

## Original Article

# Microsatellite instability of gastric cancer and precancerous lesions

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Received March 18, 2015; Accepted July 1, 2015; Epub November 15, 2015; Published November 30, 2015

**Abstract:** Objective: To investigate whether microsatellite instability (MSI) of gastric cancer and precancerous lesions were existed and its effect. Methods: Laser microdissection was used. Gastric, intestinal metaplasia, dysplasia and normal mucosa were collected respectively. Five microsatellite loci were selected and MSI was detected by denaturing high-performance liquid chromatography. Results: In the five microsatellite loci REF-positive phenotype, intestinal metaplasia MSI was 20.7%. Dysplasia MSI was 22.4%. Gastric MSI was 47.9%, and there was no MSI in normal gastric mucosa. Conclusion: MSI gradually increased from precancerous lesions to gastric cancer. The early detection of MSI may be a potential early warning indicator for early diagnosis of gastric cancer.

**Keywords:** Gastric cancer, precancerous lesions, laser micro dissection, denaturing high performance liquid chromatography, microsatellite instability

## Introduction

Advances in molecular genetics and molecular pathology of malignancies have long been affected by the diversity of tumor tissue components and the accuracy of sampling [1]; and the histogenesis, differentiation and pathological changes are complex processes of gene regulation, therefore cell heterogeneity has become a major obstacle in the studies of normal or pathological tissue at the molecular level [2]. Recently, Germany PALM company laser capture micro dissection (LCM) system has successfully solved the problem of obtaining pure cell from different components of required samples [3]. LCM technology is the technology to isolate and purify population of cells of a single type or a single cell from tissue sections under a microscope [4]. This method can be used for archiving retrospective studies of paraffin-embedded tissue, especially with the advantages of accurate positioning and non-contamination of non-target cells; in recent years, it has been highly regarded in the field of medical research in foreign, and has been widely used in tumor heterogeneity, cell mutation analysis, chromosome structure analysis,

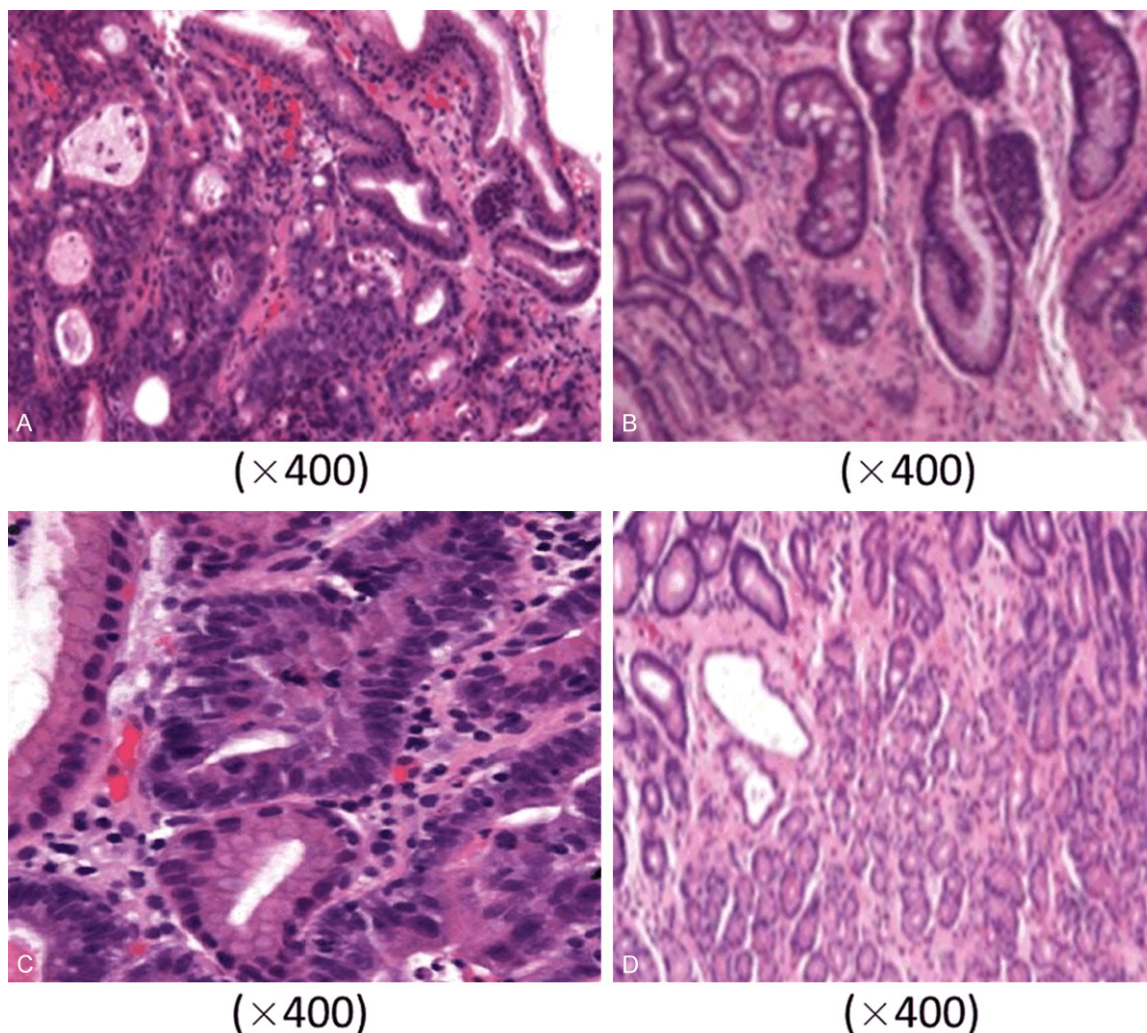
new gene discovery and the etiology and diagnosis research of certain diseases [5].

Genetic abnormalities were existed in gastric cancer. However, genetic changes on precancerous lesions were still poorly understood [6, 7]. While there was less relevant researches the application of laser micro dissection to obtain pure tumor tissue and precancerous lesions using denaturing high-performance liquid chromatography. To further investigate whether microsatellite instability (MSI) of gastric cancer and precancerous lesions were existed and its effect, in this study, we used micro dissection and denaturing high-performance liquid chromatography (DHPLC) to detect MSI.

## Materials and methods

### Source of specimen

73 cases of gastric tissue, 49 cases of atypical hyperplasia and 29 cases of intestinal metaplasia were collected from January 2013 to December 2014 which were diagnosed from Department of Surgical Oncology, General Hospital of the People's Liberation Army. All tis-



**Figure 1.** Representative histology from the study population demonstrating (A) gastric cancer; (B) intestinal metaplasia (C) dysplasia and (D) normal tissues.

sue specimens accepted conventional treatment, formaldehyde solution fixation, paraffin embed and consecutive paraffin section. They were used for histopathological diagnosis and micro dissection. We also selected 50 normal gastric tissues as the controls (**Figure 1**).

#### *Reagents and instruments*

Xylene, ethanol, eosin, Tris base, EDTA, sodium chloride, SDS, chloroform, isoamyl alcohol and sodium acetate were purchased from Beijing Chemical Plant (Beijing, China). Proteinase K (Merck, Germany products), Taq plus DNA polymerase (2 U/μl), 10 mmol/L dNTP, 10 × PCR buffer (containing 20 mmol/L  $Mg^{2+}$ ) were purchased from Beijing Dingguo Biotechnology Center (Beijing China). Centrifuge was pur-

chased from the German Eppendorf company (German). Slides with PEN film, laser capture microdissection instruments were purchased from Germany PALM companies. PCR amplification was purchased from PE firms. Optical microscope was purchased from Japanese Olympus companies. Gel imager was purchased from France IVL companies. WAVETM nucleotide fragment analysis system was purchased from Transgenomic Company.

#### *Preparation of laser micro dissection of paraffin and the dyeing process*

Preparation of three pieces of 5 μm thick serial paraffin sections was according to conventional pathology methods. One piece was used for H&E staining to observe the distribution of can-

**Table 1.** The primer sequences of each locus

MSI locus	Chromosome	Gene	Primers sequences	Length (bp)
BAT26	2p22-21	hMSH2	5'-TGACTACTTTGACTTCAGCC-3' 5'-AACCATTCAACTTTTAAACC-3'	121~123
BAT25	4q12	c-Kit	5'-TCGCCTCCAAGAATGTAAGT-3' 5'-TCTGCATTTTAACTATGGCTC-3'	123~125
D2S123	2p16	hMSH6	5'-AAACAGGATGCCTGCCTTTA-3' 5'-GGACTTTCCACCTATGGGAC-3'	197~227
D5S346	5p21/22	APC	5'-ACTCACTCTAGTGATAAATCGGG-3 5'-GGAGATAAGACAGTATTACTAGTT-3	96~122
D17S250	17q11.2-q12		5'-GGAAGAATCAAATAGACAAT-3' 5'-GCTGGCCATATATATTTAAACC-3'	151~169

cer nests, dysplasia and intestinal metaplasia and marked their position; Other two slices were processed with 40°C conventional methods and affixed on the slide with the PEN film. To dry the slices at 56°C overnight, and treat the slides according to the following steps without mounting for micro dissection: Xylene dewaxing 2 × 2 min; Anhydrous ethanol hydration for 1 min; 96% ethanol hydration for 1 min; 70% ethanol hydration for 1 min; double distilled water rinse; eosin for 1 min; double distilled water rinse; 96% ethanol dehydration for 1 min for 2 times; ethanol dehydration for 1 min for 2 times; 37°C for 30 min to dry.

#### *Laser micro dissection*

Germany PALM company laser capture micro dissection (LCM) system was used for cutting. The tissue sections waiting to be cut were placed on inverted microscope stage. As for the controlled HE sections, we should select the area waiting to be cut under the microscope, adjust this area to the vision center and carried out micro dissection with a UV laser micro beam. Tumor tissue, dysplasia and intestinal metaplasia were completely separated from their nature intact surrounding stroma, and cut the normal gastric mucosa as controls.

After cutting, laser pressure ejection was used to separate the cleaved cells and slides; they were ejected into the Eppendorf tube with 2 µL lysate, and the ejected cells will stick to the inner surface of the tube cap. Cap the tube and centrifuge for 1 min (centrifuged radius of 10 cm, 13 000 r/min), then add the same lysis buffer containing proteinase K into Eppendorf tube to 50 µL; after digestion by proteinase K overnight at 55°C, boil them at 99°C for 10 min, inactivate proteinase K, and save samples at 4°C.

#### *Genomic DNA extraction of micro-dissected tissue*

In the above-described cutting samples, an equal volume of chloroform and isoamyl alcohol mixture (24:1) was added, and centrifuged at room temperature for 15 s. The aqueous phase was transferred into a new tube; if there was white precipitate at the junction, the organic phase was re-extracted, and aqueous phase was combined. 1/10 volume of sodium acetate (3 mol/L, pH 5.2) was added and mixed. 2~2.5 times the volume of ice ethanol was added. Samples were placed on dry ice for 5 min or longer, and then centrifuged for 5 min; the supernatant was discarded. 1 ml 70% ethanol was added at room temperature, up and down for several times; after centrifuged for 5 min, discard the supernatant, dry the precipitate, dissolve in TE.

#### *Primers*

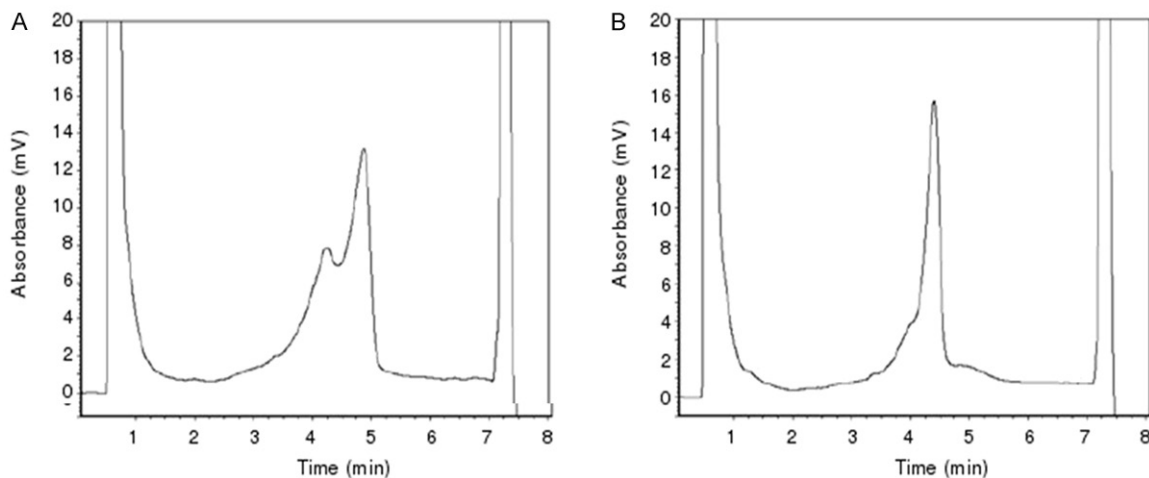
A set of standard microsatellite loci (BAT. 26, BAT-25, D2S123, D5S346, and D17S250CA) recommend by HNPCC international cooperation group and the US National Cancer Institute were selected; primer sequences were from Gene data base (<http://www.gdb.org/gdb>), and were synthesized by the Shanghai Sangon Biological Technology Co., Ltd. (Shanghai, China), shown in **Table 1**.

#### *PCR reaction*

**Reaction System (20 µl):** 10 × PCR buffer 2.0 µL, 10 mmol/L dNTP 0.5 µL, 10 µmol/L upstream primer 0.5 µL, 10 µmol/L downstream primer 0.5 µL, 2 U/µl TagDNA polymerase 1.5 µL, 100 ng/µ DNA template 1.0 µL, ddH<sub>2</sub>O 14 µL.

**Table 2.** Reaction conditions for PCR

MSI locus	Pre-denaturation	Denaturation	Annealing	Extension	Cycles
BAT26	95°C, 10 min	95°C, 45 s	55°C, 1 min	72°C, 30 s	30
BAT25	95°C, 10 min	95°C, 30 s	55°C, 1 min	72°C, 30 s	30
D2S123	95°C, 10 min	95°C, 45 s	55°C, 1 min	72°C, 30 s	30
D5S346	95°C, 10 min	95°C, 45 s	55°C, 1 min	72°C, 30 s	30
D17S250	95°C, 10 min	95°C, 45 s	52°C, 1 min	72°C, 30 s	30

**Figure 2.** A double peak for the MSI-H sample (A) and a single peak for the MSS sample (B) on DHPLC.

**Reaction conditions:** As shown in **Table 2**, BAT-26 microsatellite loci: 95°C pre-denaturation for 10 min; 95°C denaturation for 45 s, 55°C annealing for 1 min, 72°C extension for 30 s, a total of 30 cycles. BAT-25 microsatellite loci: 95°C pre-denaturation for 10 min; 95°C denaturation for 30 s, 55°C annealing for 1 min, 72°C extension for 30 s, a total of 30 cycles. Reaction conditions for D3S123 and D5S346 microsatellite loci were the same as those of BAT-25. D17S250 microsatellite loci: 95°C pre-denaturation for 10 min; 95°C denaturation for 30 s, 52°C annealing for 1 min, 72°C extension for 30 s, a total of 30 cycles.

**Agarose electrophoresis:** 5  $\mu$ L of above PCR product was mixed with 1  $\mu$ L of 10  $\times$  loading buffer and loaded on 2% agarose; electrophoresis (120 V voltage, 0.5  $\times$  TBE) was performed for 10 min; the PCR product was observed under ultraviolet light.

#### MSI detected by DHPLC

Three kinds of PCR products of tissues to be detected, corresponding normal tissues and one case of completely normal gastric mucosa

were used for DHPLC detection, with the completely normal gastric mucosa as an internal standard. Auto sampling; injection volume was 5  $\mu$ L/needle; mobile phase was 0.1 mol/L N-Acetamide (TEAA, HPLC grade) and different concentrations of acetonitrile were used for gradient elution; gradient was generated automatically by the control software WAVEMarker 4.0 based on nuclear nucleotide sequences; flow rate was 0.9 ml/min; detector was a UV spectrophotometer (260 nm). Reaction time and conditions: detection time was 15 min; the gradient of acetonitrile concentration was as below: Start 8.75%, in 3 min increased to 13.75%, in 7 min increased to 16.25%, maintaining for 0.5 min, then increased to 25% in 0.5 min, maintaining for 0.5 min before elution, and then dropped to 8.75% in 0.5 min, and continued 1 min for balance.

#### MSI judgment criteria

When DHPLC peaks of tissues to be detected, corresponding normal tissues, and the internal standard do not overlap, it is judged as MSI. Positive sites  $\geq 30\%$  was high MSI (MSI-H); Positive sites  $< 30\%$  was low MSI (MSI-L); no



**Table 3.** MSI in different tissues

Group	n	MSI (n, %)	MSS (n, %)
Normal tissue	50	Total: 0 MSI-L: 0 MSI-H: 0	50 (%)
Intestinal metaplasia	29	Total: 6 (20.7) MSI-L: 2 (6.9) MSI-H: 4 (13.8)	23 (79.3)
Dysplasia	49	Total: 11 (22.4) MSI-L: 4 (8.2) MSI-H: 7 (14.2)	38 (77.6)
Gastric cancer	73	Total: 35 (47.9) MSI-L: 12 (16.4) MSI-H: 23 (31.5)	38 (52.1)

positive loci was defined as microsatellite stability (MSS).

#### Statistical methods

SPSS 13.0 was used for statistical analysis; frequency was compared using  $\chi^2$  (chi-square) test;  $P < 0.05$  was considered statistically significant.

#### Results

##### MSI chromatogram in DHPLC detection

MSI analysis for the PCR products of test sample and corresponding normal tissue was performed under non-denaturing conditions, and the results showed that the microsatellite repeat segments of test samples were different from those of their corresponding normal tissues; non-overlapping peaks appeared in DHPLC (Figure 2).

##### MSI of gastric cancer, dysplasia, intestinal metaplasia and normal gastric mucosa tissues

As shown in Table 3, normal gastric tissue had no MSI. MSI accounted for 47.9%, 20.7%, and 22.4% in gastric cancer, dysplasia, and intestinal metaplasia, respectively. Compared with normal tissues, the differences were significant. In gastric cancer tissues, 16.4% (12/73) of MSI was MSI-L, and 31.5% (23/73) was MSI-H.

As shown in Table 4, in 35 cases of gastric cancer with MSI, the proportion of intestinal type gastric cancer was 71.4% (25/35); 28.6% was

diffuse gastric cancer (10/35); highly-differentiated gastric cancer (26 cases) accounted for 74.3%; 9 cases were poorly-differentiated gastric cancer, accounting for 25.7%. In 49 cases of dysplasia, positive MSI in one or more sites had been found in 11 cases (7 cases of severe dysplasia, 4 cases of moderate dysplasia); In 29 cases of intestinal metaplasia, positive MSI in one or more sites had been found in 6 cases (all 6 cases were incomplete intestinal metaplasia).

#### Discussion

In this study, the LCM technology (German PALM Company) was used to attach paraffin tissue sections on glass slides with a PEN film (slide thickness was 0.17 mm; PEN thickness was 1.35  $\mu\text{m}$ ; PEN film provided a back effect in the laser ejection process), and then dewaxing, hydration, eosin staining and UV laser cutting under a microscope were performed. Computer-controlled laser can cut the along the edge of selected cells on the monitor in accordance with the requirements of the operator to completely separate tumor tissue, intestinal metaplasia and dysplasia from surrounding stroma; the cutting tissue and cells directly fall into the following Eppendorf tube. Gastric cancer, dysplasia, intestinal metaplasia and normal gastric mucosa cells were collected. The cleaved tissues in Eppendorf tube can be directly observed under a long focus microscope. Thus contamination was avoided; at the same time, damage to DNA, RNA or protein was also avoided, achieving a rapid, simple and precise micro-dissection and pollution-free extraction of target cells; and the deviation caused by cell heterogeneity was effectively relieved, achieving the unified specificity and sensitivity; Therefore, in this study, the DNA molecules extracted from the normal gastric mucosa, pre-cancerous lesions, gastric cancer and a series of microscopic visible lesions were accurate and reliable, providing clean, precise and homogenous tissue cells for the followed microsatellite studies of gastric cancer and pre-cancerous lesions.

DHPLC eliminates the need for purification [8-10]; PCR amplification products can be directly used for microsatellite analysis. DHPLC technology was pioneered by Underhill and Oeffner in Stanford University in 1995 [11], with the advantages of semi-automatic, fast,

**Table 4.** Histology of MSI positive tissues

Group	n	Histology (n, %)	
Dysplasia	11	Severe dysplasia	7 (63.6)
		Moderate dysplasia	4 (36.4)
		Mild dysplasia	0 (0)
Intestinal metaplasia	6	Incomplete intestinal metaplasia	6 (100)
		Complete intestinal metaplasia	0 (0)
Gastric cancer	35	Intestinal type gastric cancer	25 (71.4)
		Diffuse gastric cancer	10 (28.6)
		Highly-differentiated gastric cancer	26 (74.3)
		Poorly-differentiated gastric cancer	9 (25.7)

high detection rate and suitable for large fragments, which can be used for the detections of MSI, mutations, loss of heterozygosity and single nucleotide acid polymorphism. The DHPLC WAVE nucleotide system does not require the steps of Glue preparation, sampling, and radio-nuclides or fluorescent labeling, saving time and effort, and the repetition rate is up to 100% (error rate is less than 0.03%), enabling non-gel laboratory nucleotide analysis and injecting new vitality to molecular biology, clinical diagnosis and other areas [12]. In this study, the sizes of all sites of amplified primer fragments were consistent with the designed fragment sizes, without non-specific bands. DHPLC detection is a new detecting technology of MSI, which has been widely carried out in foreign countries.

MSI is that the size of tumor DNA structural allele changed compared with the corresponding normal tissues, also known as replication error positive (RER+). MSI was firstly discovered in hereditary non-polyposis colorectal cancer and was found later in a variety of sporadic tumors [13-16]. Whether MSI can be used as a marker of gastric cancer to discover high-risk population, whether it can be used as an indicator of early diagnosis of gastric cancer, and the relationship between MSI and the clinic pathological biological behavior of gastric cancer still need a lot of research. MSI in gastric cancer still lacks deep and comprehensive study in domestic, and genetic changes related with precancerous lesions are poorly understood.

In view of this, in order to further investigate the existence of MSI and its role in precancerous lesions in the pathogenesis of gastric cancer, this study used LCM technology to collect gas-

tric cancer, intestinal metaplasia, dysplasia and normal mucosa tissues, in order to obtain pure cells; three dinucleotide markers and two single nucleotide markers, a total of five microsatellite loci, were studied. It was found that no MSI was found in normal gastric tissue, and MSI had been found in 47.9% of gastric cancer, 22.4% of dysplasia, and 20.7% of intestinal

metaplasia, thus indicating that intestinal metaplasia and dysplasia had genetic instability; the whole incidence and development process of gastric cancer were closely associated with the genetic instability [17, 18]; this instability occurred in identifiable precancerous lesions, and showed a gradual increase from precancerous to cancerous MSI, indicating that early involvement and accumulation of MSI in gastric mucosal cells were involved in gastric cancer multi-step pathway; MSI was an early event [19, 20]; the accumulation of MSI helped the development of gastric cancer. Precancerous MSI detection may be a potential warning indicator for early diagnosis of gastric cancer; MSI is a useful biomarker for clinical evaluation of the malignant potential of non-neoplastic gastric mucosa; MSI identification in precancerous lesions will help the screening of high-risk patients.

#### Disclosure of conflict of interest

None.

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