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Comparative features of colorectal and gastric cancers with microsatellite instability in Chinese patients*

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Abstract: Objective: The purpose of this study was to determine the unique and universal features of microsatellite instability-high (MSI-H) colorectal cancer (CRC) and MSI-H gastric cancer (GC) in the Chinese population. Methods: A new panel of mononucleotide MSI markers, BAT25, BAT26, NR21, NR24, and MONO-27, was used to define MSI status in 303 CRC and 288 GC subjects. Clinicopathological features of both types of MSI-H tumors were analyzed. Methylation analysis in the *hMLH1* promoter region by methylation specific polymerase chain reaction (PCR) and mutation detection of *hMSH2*/*hMLH1* genes by denaturing high-performance liquid chromatography (DHPLC) were carried out simultaneously. Results: MSI-H CRCs and MSI-H GCs account for 11.9% and 8.0% of unselected sporadic CRCs and GCs, respectively. MSI-H CRCs are strongly characterized by early onset, right-side location, low differentiation, mucinous tumor, less infiltration, less lymphatic metastasis, and more often familial tumor. MSI-H GCs only showed site preference for the antrum and less lymphatic metastasis. Genetic and epigenetic analyses were positive in 6/36 MSI-H CRCs and 0/23 MSI-H GCs with pathological mutation in major mismatch repair genes, and in 7/36 MSI-H CRCs and 18/23 MSI-H GCs with methylated *hMLH1* promoter (P<0.01), respectively. Conclusions: Although there are many differences in the genetic basis and clinicopathological features between MSI-H CRC and MSI-H GC, when compared with their microsatellite stable (MSS) counterparts, site preference and lymphatic metastasis are features common to both types of MSI-H tumors.

Key words: Microsatellite instability, Colorectal cancer, Gastric cancer, Mutation, DNA methylation
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1 Introduction

Microsatellite instability (MSI) is the term given to deletions or insertions of short repeat nucleotides sequence in the genome (Oda *et al*., 2005). MSI can be divided into microsatellite instability-high (MSI-H) and microsatellite instability-low (MSI-L) according to the appearance of a number of unstable MSI markers (Pawlik *et al*., 2004). The causes of MSI-H tumors have been linked to several genetic and epigenetic changes in mismatch repair (MMR) genes, suggesting that an MSI-H tumor is caused by an impotent MMR system (Grady, 2004; Li and Lai, 2009). MSI-H tumors are often seen in gastrointestinal cancer. In fact, there are a number of studies of MSI-H colorectal cancer (CRC) and MSI-H gastric cancer (GC) in recent decades (Lubbe *et al*., 2009; Seo *et al*., 2009). However, to date, there have been no comparisons made of these two types of MSI-H cancers. Presumably, since the MSI-H tumor is caused by MMR gene deficiency, MSI-H tumors may share similar cancer genesis pathways, which may lead to similar clinical outcomes. If there exist common features in MSI-H tumors, this will help to understand better the relationship between MSI genotype and clinicopathological phenotypes. Moreover, it will

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help to distinguish MSI-H tumors from microsatellite stable (MSS) tumors more easily. In an attempt to discover similarities and differences between MSI-H CRC and MSI-H GC, we studied clinicopathological features as well as conducted genetic analyses of both MSI-H CRC and MSI-H GC in a Chinese population.

2 Materials and methods

2.1 Subjects and specimens

This study was approved by the ethics committee of our institution, following the ethical guidelines of the 1975 Declaration of Helsinki (Forster *et al*., 2001). In total, 303 patients diagnosed with CRC and 288 patients diagnosed with GC underwent curative surgical resection between 2000 and 2004 in the Second Affiliated Hospital, School of Medicine, Zhejiang University, China. These patients served as the study population. During surgical resection, about 1 cm^3 of paired tumor and normal tissue were resected from the removed gastrointestinal tract and immediately preserved in −20 °C for future use. Each resection and preservation took place with the informed consent of the patients. Each tumor sample was pathologically verified by two experienced pathologists. Genomic DNA of each tissue was extracted by a standard phenol-chloroform method and was preserved in −20 °C.

2.2 MSI analysis by denaturing high-performance liquid chromatography (DHPLC)

The DHPLC instrument used was the WAVE system (Transgenomic Inc., Omaha, NE, USA). Five MSI markers, BAT25, BAT26, NR21, NR24, and MONO-27, were used to determine microsatellite status according to the methodology previously reported (Murphy *et al*., 2006; Berginc and Glavac, 2009).

Polymerase chain reaction (PCR) primers for these five MSI markers were designed. DNA was amplified in a 25-μl volume system containing 100 ng of sample DNA, 200 μmol/L deoxynucleotide triphosphates (dNTPs), 2 mmol/L $MgCl_2$, 15 pmol of each primer, and 0.25 U *Taq* DNA polymerase in a transgenomic recommended buffer. The reaction was incubated at 95 °C for 5 min, followed by 35 cycles of 95 °C (primer specific annealing temperature) and 72 °C for 50 s each, and then 8 min of final extension at 72 °C. PCR products were sent to DHPLC analysis without any purification.

MSI analysis was carried out on an automated HPLC device equipped with a DNA separation column. In total, 6–10 μl of each PCR product was separated at a flow rate 0.9 ml/min by means of a linear acetonitrile gradient. The column temperature was 50 °C. The detection range was set as ±100 bp of specific DNA fragment. In the DHPLC, MSI-H sample shows a double peak and MSS one shows a single one (Fig. 1). A tumor sample was considered to be MSI-H if two or more of the five markers demonstrated instability, and was considered to be MSI-L if only one marker demonstrated instability.

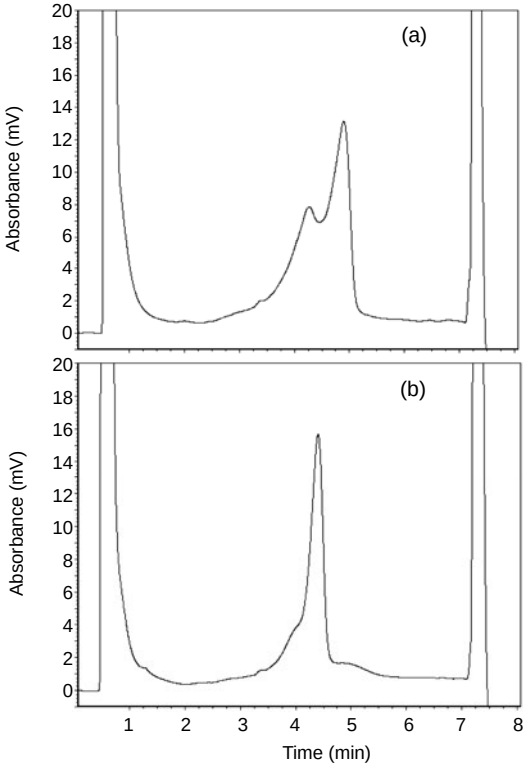

Fig. 1 A double peak for the MSI-H sample (a) and a single peak for the MSS sample (b) on DHPLC

2.3 Methylation analysis of *hMLH1* promoter region

After MSI-H CRC and MSI-H GC were determined, methylation specific PCR (MSP) was used to determine the methylation status of the *hMLH1* promoter region in both MSI-H tumor types. Methylation specific primers were designed as previously reported

(Deng *et al*., 1999). The PCR amplification procedure was similar with that in MSI detection. Each promoter region amplified by PCR has decisive roles in gene expression. MSP product was separated by DHPLC instead of agarose gel. If the DNA sequence was methylated, MSP would amplify methylated alleles, which would appear on DHPLC as a peak. If the DNA sequence was not methylated, there was no appearance of peaks on DHPLC.

2.4 Mutation analyses of coding sequences of *hMSH2* and *hMLH1*

For those MSI-H tumors without methylation in the *hMLH1* promoter region, further detection of somatic mutations in coding sequences of *hMSH2* and *hMLH1* was carried out by DHPLC. A total of 35 exons of both genes for every single methylation-negative MSI-H tumor were examined according to the methodology previously reported (Yuan *et al*., 2004). When a mutation was found by DHPLC and was verified by DNA sequencing, it was soon sent to a verification procedure to determine if it was a pathological mutation. Verification was settled in two steps. The first step was to align the mutant sequence with published dbSNP database in order to exclude the possibility of being known single nucleotide polymorphisms (SNPs). If the mutation was unpublished, the second step was carried out, in which DNA from 100 healthy individuals was examined for the same mutation. If the positive rate of a specific mutation in healthy individuals was less than 1%, it was considered to be a pathological mutation; otherwise it was regarded as a single nucleotide polymorphism with no significant pathological meaning.

2.5 Data analysis

Medical data for every subject were collected. The database was designed to preserve clinical and pathological statistics, MSI status, and genetic and epigenetic information. χ^2 statistic analysis was conducted by SPSS software.

3 Results

3.1 Frequency of MSI tumors

MSI analyses of 303 CRCs resulted in 36 (11.9%) MSI-H and 262 (86.5%) MSS tumors. Three DNA fragments from BAT26 PCR products were sent to DNA sequencing, and all the repeated mononucleotides showed shortened (2–6 bp) alleles. Of the 288 GCs in which MSI analyses were completed, 23 (8.0%) were MSI-H and 253 (87.8%) were MSS.

The DHPLC evaluation of mononucleotide instability is convenient, effective, and timesaving. It took less than 8 min per sample, allowing rapid throughput of a large numbers of samples in minimal time. However, it is difficult to evaluate dinucleotide markers D5S346, D2S123, and D17S250 by DHPLC. Despite the comparison of DNA from both paired cancer and normal tissue to define MSI status in dinucleotide evaluation, it was difficult to distinguish instability from stability by DHPLC for these dinucleotide markers.

3.2 Clinicopathological features of MSI-H CRC and MSI-H GC

Statistical analysis showed (Table 1) that there were significant differences between MSI-H CRC and MSS CRC in several clinicopathological characteristics. However, differences between MSI-H GC and MSS GC were not apparent. MSI-H CRCs were more likely from younger subjects, were located in the right colon, and have a mucinous phenotype, less local aggressiveness, and less lymphatic metastasis. However, MSI-H GC tended only to locate in distal parts of the stomach.

3.3 Genetic and epigenetic alterations of MMR genes in MSI-H gastrointestinal tumors

hMLH1 promoter methylation was present in 18 out of 23 MSI-H GCs and 5 of 30 MSS GCs, while only 7 out of 36 MSI-H CRCs and 1 of 30 MSS CRCs showed methylated alleles in the *hMLH1* promoter region, significantly lower (P<0.01) than those in GCs. After methylation analysis, 5 MSI-H GCs and 29 MSI-H CRCs without methylation were sent for further mutation detection in *hMSH2*/*hMLH1* genes.

Mutation detection of *hMSH2* and *hMLH1* indicates 37 single nucleotide changes in 34 MSI-H tumors. However, only six mutations proved pathological in MSI-H CRC (Table 2). No pathological mutations were found in MSI-H GC.

Table 1 Clinicopathological features of MSI-H CRC and MSI-H GC in a Chinese population

Parameter	CRC			GC		
	MSI-H (36)	MSS (262)	*P*	MSI-H (23)	MSS (253)	*P*
Sex			0.517			0.695
Male	24	160		15	175	
Female	12	102		8	78	
Average age (year)	54.6	60.9	0.035	62.3	59.3	0.415
Site						
Right side[1]	20	63	0.000	−	−	−
Rectum	5	126	0.031	−	−	−
Antrum[2]	−	−	−	19	142	0.014
Differentiation			0.007			0.869
High	21	147		3	38	
Middle	10	107		10	96	
Low	5	8		10	119	
Mucinous tumor[3]			0.002			0.645
+	10	24		3	43	
−	24	204		16	17	
Infiltration			0.002			0.422
Muscle	6	47		6	51	
Serosa	28	131		11	101	
Invade through serosa	2	84		6	101	
Lymphatic metastasis[4]			0.011			0.089
N0	29	144		3	56	
N1	6	79		10	81	
N2	1	39		9	73	
N3	−	−	−	1	43	
TNM stage			0.024			0.468
I	6	37		3	48	
II	23	105		7	53	
III	6	102		11	106	
IV	1	18		2	46	
Family history[5]			0.001			0.100
Yes	8	11		3	11	
No	28	251		20	242	

[1] Right side means that colorectal cancers (CRCs) were located in the cecum, ascending colon, and right half of transversum colon; [2] 19 microsatellite instability-high (MSI-H) gastric cancers (GCs) were located at antrum while four were located in another part of the stomach; [3] Partial mucinous differentiation was excluded from mucinous tumor category; [4] Number of metastatic lymph nodes (N0: no, N1: 1–6, N2: 7–15, N3: >15); [5] Family history includes hereditary nonpolyposis colorectal cancer related tumor in any of subject's immediate relatives

Table 2 Six pathological somatic mutations in MSI-H CRC

Sample No.	Gene	HGVS description	Amino acid change	Population frequency
CRC03	*hMLH1*	GI:13905125 c.8+5 T>G	Splice site	<1%
CRC07	*hMLH1*	GI:13905125 c.244 G>A	Ala>Thr	<1%
CRC14	*hMLH1*	GI:13905125 c.1477 C>G	Pro>Ala	<1%
CRC15	*hMLH1*	GI:13905125 c.1449 delA	Frame shift	<1%
CRC20	*hMLH1*	GI:13905125 c.2250 G>C	Termination	<1%
CRC31	*hMSH2*	GI:18204305 c.2516 G>A	Asp>His	<1%

MSI-H: microsatellite instability-high; CRC: colorectal cancer; HGVS: Human Genome Variation Society

4 Discussion

4.1 MSI frequencies of CRC and GC in Chinese patients

In 1997, a National Cancer Institute (NCI) workshop recommended the Bethesda panel markers for defining MSI tumor, these markers including two mononucleotide markers (BAT25 and BAT26) and three dinucleotide markers (D5S346, D2S123, and D17S250). If two of the five markers are unstable in a tumor, it is MSI-H. If only one marker is positive, it is MSI-L (Boland *et al*., 1998). In 2004, a follow-up NCI workshop further discussed MSI testing (Umar *et al*., 2004), and recognized the limitations of the original Bethesda panel due to the inclusion of dinucleotide repeats, which are less sensitive and specific than mononucleotide repeats for the identification of cancers with MMR deficiencies. Subsequent studies have shown that mononucleotide markers are sufficient to define MSI-H tumors (de la Chapelle, 1999; Suraweera *et al*., 2002; Buhard *et al*., 2004). Bacher *et al*. (2004) evaluated a set of 266 mono-, di-, tetra-, and penta-nucleotide repeats loci for potential use in MSI screening. They determined that mononucleotide loci are more sensitive and specific than dinucleotide loci for defining MSI tumors. Therefore, a new panel of markers for MSI testing was developed. This MSI analysis system includes five mononucleotide markers: BAT25, BAT26, NR21, NR24, and MONO-27. The system is now commercially available from Promega Corp. (Murphy *et al*., 2006).

In our study, a total of 303 CRCs and 288 GCs were screened by five mononucleotide markers. We confirmed 36 MSI-H CRCs and 23 MSI-H GCs. The observed MSI-H tumor frequency in CRC was 11.9%, which was similar with that reported in Korean (9%) and Australian (11%) populations (Lim *et al*., 2004). The frequency of MSI-H GC in our study was 8.0%, which was quite lower than that observed either in Japanese population (20%) or in European American (39%) (Theuer *et al*., 2002). Another study reported a frequency of 19% in GCs in Caucasians (An *et al*., 2005). However, a study in a Chinese population with a small sample (n=68) reported an 11% MSI-H frequency in GCs (Fang *et al*., 2003). A very recent study in a Korean population reported a 9.6% MSI-H GC frequency in sporadic GCs (Gu *et al*., 2009). We conclude that the frequency of MSI-H GC in Chinese populations is relatively low when compared with those in Japan and Western countries.

4.2 Universal and unique features of MSI-H gastrointestinal cancer

Many reports have provided information about clinicopathological features of MSI-H CRC. MSI-H CRC is strongly characterized by early onset, mucinous type tumors, right-side location of the colon, and better survival rates. In this study, we were interested in comparative features between MSI-H CRC and MSI-H GC. Hypothesizing that since all MSI tumors are caused by impotent DNA mismatch repair system, one may postulate that MSI tumors share similar oncogenesis pathways which in turn may lead to similar clinicopathological outcomes. However, the results turned out to be different. There were only two universal features between MSI-H CRC and MSI-H GC found in this study. First, both MSI-H CRC and MSI-H GC tend to be located at a particular site of the respective organs, though it is difficult to explain the relationships between these particular sites. Another universal feature which may exist between these two types of MSI-H tumors is local lymphatic metastasis. In an attempt to find out the relationship between lymphatic metastasis and MSI status, we first separated tumors into two groups in both the MSI-H tumor and MSS tumor groups. One was lymphatic metastasis positive and the other was free from lymphatic metastasis. As a result, MSI-H CRC showed statistically significantly less lymphatic metastasis than MSS CRC. Overall, 80% of MSI-H CRCs had no lymphatic involvement. Yet, the same feature failed to reach statistical significance for MSI-H GC, though there was a tendency that MSI-H GC had less lymphatic metastasis. Re-examining the data, we found that there was a significant difference of involved lymph node numbers between MSI-H GC and MSS GC. The average number of inspected lymph nodes per case in MSI-H GC was 23.4, almost the same as that for MSS GC (23.7). However, the average number of positive lymph nodes in MSI-H GC (5.8) was significantly (P=0.005) lower than that of MSS GC (8.3). The data were less persuasive, suggesting that both MSI-H CRC and MSI-H GC tend to have less lymphatic involvement. In a very recent study, Ma *et al*. (2009) observed a lower frequency of MSI-H in GC without lymph node metastasis when

compared with that in lymph node metastasis positive GC. Xiao *et al*. (2006)'s study in a Chinese population also found that the frequency of MSI in GC without lymph node metastasis was significantly higher than that in GC with lymph node metastasis (66.7% vs. 34.3%, $P<0.05$). We conclude that there may exist a weak relationship between MSI-H and lymph node metastasis in gastrointestinal cancer.

Apart from site preference and local lymphatic metastasis, MSI-H GC failed to show any special features when compared with its MSS counterpart. However, unique features of MSI-H CRC are apparent and well-grounded.

4.3 Clinical and biological significance

In the newest version of practice guidelines for colon cancer from the National Comprehensive Cancer Network, MSI testing is recommended for all colon cancers because stage II colon cancer will not benefit from 5-FU (fluorouracil) adjuvant chemotherapy (Sargent *et al*., 2010). Selective adjuvant chemotherapeutic treatment of stage II CRC has long been a problem for oncologists. This problem also existed in GC (Vita *et al*., 2007), and induction adjuvant chemotherapy for stage II GC will possibly depend on MSI status of the cancer. From the results of our findings, it is unreasonable to make such a hypothesis, because MSI-H CRC and MSI-H GC are so often different in both clinicopathological and genetic features. From DNA mismatch repair deficiency to MSI and tumor formation, it is difficult to conclude that there exists a definite, common oncogenesis pathway in gastrointestinal tumors. In conclusion, MSI is a phenotypic phenomenon rather than a cause of these diseases.

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