

# Microsatellite instability, MMR gene expression and proliferation kinetics in colorectal cancer with familial predisposition

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## INTRODUCTION

Genetic instability is a common property of many human cancers<sup>[1]</sup>, including those of HNPCC<sup>[2,3]</sup>. A novel form of genetic instability involving somatic alterations, such as deletions and insertions in simple repeated sequences<sup>[4]</sup>, has been found. Microsatellites are relatively short runs of tandemly repeated sequences scattered throughout the genome<sup>[5,6]</sup>. Ubiquitous alterations in these sequences were initially detected by unbiased DNA fingerprinting in a subset of colorectal cancer<sup>[7,8]</sup>, implying the presence of genome-wide genetic instability. Subsequently, amplification by polymerase chain reaction (PCR) of a few microsatellite loci should be used to reveal this microsatellite instability (MSI) in colorectal cancers<sup>[9,10]</sup> and other malignancies<sup>[11-14]</sup>.

MSI was observed to be a common feature of HNPCC<sup>[15]</sup>. Physical mapping and finding of mutations in HNPCC patients revealed that a human homologues of the *Escherichia coli* DNA mismatch repair (MMR) enzyme MutS was one of the candidate genes for HNPCC and was named hMSH2<sup>[16]</sup>. Human homologues of the *E.coli* and yeast mismatch repair enzymes hMLH1, hPMS1 and hPMS2 have also been associated with HNPCC<sup>[17]</sup>. Defects of these mismatch repair genes have been reported to produce MSI in bacteria and yeast, and a high mutation rate of microsatellites has been

observed in HNPCC patients and in cell lines established from HNPCC tumors<sup>[18]</sup>. Therefore, MSI in HNPCC is one of the results of mutations in these mismatch repair genes. In the present study, we analyzed MSI, expression of MMR genes and cell proliferation activity in colorectal cancer (CRC) patients with familial predisposition, to reveal the characteristics of these patients' genetic defects and to afford the biological basis for screening high-risk relatives of CRC.

## MATERIALS AND METHODS

### Patients

Forty-six colorectal cancer patients who underwent surgical resection between 1993 and 1995 at Nanfang Hospital in Guangzhou, China, were analyzed. These included 26 patients with familial predisposition (Group A) by summarizing clinic archives and surveying their pedigrees, and 20 randomly selected colorectal cancer patients without familial predisposition (Group B). In Group A, 4 patients were eligible HNPCC according to the Amsterdam criteria<sup>[15]</sup>.

### PCR

Genomic DNA was extracted from formalin fixed, paraffin embedded tumor tissues and corresponding normal tissues, respectively, using a modification of the method reported previously<sup>[19-21]</sup>. About 100ng of genomic DNA was used for PCR amplification of microsatellite sequences.

### Microsatellite instability

Four microsatellite loci, D2S119, D2S123, D5S107 and D17S250 were analyzed in all patients, using silver staining polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique<sup>[22,23]</sup>. We classified patients as positive for MSI when the PCR product using tumor DNA revealed the presence of extra bands or shifting bands that were not visible in the PCR product of the corresponding normal tissue DNA, and these changes were found in at least two microsatellite loci, called MSI-H<sup>[24,25]</sup>.

### Immunohistochemical staining of hMLH1, hMSH2 and proliferation cell nucleus antigen (PCNA)

hMLH1, hMSH2 enzyme was detected using a rabbit polyclonal antibody, and PCNA, a

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monoclonal antibody (Dako Co). By streptavidin-peroxidase (SP) immunohistochemical method<sup>[26]</sup>, we defined positive cases as those of hMLH1, hMSH2 that showed staining of tissues (tumor or normal) cell nuclei or cytoplasm in clusters<sup>[27]</sup>, and PCNA that showed staining of tumor cell nuclei in clusters<sup>[28,29]</sup>. The label index (LI) of PCNA was analyzed.

#### Flow cytometry for DNA analysis

Using Flow Cytometry (Type of EPICS ELITE), the DNA index (DI), heteroploid rate, proliferation index (PI) and S-phase percentage were assayed in tumor cells. We defined heteroploid cells as that DI was not between 0.90 and 1.10.

#### Statistical analysis

Two-tailed Fisher's and  $\chi^2$  exact tests were used to analyze the significance of differences between the groups. The significance level was set at  $P<0.05$ .

### RESULTS

All patients were detected for MSI in four microsatellite loci (Figure 1). MSI was detected in 20 (76.9%) out of 26 patients in Group A. The MSI-positive (MSI-H) proportion in Group A was 46.2% (12/26), significantly higher than 10% (2/20) in Group B ( $P<0.05$ ). Three of 4 HNPCC patients in Group A were MSI-positive.

We examined the association of MSI with clinicopathologic characteristics of CRC patients with familial predisposition, and elucidated the following features: ① An early age of onset (Table 1). Patients positive for MSI in Group A tended to have a younger mean age at onset than patients in Group B ( $P<0.01$ ) and patients negative for MSI in Group A. ② A preponderance of tumors in the proximal colon (Table 2). Proximal colon includes cecum, ascending and transverse colon<sup>[30]</sup>. Among patients positive for MSI, 9 (64.3%) of 14 tumors were located in the proximal colon. In contrast, among patients negative for MSI, 9 (28.1%) of 32 tumors were located in the proximal colon ( $P<0.05$ ). In MSI-negative patients, tumors in Group A also had a proclivity for the proximal colon (5/9) but tumors in Group B did not (4/14). ③ Correlation with a poorly differentiated phenotype (Table 2). Among patients positive for MSI, 11 (78.6%) of 14 tumors were poorly differentiated adenocarcinomas. It was significantly higher than that among MSI-negative patients ( $P<0.05$ ). Also in tumors of Group A, the poor differentiation rate positive MSI was higher than that of negative MSI. ④ Proclivity for extracolorectal malignancy. Among patients in Group A, 6 cancers were associated with cancers in other organs (2 in uterus, 2 in stomach, 1 in bladder, and 1 in bile duct), and 5 of these were positive for MSI. In contrast, there was no patient with cancers in other organs in Group B.

Immunohistochemically, contrast with Group B, patients in Group A tended to be negative for hMLH1 protein staining in tumor tissues, and hMLH1, hMSH2 protein staining in normal tissues (Figure 2, Table 3). Among patients positive for MSI, 6 of 14 colorectal tumors were negative for hMLH1 together with hMSH2 protein, whereas 4 of 32 tumors negative for MSI ( $P<0.05$ ). The LI of PCNA staining (Figure 3) in tumors of Group A was  $0.54\pm 0.10$ , which was lower than that of Group B ( $0.62\pm 0.07$ ),  $P<0.01$ , meanwhile in cancer tissues with MSI-positive, the LI was  $0.53\pm 0.10$ , which was lower than that of negative MSI ( $0.59\pm 0.08$ ),  $P<0.05$ .

According to flow cytometry (Table 4), the heteroploid rate of DNA in tumors of Group A was 23.1%, PI and S-phase percentage in tumors with positive MSI were  $14.34\pm 5.49$  and  $8.18\pm 2.55$ . In contrast with that of Group B and negative MSI respectively, they decreased obviously ( $P<0.05$ ).

**Table 1 MSI and mean age at onset of colorectal cancer**

Group	MSI	n	Age( $\bar{x}\pm s$ )
HNPCC		4	41.8 $\pm$ 7.7 <sup>b</sup>
A	+	12	45.4 $\pm$ 8.3 <sup>b</sup>
	-	14	49.6 $\pm$ 9.7
B	+	2	57.5 $\pm$ 9.2
	-	18	58.9 $\pm$ 11.0

<sup>b</sup> $P<0.01$  vs Group B; Fisher's test.

**Table 2 MSI and location, histologic differentiation of colorectal cancer**

Group	MSI	Location n (%)		Differentiation n (%)	
		Pr	Di	PD	WD
A	+	8(66.7)	4(33.3)	9(75.0) <sup>b</sup>	3(25.0)
	-	5(35.7)	9(64.3)	4(28.6)	10(71.4)
B	+	1(50.0)	1(50.0)	2	0
	-	4(22.2)	14(77.8)	5(27.8)	13(72.2)
Total	+	9(64.3) <sup>a</sup>	5(35.7)	11(78.6) <sup>a</sup>	3(21.4)
	-	9(28.1)	23(71.9)	9(28.1)	23(71.9)

<sup>a</sup> $P<0.05$ , vs patients negative for MSI;  $\chi^2$  test. <sup>b</sup> $P<0.05$ , vs patients negative for MSI in Group A;  $\chi^2$  test.

Pr: proximal colon; Di: distal colon (descending, sigmoid colon and rectum); PD: poorly differentiated adenocarcinoma; WD: well and moderately differentiated adenocarcinoma.

**Table 3 Negative for hMLH1, hMSH2 protein staining in CRC patients' tissues (n,%)**

	Group A (26 cases)		Group B (20 cases)	
	Tumor	Normal	Tumor	Normal
hMLH1 -	16(61.5) <sup>a</sup>	17(65.4) <sup>b</sup>	6(30.0)	5(25.0)
hMSH2 -	14(53.9)	16(61.5) <sup>b</sup>	9(45.0)	6(30.0)

<sup>a</sup> $P<0.05$ , vs tumor tissues in Group B;  $\chi^2$  test.

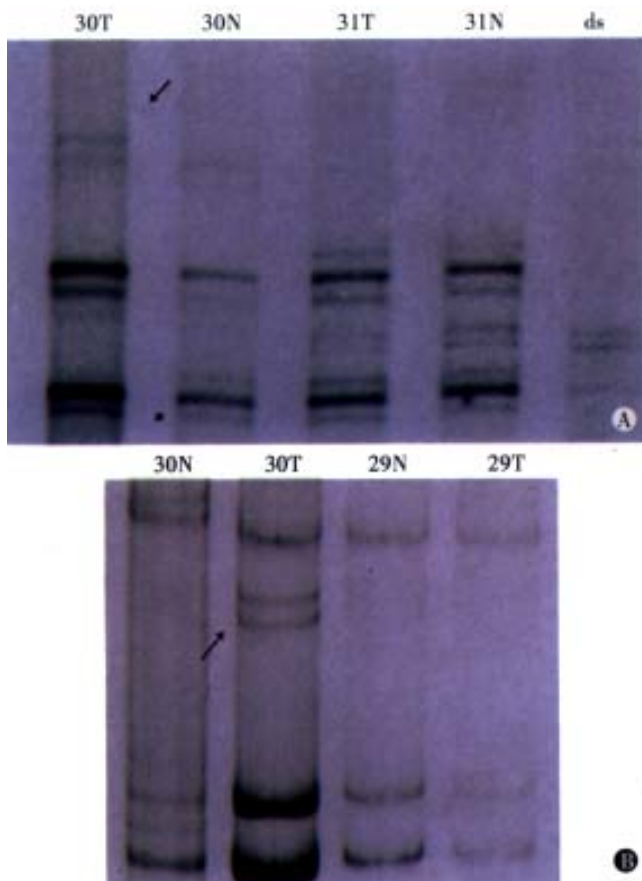
<sup>b</sup> $P<0.05$ , vs normal tissues in Group B;  $\chi^2$  test.

**Table 4 MSI and DNA analysis in CRC tumor cells by flow cytometry ( $\bar{x}\pm s$ )**

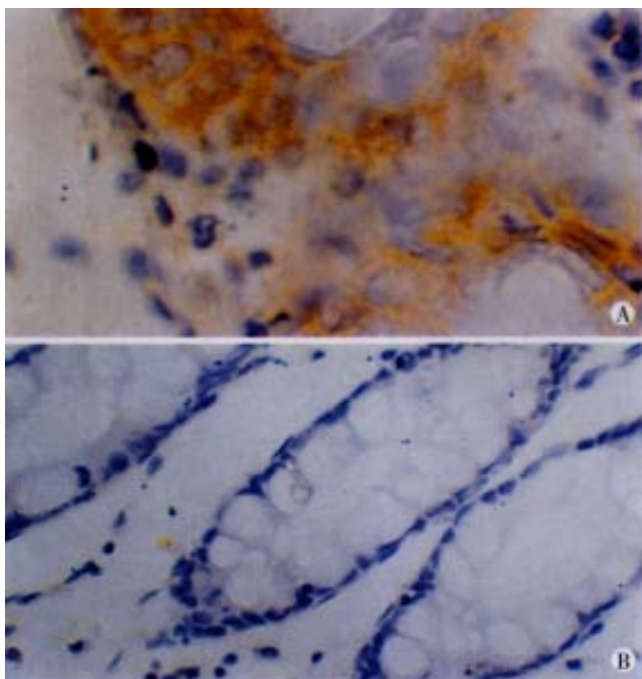
Group	Cases	Heteroploid (n,%)	PI	S-phase
HNPCC	4	1(25.0)	14.58 $\pm$ 3.12	8.70 $\pm$ 1.18
A	26	6(23.1) <sup>a</sup>	17.57 $\pm$ 6.51	9.47 $\pm$ 2.85
	18	10(55.6)	17.94 $\pm$ 7.51	10.38 $\pm$ 3.89
MSI+	14	4(28.6)	14.34 $\pm$ 5.49 <sup>b</sup>	8.18 $\pm$ 2.55 <sup>b</sup>
MSI-	30	12(40.0)	19.30 $\pm$ 6.93	10.62 $\pm$ 3.36

<sup>a</sup> $P<0.05$ , vs tumor cells in Group B; Fisher's test.

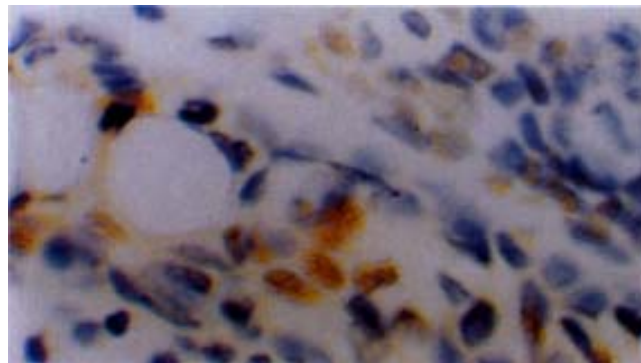
<sup>b</sup> $P<0.05$ , vs tumor cells negative for MSI; Fisher's test.



**Figure 1** Patient of No.30 positive for MSI. Lane N: corresponding normal tissue, Lane T: tumor tissue, Lane ds: di-strand control. MSI is defined as showing the presence of extra or shifting bands in PCR products using tumor DNA that are not visible in the products from corresponding normal tissue. (Left)D17D250, (Right)D2S123.



**Figure 2** Immunohistochemistry of hMLH1. (Left) Patient positive for hMLH1 immunostaining. ×40. (Right) Patient negative for hMLH1 immunostaining.×20.



**Figure 3** Immunostaining for PCNA in colorectal cancer tissue of patient with familial predisposition. ×40.

**DISCUSSION**

MSI is the phenotype of a profound genomic instability caused by a mutator phenotype mechanism for cancer<sup>[31,32]</sup>. Four microsatellite loci (D2S119, D2S123, D5S107 and D17S250) used in our study resemble to the loci recommended by NCI workshop<sup>[25]</sup>. The ratio of MSI-positive patients in Group A (46.2%) was significantly higher than that among randomly selected patients in Group B (10%). These results showed that MSI was an important contributor in CRC with familial predisposition. Colorectal tumors with MSI exhibit several clinicopathologic characteristics. In this study, we confirmed that the age at onset of cancer in Group A was younger than that in Group B, especially in HNPCC patients and patients with MSI-positive in Group A. We also confirmed that the ratio of proximal colon tumors, complication by extracolorectal malignancies and poorly differentiated carcinomas in patients with positive MSI were higher than those with negative MSI, and these characteristics were significant in Group A. These results suggested that it was possible and effective to identify high-risk relatives of CRC by applying pedigree survey together with MSI detection.

Leach *et al* established the method for detecting the products of MMR gene mutation<sup>[33]</sup>. If the MMR genes mutate, there may be a kind of methylation on promoter in transcription. And it brings the products in C-termination which block the normal expression of MMR genes. Therefore, negative for hMLH1, hMSH2 staining means potential mutation of MMR genes in tumor or normal tissues, and positive means no mutation<sup>[27,34]</sup>. In our study, we found that in Group A the incidence of negative hMLH1 staining in tumor tissues and hMLH1, hMSH2 staining in normal tissues was significantly high (Table 3). Among patients positive for MSI, ratio of negative hMLH1 together with hMSH2 staining in tumors was also higher than that among patients negative for MSI. It suggested that the rate of hMLH1, hMSH2 abnormality in CRC patients with familial

predisposition increased in colorectal tumors and normal tissues. The colorectal epithelium of this group could have wide gene abnormality. And these gene changes had obviously been related with MSI.

According to the previous studies, the expressions of PCNA<sup>[35,36]</sup>, DNA ploidy and proliferation phases of cells<sup>[37,38]</sup> are the important markers for diagnosing and prognosticating cancers. We found that the LI of PCNA staining of tumor cells in Group A and MSI-positive group were lower than that in Group B and MSI-negative group respectively. According to Flow Cytometry, the heteroploid rate of DNA in tumor cells of Group A was lower than that of Group B. PI and S-phase percentage in tumor cells with positive MSI were also lower than those in negative MSI. It was reported that the metastasis and recurrence in colorectal cancer with MSI (due to replication error, RER) were less than in sporadic cancer. The low invasion of cancer could be explained that the mutation was too frequent and complicated to express the phenotype of metastasis and recurrence<sup>[18]</sup>. Our study showed that this low invasion was also associated with the decreased proliferation activity in colorectal cancer with familial predisposition and MSI.

Studies on MSI, MMR gene expression and proliferation kinetics showed the characteristics of colorectal cancer with familial predisposition. According to this, the early diagnosis and warning system<sup>[39]</sup> of colorectal cancer can be established. It is important to screen high-risk relatives of colorectal cancer and diagnose colorectal cancer early<sup>[40]</sup>.

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