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MICROSATELLITE ALTERATION AND ITS CHARACTERISTICS IN COLORECTAL CARCINOMA*

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Abstract: Objective: To determine the role of microsatellite alterations in carcinogenesis of colorectal carcinoma (CRC). Methods: Alterations of 10 microsatellite loci from 5 different chromosomes were detected in 92 colorectal cancers and their paired normal mucosa by PCR, denatured polyacrylamide gel electrophoresis and silver staining. Associations of microsatellite alterations with clinopathologic parameters were statistically clarified. Results: Alterations of microsatellite were classified into microsatellite instability type I, type II and loss of heterozygosity (LOH). The carcinoma with $\geqslant 30\%$ loci microsatellite alterations was defined as replication error(RER) positive tumors. Of 92 cases, 14 were RER +. Microsatellite alterations of $P53_{(1)}$ and D18S363 loci (64.29%) was most commonly identified in the RER + tumors. RER + were more commonly seen in poorly differentiated carcinomas and tended to occur in mucoid carcinomas. The type of microsatellite alterations varied in different histological types of CRC. Conclusions: Microsatellite alteration is a common molecular event in CRC. Different microsatellite loci showed various biologic significance. $P53_{(1)}$ and D18S363 should be essentially detected loci that can show the RER status of tumors.

Key words: microsatellite instability, replication error, colorectal carcinoma
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INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common malignancies whose incidence increases year by year in China (Fan et al., 1993). The molecular genetics of CRC is among the best understood of common human cancers. The development of colorectal carcinoma involves a multistage process in which a number of oncogenes and tumor suppressor genes play a role. Genetic instability, microsatellite instability (MSI) and chromosomal instability, are the molecular characteristics of colorectal carcinoma. MSI, which occurs in 90% of hereditary nonpolyposis colorectal cancer (HNPCC) and 15% - 20% of sporadic colorectal carcinomas, is the primary characteristic of the mutator phenotype in tumors constituting HNPCC. MSI results from defects in DNA mismatch repair leading to nucleotide additions or deletions in simple repeated sequences known as microsatellite alterations throughout the human genome. MSI, as the new mechanism of carcinogenesis, is a hot spot in the field of tumor genetics. MSI and the genetic defect responsible for this phenotype in CRC in China has yet to be clearly pinpointed (Gao et al., 1998; Zhang et al., 1999; Xu et al., 1998). In this study, 10 microsatellite loci were analyzed in 92 colorectal carcinoma samples to determine the type of microsatellite alterations and to explore their association with a number of clinical and pathological features.

MATERIALS AND METHODS

Materials

Fresh samples of colorectal carcinomas were obtained from 92 patients during surgery at the first and the second Affiliated Hospital of Zhejiang Medical University and Hangzhou Railway Hospital from October, 1995, to October, 1997. Fresh paired normal colorectal tissues

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were also obtained. The tissue was then stored in liquid nitrogen. Among 92 cases of colorectal carcinomas (36 cases of colonic cancer and 56 cases of rectal cancer), 59 were males and 33 females. The mean age was 58 years (28 - 79).

METHODS

1. Microsatellite analysis

Extraction of genomic DNA was by standard phenol/chloroform extraction and precipitation with precold ethanol. The occurrence of MSI and LOH (Loss of heterozygosity) was studied at 10 loci from 5 chromosomes. The primer sequences are given in Table 1. The primers of $P16_{(1)}$ and $P16_{(2)}$ were devised by ourselves. $P16_{(1)}$ was a dinucleotide repeat sequence, TA_{23}. $P16_{(2)}$ was a mononucleotide repeat sequence, A_{22}. Other PCR primer sets were synthesized according to the published sequences. Polymerase chain reactions(PCRs) were performed in a 25 μl reaction volume containing 2.5 μl 10 × reaction buffer (Promega), 10 - 200 ng of gemome DNA, 4 × dNTPs 200 μ mol/L, different primers(ANK_1 50 pmol, NF1 30 pmol, $APC_{(1)}$, $P16_{(2)}$ and $P53_{(1)}$ 10 - 15 pmol, D18S46 and D9S162 20 pmol, D18S363 40 pmol, D9S171 and $P16_{(1)}$ 25 pmol), 1.5 mmol/L magenesium chloride (D9S162 2 mmol/L, $P16_{(1)}$ 2.5 mmol/L), and 0.75 μ Taq DNA polymerase(Promega)($P16_{(2)}$ 1 μ). This was overlaid with mineral oil. The target DNA sequences were amplified in an MJ Research Thermal Controller by touch down program ($P53_{(1)}$, $APC_{(1)}$ and $P16_{(2)}$) and three temperature procedures(other loci). The annealing temperatures are shown in Table 1. The cycle number was optimised for each microsatellite locus to ensure that the PCR products were detectable and specific.

Table 1 Microsatellite loci, primer sequences and annealing temperatures*

Loci	Chromosome	Sequence	Annealing temperature(℃)
ANK_1	8p11.1 - 21.1	5'TCC CAG ATC GCT CTA CAT GA 3' 5'CAC AGC TTC AGA AGT CAC AG 3'	55
$APC_{(1)}$	5q21	5'GTA AGC AGG ACA AGA TGA CAG 3' 5'GCT ATT CTC TCA GGA TCT TG3'	55 ~ 60
$P53_{(1)}$	17p13	5'AGG GAT ACT ATT CAG CCC GAG GTG3' 5'ACT GCC ACT CCT TGC CCC ATTC 3'	56
NF_1	17p11	5'CAG AGC AAG ACC CTG TCT3' 5'CTC CTA ACA TTT ATT AAC CTT A3'	50.5
D18S46	18q21.1	5'GAA TAG CAG GAC CTA TCA AAG AGC3'	53
D18S363		5'CAG ATT AAG TGA AAA CAG CAT ATGTG3' 5'GCT TCA TTC TCT CAC TGG AT 3' 5'TTG GGA ACT GCT CTA CAT TC 3'	
D9S162	9p21 - 22	5'GCA ATG ACC AGT TAA GGT TC3' 5'AAT TCC CAC AAC AAA TCT CC 3'	53
D9S171		5'AGC TAA GTG AAC CTC ATC TCT GTCT3' 5'ACC CTA GCA CTG ATG GTA TAG TCT3'	55 ~ 57
$P16_{(1)}$		5'CTT GCA TAC GCA TGT TTA TAG CA3' 5'TTC AGG CCA CTG CAA ATG CTG TA3'	50
$P16_{(2)}$		5'AGG AGA CTC GCT TGA ACC TG 3' 5'CAC CTC CAG TTA TGA GAA GA 3'	51 ~ 55

* D18S46 and D18S363 were from Research Genetics Inc. USA. ANK_1, $APC_{(1)}$, $P53_{(1)}$ and NF_1 were from Shanghai Institute of Cell Biology. D9S162, D9S171, $P16_{(1)}$ and $P16_{(2)}$ were from Shanghai Sangon.

2. Detection of amplification products

After successful PCR, PCR products were analyzed on 2% agrose gel and separated using 8% denatured polyacrylamide gel and then were stained by silver salt(Gao et al., 1998; Zhang et

al., 1999). Some of the products were detected by PHARMACIA Genephor automatic system. The positive samples were assayed at least twice in order to rule out false positive results

RESULTS

Microsatellite alteration pattern

MSI is regarded as a change in allele size(s) in tumor DNA from the constitutional allele pattern observed in the corresponding normal DNA sample. Tumor without MSI shows band pattern similar to that of paired normal mucosa in denatured polyacrylamide gel (Fig. 1). In our comparisons between tumor and normal DNA samples from the same patient, at least three distinct subtypes of microsatellite alterations were evident.

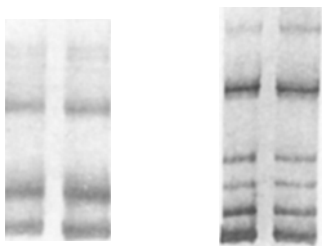

Fig. 1 Microsatellite instability negative (microsatellite stability)

1. Microsatellite Instability type I(MSI type I): The type I includes type Ia and type Ib. Ia (Fig. 2a) shows that novel allele band(s) appears in tumor DNA compared with normal DNA. Type Ib(Fig. 2b) shows the band(s) disappears in tumor DNA compared with normal DNA. In some cases, Ia and Ib coincide in the same case(Fig. 2d).

2. Microsatellite Instability type II(MSI type II): Band shift appears in tumor DNA compared to those of corresponding bands in normal DNA (Fig. 2c).

3. Loss of Heterozygosity(LOH). One allele band disappeared(Fig. 3).

4. Other types of complicated changes including I + II(Fig. 2e), Ib + LOH(Fig. 4), etc.

Detected results in 92 cases in 10 loci are given in Table 2 – 3. From Table 2 and 3, in 917(except for three PCR failures) times of detection, 124 changes occurred, at alteration rate of 13.52%. As to the type of alterations, type Ia occurred most frequently, at rate of up to 30.65%. Next was Ib, at rate of 25.00%. The occurrence frequency of LOH was 19.35%, and of type II was 16.13%. The occurrence frequencies of allele imbalance were: $P53_{(1)}$ (23.91%), D18S363 (19.57%), D18S46 (15.56%), NF_1 (16.48%), $P16_{(2)}$ (15.22%), ANK_1 and $APC_{(1)}$ (13.04%), D9S162 (9.78%), D9S171 (7.61%). There was only one case of LOH ($P16_{(1)}$).

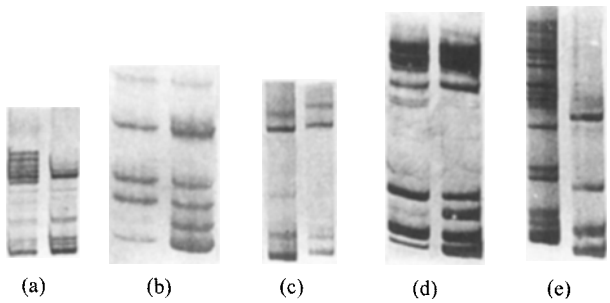

Fig. 2 Microsatellite instability positive
(a) Novel band(s) appears in tumor DNA compared with the corresponding normal DNA;
(b) Band(s) disappears in tumor DNA compared with the corresponding normal DNA;
(c) band shift appears;
(d) both a and b; (e) both a and c.

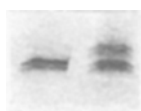

Fig. 3 Loss of heterozygosity

Fig. 4 Microsatellite instability and loss of heterozygosity
Left: tumor tissue; Right: paired normal tissue

Table 2 Microsatellite alterations in total*

	D9S162	D9S171	$P16_{(1)}$	$P16_{(2)}$	ANK_1	$APC_{(1)}$	$P53_{(1)}$	NF_1	D18S46	D18S363	Total
Ia	1	4		2	6	8	5	3	1	8	38
Ib				1	3	2	11	6	5	3	31
II	3			3	1	1	3	2	5	2	20
LOH	4	3	1	6		1	1	2	3	3	24
Ia + Ib					2		1	2		1	6
Ia + II										1	1
Ib + II	1			1			1				3
Ib + LOH				1							1
Total	9	7	1	14	12	12	22	15	14	18	124

* Among 18 cases of mucous adenocarcinoma, one case in NF1 and two in D18S46 showed PCR failures.

Table 3 Comparison of microsatellite alterations in mucous adenocarcinoma with those in other histology types

Histology type	Case number	Detection times	Changes	Microsatellite alteration types							
				Ia	Ib	Ia + Ib	II	Ia + II	Ib + II	LOH	Ib + LOH
Mucous Adenocarcinoma	18	177	28	12	6	2	1	0	1	6	0
Other types	74	740	96	26	25	4	19	1	2	18	1
Total	92	917	124	38	31	6	20	1	3	24	1

Clinicopathological correlation with RER + status

In 92 colorectal cancers, among 10 loci detected, 57.61% (53/92) colorectal carcinomas showed some degree of microsatellite alterations ($\geqslant$1 loci); 32.61% (30/92) of cases showed microsatellite alterations in more than two loci; 15.22% (14/92) of cases showed microsatellite alterations at more than 3 loci; 4 cases showed microsatellite alterations at 4 loci.

RER + tumors were defined as those with microsatellite alterations at more than 30% of the loci . The correlation of clinicopathological parameters with RER + status is shown in Table 4. The RER + rate in mucous adenocarcinomas was 27.78% (5/18), and was 12.16% (9/74) in other histiologic types ($P = 0.098$).

Table 4 Replication error and clinical pathological parameters*

		n	RER +	RER −		P
Total		92	14	78		
Gender	Male	59	11	48		n.s
	Female	33	3	30		
Age	< 40	9	1	8		n.s
	40 −	30	5	25		
	$\geqslant$60	51	8	43		
Size	< 4	29	4	25		n.s
	4 −	50	8	42		
	$\geqslant$8	7	2	5		
Site	Colon	36	8	28		n.s
	Rectum	56	6	50		
Differentiation	High-moderately	72	8	64	$X^2 = 4.33$	$P < 0.05$
	Poorly	20	6	14		
Lymphocyte	Intense	49	10	39		
Infiltration	Moderate	43	4	39		n.s
Dukes	A	18	2	16		
	B	38	7	31		n.s
Stage	C	36	5	31		

* 1. Lacking 2 cases of age data and 6 cases of tumor size data

2. Adenoma-carcinoma transformation, papillary adenocarcinoma and highly differentiated adenocarcinoma are clarified as highly-moderately differentiated group, while poorly differentiated adenocarcinoma, mucous adenocarcinoma, signet − ring cell carcinoma and undifferentiated carcinoma are classified as poorly differentiated carcinoma group.

3. In lymphocyte infiltration item: $X^2 = 4.46$, $P < 0.05$, $X_c^2 = 1.41$, $P > 0.05$

4. The unit of tumor size is cm in diameter.

In 14 RER + cases, the occurrence frequency of alterations in $p53_{(1)}$ and D18S363 was highest, 64.29% (9/14), followed by NF1 (42.86%, 6/14), D18S46, D9S162 and $p16_{(2)}$ (35.71%, 5/14), ANK_1 (28.57%, 4/14), D9S171 and $APC_{(1)}$ (21.43%, 3/14).

DISCUSSIONS

Researchers have paid close attention to the important role of MSI in carcinogenesis and MSI study has become a hot spot in tumor molecular genetics (Lai, 1997). It has been shown that MSI exists not only in colorectal carcinoma, but also in a variety of human tumors (both familial and sporadic), including cancers of the endomatrium, stomach, pancreatobiliary system, ovary, prostate, breast, lung, renal pelvis and ureters (Arzimanoglou et al., 1998). To date, 6 human DNA mismatch repair genes, including $hMSH_2$, $hMSH_6$ (GTBP), $hMSH_3$ (DUG), $hMLH_1$, $hPMS_1$ and $hPMS_2$, have been identified. Mutation in any of the genes may thus cause MSI. Further study showed that some tumors with MSI were not associated with somatic mutations in the mismatch DNA repair genes, which suggests that there may be another mechanism leading to MSI or that other unidentified mismatch repair genes do exist. It is widely accepted that MSI is a marker of genomic instability and a dynamic process that gradually develops in MSI tumors during cell proliferation. An initial inactivation of the mismatch DNA repair system controlling the fidelity of DNA replication results in genomic instability that enhances the random mutation rate and contributes to tumorigenesis (Arzimanoglou et al., 1998).

Our study showed that there were three distinct subtypes in CRC with microsatellite alterations on gel; that is MSI type I, II, and LOH. Few cases had the complicated pattern of the above three types. The mechanisms of different ratio in three types in different loci and their biological meanings were not clear, and are being studied in our laboratory. The occurrence frequency of microsatellite alterations at different loci varied, with that of $P53_{(1)}$ being highest (23.91%), which implies the change of P53 play an important role in colorectal carcinogenesis. The correlation of P53 and mismatch repair system changes needs further clearification.

Definitions of RER + and MSI tumors differ (Thibodeau et al., 1998). Someone defined MSI on equal terms with RER +, but most scientists distinguish MSI from RER +. A tumor with MSI at one locus was once called an MSI tumor; although RER + should be assigned to cases with evidence of microsatellite instability at a certain ratio of loci. There is currently no consensus as to the optimal number and type of microsatellite markers that should be analyzed (Dietmaier et al., 1997; Cawkwell et al., 1995). That is why the rate of RER + ranges from 12% to 40% in sporadic colorectal cancers. The differing rate of RER + may be correlated with loci used, the location of the tumor, and different population. Our result suggested that MSI tumors in more than 30% of loci could suitably be defined as RER + tumors. Especially in colorectal carcinoma, which has genetic instability, the possibility of MSI at one locus increases when the number of detected loci increases (Thibodeau et al., 1998).

Our analysis of the relationship between RER + status and clinopathologic parameters showed that there was a tendency to higher frequency of microsatellite alterations in poorly differentiated tumors ($P < 0.05$). No statistically significant differences were found between RER + status and other parameters. The results implying that replication error is more often seen in poorly differentiated colorectal carcinoma accords with other reports (Arzimanoglou et al., 1998; Aaltonen et al., 1993). RER + occurred more commonly in mucous adenocarcinoma than in other tumor type, but the difference was not statistically significant. Analysis of microsatellite alteration patterns in mucous adenocarcinoma showed that Ia/Ia + Ib were the major alteration types (50%, 14/28), but were less often seen in other types (31.25%, 30/96); and that the occurrence frequency of LOH in each group was similar, 21.43% (6/28) and 18.75% (18/96) respectively. Therefore the molecular genetics in mucous adenocarcinoma is different from that in other histologic type tumors. We will study further the molecular characteristics of mucous adenocarcinoma using microdissection.

This preliminary study showed that microsatellite alterations are common in colorectal carci-

noma in a Chinese population. The tumorigenesis is a process involving multiple gene alterations. The different microsatellite loci may have a different role in the process of carcinogenesis. The etiology and importance of MSI in colorectal carcinoma are unknown and require further investigation.

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