SPSS 8 statistical software.

Results

Since the initial reports of homozygous deletion of chromosome 9p21 in human cancer cell lines, numerous studies have shown varying, but in general much less frequent, abnormalities of p16 in primary tumours of these cancers [3, 21]. Analysis of the region that encodes for p16 by deletion mapping and SSCP analysis has not identified which specific marker of the many used till now correlates exactly with p16. LOH occurs predominately between IFNα and D9S171 markers in most tumour types: this small region, 4 cM long, contains at least four TSGs: p16, p15, p16β and MTAP. Marker D9S1747 is located about 150 kb telomeric to p16 and LOH was found most frequently at D9S1747 in a series of 46 small cell lung cancers [15]. Indeed, these authors chose this marker for the determination of the homozygous deletion of p16. Because this region has been studied in detail, we screened 40 RCCs for LOH with the D9S1747 marker; the tumours were predominantly low stage. Detailed information on patient and tumour characteristics is given in Table 2. Nine of the 39 informative cases (23.1%) showed partial deletion of 9p21. No homozygous deletion was noticed (Table 3). A similar level of LOH was observed in the 40 RCCs regardless of histology, grade or stage. The frequency of chromosome 9 loss rivals that reported previously for any chromosome other than 3p in RCC [20, 24]. 9p21–22 LOH in renal cancer was first described by Cairns et al. [1]: 11/42 RCCs showed LOH, but only two homozygous deletions were observed. According to the results described above, 9p21 allele loss in RCC is not a frequent event; it is possible that 23.1% is an underestimation, however tumour tissues in our experiments contained a minimal amount of normal cells. It is also possible that other TSG in the vicinity of the lost allele (rather than p16 or p15 at the 9p21 region) may be involved in RCC development as suggested by others [1].

Table 1 PCR primers used for LOH and methylation analyses on 9p21. p16-W: wild DNA primer, p16-M and p16-M2 modified and methylated DNA primers, p16-U and p16-U2: modified and unmethylated DNA primers. Differences between modified methylated and unmethylated DNA primers are underlined

Locus	Primers: sense 5′ → 3′	Primers: antisense 5′ → 3′	Annealling temperature (°C)	Size (bp)
D9S1747	ATTCAACGAGTGGGATGAAG	TCCAGGTTGCTGCAAATGCC	55	150
p16-W	CAGAGGGTGGGGCGGACCGC	CGGGCCGCGGCCGTGG	65	140
p16-M	TTATTAGAGGGTGGGG*C*GGAT*C*G*C*	*G*ACCCC*G*AACC*G*C*G*ACC*G*TAA	65	150
p16-U	TTATTAGAGGGTGGGG*T*GGAT*T*G*T*	C*A*ACCCC*A*AACC*A*C*A*ACC*A*TAA	60	151
p16-M2	TTATTAGAGGGTGGGG*C*GGAT*C*G*C*	CCACCTAAATC*G*ACCTCC*G*ACC*G*	65	234
p16-U2	TTATTAGAGGGTGGGG*T*GGAT*T*G*T*	CCACCTAAATC*A*ACCTCC*A*ACC*A*	60	234

Table 2 9p21 LOH and methylation status according to histopathological features

	Informative cases/total patients	9p21LOH/ informative cases	p16 methylation/ informative cases	LOH and methylation/ informative cases
Tumour size				
T1	23/40	6/22	4/23	10/23
T2	2/40	0/2	1/2	1/2
T3	15/40	3/15	3/15	5/15
Nodal status				
N0	37/40	9/36	8/37	16/37
N1	3/40	0/3	0/3	0/3
Distant metastases	1/40	0/1	0/1	0/1
TNM stage				
I	23/40	6/22	4/23	10/23
II	1/40	0/1	1/1	1/1
III	15/40	3/15	3/15	5/15
IV	1/40	0/1	0/1	0/1
Histological type				
Clear cell carcinoma	27/40	7/26	5/27	11/27
Papillary carcinoma	4/40	1/4	0/4	1/4
Chromophobe carcinoma	4/40	0/4	2/4	2/4
Unclassified carcinoma	1/40	0/1	0/1	0/1
Collecting duct carcinoma	4/40	1/4	1/4	2/4
Fuhrman grade				
Histological grade I	9/40	2/9	3/9	5/9
Histological grade II	21/40	5/20	3/21	7/21
Histological grade III	9/40	2/9	2/9	4/9
Histological grade IV	1/40	0/1	0/1	0/1

Table 3 Allele loss and methylation of 9p21 in 40 sporadic RCCs. ■: LOH; ❑: retention of heterozygosity; ☆: instability; ⊙ non informative; ●: methylated; ❍: unmethylated. *p16met = p16 methylation

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
D9S1747	❑	■	❑	❑	■	■	❑	❑	❑	❑	❑	☆	❑	❑	■	❑	❑	❑	■	❑	❑	❑	❑	❑	■	❑	■	❑	❑	❑	❑	■	⊙	❑	❑	❑	■	❑	❑	❑
p16 met*	●	❍	❍	❍	●	❍	❍	❍	❍	❍	●	❍	❍	❍	❍	❍	●	❍	❍	❍	❍	❍	❍	❍	❍	●	❍	●	❍	❍	❍	❍	❍	❍	❍	●	❍	●	❍	❍

Discussion

Given that there is no evidence for a dominant-negative mutation of p16, it must be inactivated in other ways. Hypermethylation of the promoter regions of the p16 gene has been shown to be one of the major mechanisms for the inactivation of this TSG in human cancers [19]: p16 can be silenced by the methylation of either one or both alleles of the gene, as demonstrated for the VHL gene in human RCC [12]. Different methylation assays have been investigated yielding concordant results in primary melanomas [9]. Bisulphite modification of DNA has made it possible to use PCR-based methods for the accurate detection of methylated DNA molecules present in tumour samples [11]. PCR-based approaches have several obvious advantages, including efficiency, ease, and a requirement for only a small amount of material. Our current study, the first to analyse methylation of p16, revealed methylation in eight RCCs (20%). This is in line with 19% methylation of the VHL gene in RCCs previously described [12]. However, the frequency of this event is not high (20%) and contrasts with those from other tumour types (e.g. breast, colon and bladder cancers) where the potential contribution of methylation-associated gene silencing of p16 has been reported to be between 31–67% [8, 12]. This is in line with the finding that hypermethylation of p16 is a selective phenomenon depending on the tissue of origin. There was no significant relationship between methylation status and histological type, grade or stage, although none of the four papillary tumours was methylated. No correlation was found between hypermethylation of either one or both alleles of the p16 gene and allelic loss on chromosome 9p21: only one RCC showed inactivation of both copies of p16 via LOH and methylation in our study, which suggests that p16 inactivation is not a major event in sporadic RCC development and progression. However, these results, together with the fact that renal cancer usually occurs in adulthood even in individuals that inherit a mutant VHL gene, suggest that alterations of TSGs on other chromosomes are of considerable significance in the aetiology of malignant renal disease.

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