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Analysis of genetic alterations in renal cell carcinoma using the polymerase chain reaction

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Abstract Very frequent loss of heterozygosity (LOH) on chromosome 3p has been found in human renal cell carcinoma (RCC). In the present study, we examined LOH at the retinoblastoma (RB), mutated in colorectal cancer (MCC) and adenomatous polyposis coli (APC) tumour suppressor genes loci, and mutations of the H-, K-, and N-ras oncogenes. We performed these studies using the polymerase chain reaction (PCR) method followed by restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) analyses. LOH was detected in 2 of 11 (18.2%), and 2 of 14 (14.3%) informative cases at the MCC and APC loci, respectively, and in none of 15 informative cases at the RB locus in 25 RCCs. LOH at the MCC was accompanied by LOH at the APC locus in two RCCs. No mutation was detected in H-, K-, and N-ras genes in 39 RCCs. Thus, alterations of the known tumour suppressor genes and the ras oncogenes were infrequent events in RCC. The results suggest that the genetic pathway in the genesis of RCC differs considerably from that of other common human carcinomas.

Key words Tumour suppressor genes · ras oncogene
Renal cell carcinoma · Polymerase chain reaction

Introduction

Several genetic alterations which accumulate commonly in human carcinomas support the hypothesis of multistep carcinogenesis [41]. Frequent loss of heterozygosity (LOH) on chromosome 3p in renal cell carcinoma (RCC) has been reported genetically and cytogenetically [17, 23, 40, 42, 43]. Aberrations of the retinoblastoma tumour suppressor gene (RB), which is located on chromosome 13q, play a significant role in the genesis of retinoblastoma, osteosarcoma and soft tissue sarcoma as well as breast and hepatocellular carcinomas [8, 19, 25]. LOH on chromosome 5q, where the mutated in colorectal cancer (MCC) and adenomatous polyposis coli (APC) genes are mapped [3, 15, 20], has been frequently detected in human colorectal, oesophageal, gastric, lung, and hepatocellular carcinomas [5, 6, 7, 21, 38]. These observat ions suggest that these genes play a significant role as tumour suppressors in the genesis of diverse human carcinomas, similar to the p53 gene as previously described [12, 28]. Mutations of the ras oncogenes have also been reported to play important roles in carcinomas of colorectum, pancreas, lung and endometrium [2, 4, 22, 33].

To clarify the role of genetic alterations, which are common in human malignancies in RCC, we have investigated LOH at RB, MCC and APC genetic loci and searched for mutations of the ras oncogenes using the polymerase chain reaction (PCR) [4].

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Materials and methods

A total of 39 carcinoma tissues were obtained surgically from patients with RCC at Iwate Medical University Hospital or its affiliated clinics between May 1991 and March 1993. In 25 patients, corresponding normal tissues were also obtained. The patients included 27 men and 12 women (average age, 54 years at diagnosis) with RCC. The histological classification, stage and grade are summarized in Table 1. Classification was determined according to the criteria of the tumour-node-metastasis (TNM) system of malignant tumours of UICC [11] and the Robson staging protocol [31]. Specimens were frozen immediately after resection and stored at −80° C. DNA was extracted from these tissues by the phenol/chloroform method [35].

In the polymerase chain reaction

Oligonucleotide primers for RB, MCC, APC and ras genes were synthesized by the phosphoramidite method using a DNA synthesizer (MilliGen/Biosearch, Division of Millipore, Mass., USA) and purified on Oligo-pak columns (MilliGen/Biosearch, Division

Table 1 Microscopic findings in 39 renal cell carcinomas

Classification	No. of Patients
Histological classification	
Clear cell carcinoma	26 (66.6%)
Granular cell carcinoma	4 (10.3%)
Mixed clear and granular cell carcinoma	6 (15.4%)
Spindle cell carcinoma	2 (5.1%)
Pleomorphic carcinoma	1 (2.6%)
Histological grade	
1	20 (51.3%)
2	18 (46.1%)
3	1 (2.6%)
Robson stage	
I	22 (56.4%)
II	5 (12.8%)
III	4 (10.3%)
IV	8 (20.5%)

Table 2 Primer sets used in polymerase chain reaction (PCR)

Priming region	Primer sequence
RB intron 25	5 -ATCGAAAGCATCATAGTTAC-3
	5 -TAACGAAAAGACTTCTTGCA-3'
MCC exon 10	5 -TACGAATCCAATGCCACA-3
	5 -CTGAAGTAGCTCCAAACA-3'
APC exon 11	5 -GGACTACAGGCCATTGCAGAA-3
	5 -GGCTACATCTCCAAAAGTCAA-3
APC[a] exon 15	5 -AAGACTTATTGTGTAGAAGATAG-3
(codons 1260–1410)	5 -ATGGTTCACTCTGAACGGA-3
exon 15	5 -TCTGTCAGTTCACTTGATAG-3
(codons 1389–1547)	5 -CATTTGATTCTTTAGGCTGC-3
H-*ras* exon 1	5 -CAGGCCCCTGAGGAGCGATG-3
	5 -TTCGTCCACAAAATGGTTCT-3
exon 2	5 -TCCTGCAGGATTCCTACCGG-3
	5 -GGTTCACCTGTACTGGTGGA-3
K-*ras* exon 1	5 -GGCCTGCTGAAAATGACTGA-3
	5 -GTCCTGCACCAGTAATATGC-3
exon 2	5 -TTCCTACAGGAAGCAAGTAG-3
	5 -CACAAAGAAAGCCCTCCCCA-3
N-*ras* exon 1	5 -GACTGAGTACAAACTGGTGG-3
	5 -GGGCCTCACCTCTATGGTG-3
exon 2	5 -GGTGAAACCTGTTTGTTGGA-3
	5 -ATACACAGAGGAAGCCTTCG-3

[a] The primer sets were used to detect mutations of the APC gene by PCR – single-strand conformation polymorphism (SSCP) analysis

of Millipore). Intron 25 of the RB contained a nucleotide polymorphism, exon 10 of the MCC contained a variable insertion polymorphism, and exon 11 of the APC contained a Rsa I restriction fragment length polymorphism and exons 1 and 2 of H-, K-, N-ras genes were amplified by PCR. The sequence of each primer used for PCR is shown in Table 2. Genomic DNA (100 ng) was amplified in a total volume of 20 μ l in the buffer recommended by Perkin-Elmer Cetus which contained 1mM magnesium chloride. One microlitre of [α ^{32}P] dCTP (3000 Ci/mmol, 10 Ci/ml) was added for single-strand conformation polymorphism (SSCP) analysis. Thirty-five cycles, each consisting of denaturation at 94° C for 40 s, annealing at 55° C for 40 s and extension at 72° C for 1 min, were performed with a temperature cycler (Hybaid, Middlessex, UK).

LOH at the APC and MCC genes were analysed by restriction fragment length polymorphism (RFLP). To analyse the APC gene, PCR products were digested with 3 μ l (10 U/μ l) of Rsa I (Toyobo, Osaka, Japan) at 37° C for 12 h. For the MCC gene, no endonuclease digestion was necessary. Each product was then electrophoresed on 3% NuSieve/1% Seakem agarose gel (FMC Bioproducts, Rockland, Me., USA), and stained with ethidium bromide. LOH was defined as disappearance or reduction in intensity of one band for the MCC gene, and one or two bands corresponding to one allele for the APC gene.

LOH at RB and mutations of the ras genes were analysed by SSCP [30]. A volume of 2 μ l of the PCR product was diluted 100-fold with a sequencing gel-loading buffer (98% deionized formamide, 10 mM EDTA pH 8.0, 0.025% xylene cyanol, 0.025% bromophenol blue), and then applied (1 μ l/lane) to a 6% neutral polyacrylamide gel. Electrophoresis was performed at 25–40 W for 1–6 h at 4° C. The gel was dried and exposed to Kodak XAR film (Rochester, N.Y., USA) at –80° C for 6–12 h. We also examined mutations in the "mutation cluster region (MCR)" of the APC gene in two cases in which LOH at APC and MCC genetic loci were detected. The primer sets used for this analysis are shown in Table 2.

Results

Tumour characteristics and analyses of LOH at the RB, MCC and APC gene loci in 25 primary renal cell carcinomas were summarized in Table 3. Fifteen of 25 (60.0%) cases were heterozygous (informative) at the RB locus. LOH at the RB gene locus was detected in none of 15 informative cases using PCR-SSCP analysis (Fig. 1). LOH at MCC and APC loci was found in 2 of 11 (18.2%) and in 2 of 14 (14.3%) informative cases, respectively (Fig. 2). Although two cases (Cases 2 and 19) with MCC and APC-LOH were diagnosed histologically as clear cell subtype and garde 1, no significant correla tion was observed between LOH of these genes and any clinicopathological characteristic. In these two cases (Cases 2 and 19), we performed PCR-SSCP analysis, but detected no mutation in the "mutation cluster region" of the APC gene as no mobility shift was detected.

No mutation of an H-, K- or N-ras gene was detected in any of 39 RCCs (Fig. 3). Incidences of the genetic alterations detected in the present study are shown in Table 4.

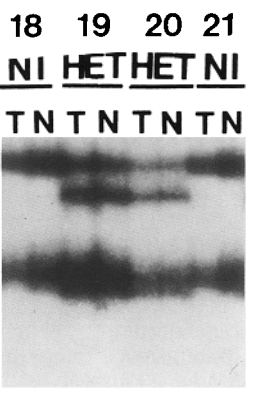

Fig. 1 PCR-SSCP analysis of intron 25 of the RB gene (*NI* not informative; *HET* heterozygosity. *T* tumour DNA; *N* normal DNA)

Table 3 Results of loss of heterozygosity (LOH) analysis at RB, MCC and APC loci in 25 renal cell carcinomas (RCC)

Case	Sex	Age (years)	Stage	Histological		Polymorphic sites		
				type[a]	grade[b]	RB	MCC	APC
						intron 25	exon 10	exon 11
1	M[c]	57	I	m[e]	G1	HET[g]	NI	NI
2	M	63	II	c[f]	G1	NI[h]	LOH[i]	LOH
3	M	57	I	m	G1	HET	HET	HET
4	M	73	I	c	G2	NI	NI	NI
5	M	87	III	m	G2	NI	HET	HET
6	F[d]	57	I	c	G2	HET	NI	NI
7	M	65	I	c	G1	HET	NI	NI
8	M	66	II	m	G2	NI	NI	HET
9	M	79	I	c	G1	NI	HET	NI
10	M	47	I	c	G1	HET	HET	HET
11	M	55	II	c	G1	NI	NI	NI
12	M	47	IV	c	G1	NI	NI	HET
13	F	80	I	c	G2	HET	HET	NI
14	M	61	III	c	G1	HET	HET	HET
15	F	44	I	c	G1	HET	HET	NI
16	F	32	I	c	G1	NI	NI	NI
17	F	43	IV	c	G2	HET	HET	NI
18	M	40	I	m	G1	NI	NI	HET
19	M	56	II	c	G1	HET	LOH	LOH
20	M	84	IV	c	G1	HET	HET	HET
21	M	55	III	m	G2	NI	NI	NI
22	F	63	I	c	G1	HET	NI	HET
23	F	57	IV	c	G2	HET	NI	HET
24	M	72	I	c	G2	HET	NI	HET
25	M	62	II	c	G1	HET	NI	HET

[a,b] Histological type and grade were classified according to the criteria of TNM system of malignant tumors of the UICC [11]
[c] Male
[d] Female
[e] Mixed clear and granular cells subtype
[f] Clear cell subtype
[g] Heterozygosity
[h] Not informative
[i] Loss of heterozygosity

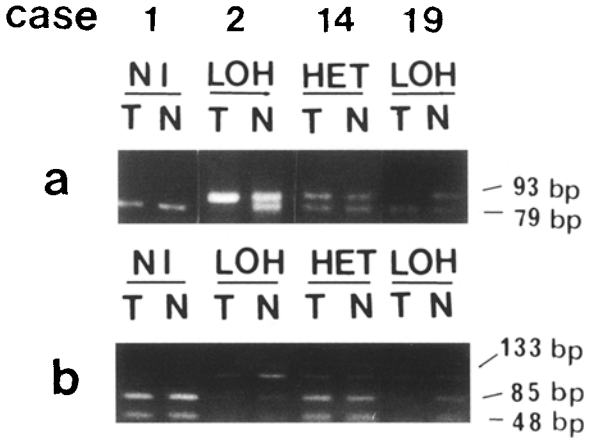

Fig. 2 PCR-restriction fragment length polymorphism (RFLP) analysis of MCC (**a**) and APC (**b**) genes. Two cases (No. 2 and 19) exhibited LOH at both genetic loci (*NI* not informative; *HET* heterozygosity; *LOH* loss of the heterozygosity; *T* tumour DNA; *N* normal DNA)

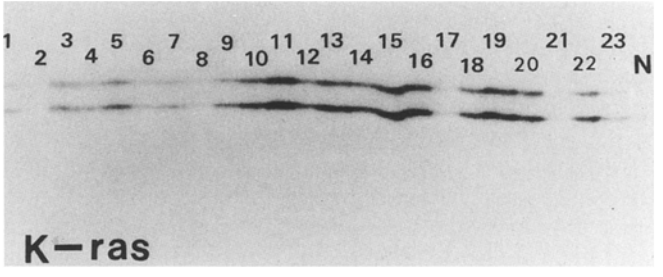

Fig. 3 PCR-SSCP analysis of exon 1 of the K-ras gene in 23 RCCs. No mobility shift was observed. Lanes: 1–23, RCCs; *N* normal DNA

Discussion

Allele typing studies have documented high incidences of deletion on chromosome 3p [23, 42, 43], and relatively frequent (15%–41%) deletions on 5q, 6q, 10q, 13q,

Table 4 Incidence of genetic alterations in RCC

Genetic alteration	Prevalence
RB-LOH	0% (0/15)
MCC-LOH	18.2% (2/11)
APC-LOH	14.3% (2/14)
APC-mutation	0% (0/2[a])
ras-mutation	0% (0/39)

[a] Mutations were analysed in two cases with LOH at the APC gene locus

17p and 19p in human RCC [23, 24]. These results suggested that tumour suppressor genes associated with RCC may exist on these chromosome arms. Although the deletions on chromosomes 3p and 5q were mapped in detail, no tumour suppressor genes associated closely with RCC have been identified [24, 42]. Recently, a candidate tumour suppressor gene of the von Hippel-Lindau disease, which is involved in the development of RCC has been identified at chromosome 3p25-26 [18].

Inactivation of the RB gene [1], located on chromosome 13q, plays an important role in the genesis of not only retinoblastoma but also osteosarcoma, soft tissue sarcoma, as well as breast and hepatocellular carcinomas [8, 19, 25]. However, LOH at the RB gene locus was detected in only 2 of 30 (6.7%) informative cases of RCCs by ordinary Southern blotting analysis [14]. Although the incidence was relatively low, LOH at the RB locus is believed to have a role in the development of certain RCCs [14].

LOH at the MCC and APC genes, located on chromosome 5q, was found frequently in colorectal, oesophageal, gastric, lung, and hepatocellular carcinomas [5, 6, 7, 21, 38]. More recently, frequent mutations of the APC gene were detected in pancreatic carcinoma [13]. In RCCs, the deletion in chromosome 5q includes the L5.71 locus, which is a genomic clone of the MCC gene, was defined as the commonly deleted region in 64 RCCs upon a close examination [24]. However, no mutation of the APC gene were detected among 14 RCCs with LOH at this locus [13]. These findings suggest that the MCC, not APC, gene may be a candidate suppressor gene in RCC.

In this study, LOH was detected at the MCC in 18.2% (2/11 informative cases), at the APC in 14.3% (2/14 informative cases), and was not detected at the RB locus. Analysis of LOH at the RB locus was practical because of the existence of nucleotide polymorphism in intron 25 of the RB gene [25] – we could not detect LOH at the RB locus in any of 15 informative RCCs. Therefore, the RB gene is not the tumour suppressor gene in RCC and other tumour suppressor genes may exist on chromosome 13q as allelic deletion of chromosome 13q was found to occur at a relatively high rate [23]. LOH at the MCC and APC genetic loci were found to occur simultaneously in two cases in the present study. These aberrations have also been found in oesophageal, gastric and lung carcinomas [5, 6, 38]. However, no mutation of the APC gene was detected in the two cases using PCR-SSCP analysis. From our observations and previous reports, the APC gene seems not to be a tumour suppressor in RCC. The MCC gene, however, is one possible candidate. It is also possible that another cellular oncogene such as fms [10] or unknown tumour suppressor gene(s) on chromosome 5q are involved in the development of RCC because chromosome 5q is very often showed translocation between chromosome 3p and other chromosomes in RCC [16].

It has been known that various oncogenes play important roles in human carcinogenesis [36]. Mutations of the ras family of oncogenes have been detected frequently in colorectal and lung carcinomas [4, 33]. In particular, mutations of the K-ras gene occur in more than 90% of pancreatic carcinomas [2]. A previous report indicated that genetic lesions affecting ras genes do occur in RCC [9]. However, a mutated H-ras gene was found in one of 51 (2%) primary and metastatic renal cell carcinomas, including three oncocytomas [27]. No mutation of H-, K- or N-ras genes was detected in 16 RCCs by dot blot hybridization assays [26]. It is possible that the absence of mutations of ras oncogenes can be attributed to the fact that we did not analyse enough high grade RCCs, however, no mutations of ras oncogenes in 20 grade 3 RCCs were reported previously [32]. In addition, cytogenetic aberrations of chromosomes 1, 11 and 12, where the N-, H- and K-ras genes are located, have been reported to be infrequent [16]. From these reports and our present results, mutations of the ras family genes occur as infrequent events, and do not play an important role of carcinogenesis in human RCC.

Mutations of the p53 gene, located on chromosome 17p, which are frequent in various human carcinomas, was found in only one of 23 RCCs in our previous study [37]. The rarity of mutations of the p53 gene in RCC [29, 39] suggest that it does not have a significant role in the genesis of RCC.

In conclusion, our study suggests that alterations of the RB, MCC and APC tumour suppressor genes as well as the ras oncogenes, are infrequent events in RCC. Other tumour suppressor genes and oncogenes should play a key role in the genesis of RCC, and the genetic mechanism in the genesis of RCC may be considerably different from that of other common human carcinomas.

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