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Simultaneous analysis of microsatellite instability and loss of heterozygosity by capillary electrophoresis with a homemade kit

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Abstract

Microsatellite instability (MSI) and loss of heterozygosity (LOH), the alteration in length and strength of short tandem repeat sequences are an important molecular characteristic of many human tumors. MSI and LOH analysis has become an attractive method for diagnostic and tumor research purposes. A method for the simultaneous analysis of MSI and LOH at the five microsatellite loci (BAT-26, D17S261, D3S1283, D2S123 and D3S1611) was developed employing a cheap homemade kit to replace the expensive commercial kit on ABI 310 capillary genetic analyzer. After studying the effect of temperature and urea denaturant on microsatellite analysis, 8 mol/L urea and 60 °C were selected for assessing accurately fragment size of microsatellite alleles. Based on this method, 52 sporadic gastric cancers were screened, and MSI and LOH, at least one locus was observed in 15 of 52 (28.8%) patients. Moreover, it is found that a statistically significant association exists between MSI and LOH and tumor-differentiated level.

Keywords: Microsatellite instability; Loss of heterozygosity; Capillary electrophoresis

1. Introduction

Microsatellites are short tandem repeat sequences of 1–6 bp, occurring throughout the genome. Because of their repetitive nature, they are prone to errors caused by slippage or stuttering during replication. Alterations in the length or strength of microsatellite alleles in tumor tissue compared with normal tissue from the same individual are referred to as microsatellite instability (MSI) or loss of heterozygosity (LOH), which reflect a defect in DNA replication or repair. Therefore, MSI and LOH are also known as a replication error (RER) phenotype that persists throughout the lifetime of tumors and has become an attractive method for diagnostic and tumor research purposes [1,2].

Traditionally, MSI and LOH were analyzed by slab gel electrophoresis, but this method is time-consuming and labor intensive [3,4]. Recently, capillary electrophoresis (CE) has become widely accepted as a suitable DNA separation method because of its speed, reproducibility and its accurate size estimation of DNA fragments and quantitative analysis of DNA relative amounts [5–7]. Microsatellite instability has been analyzed by CE instruments with UV detection [8] or laser-induced fluorescence detection of a single color [9–11]. In these cases, the microsatellite alleles were easily interfered with the internal size standards. Recently, the dedicated instrument equipped with multicolor fluorescence detection (e.g. ABI 310 capillary genetic analyzer) resolved this question and was widely applied [12–20]. But all above reports made use of an expensive commercial kit (POP-4 matrix and capillary) on the ABI 310 capillary genetic analyzer. In our previous studies, a cheap kit including short-chain linear polyacrylamide matrix (SLPA) and neutral coated capillary was prepared in our laboratory and had been successfully applied to detect gene mutation by SSCP [21–23], CDCE [24] and SNaPshot [25]. To our knowledge, there are no reports about the homemade kit for microsatellite analysis on the ABI 310 capillary genetic analyzer, and no systematic study about the effect of urea additive and separation temperature on microsatellite analysis.

Abbreviations: SLPA, short chain linear polyacrylamide; TBE, Tris–boric acid–EDTA; MSI, microsatellite instability; LOH, loss of heterozygosity

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In this paper, five microsatellite loci of BAT-26, D17S261, D3S1283, D2S123 and D3S1611 were studied, which related to DNA mismatch repair genes hMSH2 and hMLH1, and tumor suppressor gene p53. These microsatellite loci are among those recommended in the National Cancer Institute (NCI) reference panel for MSI analysis and have been shown to assess MSI with high accuracy [26]. A method for the simultaneous analysis of microsatellite instability and loss of heterozygosity of the five microsatellite loci was developed employing a cheap homemade kit. The effect of urea additive and temperature on the size of allelic fragments was investigated in details. In the end, 52 gastric cancer samples were detected and 15 cases displayed MSI and LOH at one or more microsatellite loci.

2. Materials and methods

2.1. Apparatus and materials

ABI 310 genetic analyzer and PE Model 2700 PCR system were purchased from Applied Biosystems Co. (Foster City, CA, USA). Bare fused silica capillary with 50 μm internal diameter and 365 μm outer diameter were obtained from Applied Biosystems Co. (Foster City, CA, USA) and Yongnian Optical Fiber Factory (Hebei, China).

Acrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), ammonium peroxydisulfate (APS) and 89 mmol/L Tris–89 mmol/L boric acid–2 mmol/L EDTA (1× TBE) buffer were purchased from Sigma (St. Louis, MO, USA). PCR Amplification Kit and primers were obtained from TaKaRa Biotechnology (Dalian) Ltd. Co. (Dalian, China). Proteinase K was acquired from Merck (Darmstadt, Germany). Urea was purchased from Shenyang Lianbang Reagent Factory (Shenyang, China). Other chemicals were of analytical grade.

2.2. Collection of the samples and isolation of genomic DNA

All sporadic gastric cancer patients were residents of the northeast region of China. Their tumor and corresponding normal tissues were obtained from the affiliated hospital of Ha'erbin medical university and the affiliated hospital of Dalian medical university. Genomic DNA was obtained from these samples by proteinase K digestion and phenol–chloroform extraction.

2.3. PCR amplification

Five microsatellite loci (mononucletide repeat BAT-26 and four dinucleotide repeats D17S261, D3S1283, D2S123 and D3S1611) were studied. The forward primer for each locus were labeled with a different fluorochrome chosen according to the expected size of the amplified fragments, as shown in Table 1.

PCR was performed on a ABI PCR System 2700 in a total reaction volume of 25 μL containing 50 ng genomic DNA, 400 nmol/L of both sense and antisense primer, 0.2 mmol/L of each dNTP, 1.5 mmol/L $MgCl_2$, 1.5 U *Taq* DNA polymerase in 1× PCR buffer (10 mmol/L Tris–HCl, 50 mmol/L KCl, pH 8.3). The cycling conditions were initial denaturation step of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, with an additional extension after the last cycle for 5 min at 72 °C.

2.4. Polymer synthesis and capillary coating

Short-chain linear polyacrylamide (SLPA) was synthesized in our laboratory. In a typical procedure, a solution of acrylamide (10 g) in deionized water (110 mL) was placed in a 250 mL airtight flask, immersed in a water bath kept at 60 °C, and degassed by bubbling N_2 for 30 min. Next, 3.5 mL 2-propanol, 0.625 mL 10% (w/v) APS and 0.625 mL 10% (v/v) TEMED were added in a consecutive fashion, and the resulting mixture was kept at 60 °C under N_2 flow for 2 h to ensure anaerobic conditions. The product was then extracted with 250 mL of ethanol, washed three times with 50 mL of ethanol, and kept at 50 °C for 4 h in a vacuum drier.

The viscosity average molecular mass ($M_v = 1.9 \times 10^5$) of SLPA was measured from the specific viscosity at 30 °C. A plot of η_{sp}/C (η_{sp}, specific viscosity; C, concentration of polymer) versus the concentration of the polymer was extrapolated to zero concentration, producing $[\eta]$ (106 mL/g) as the intercept. M_v was calculated according to the Mark–Houwink equation ($[\eta] = kM_v^a$), where $k = 6.31 \times 10^{-3}$ (mL/g) and $a = 0.8$.

The inner surface of the capillary was modified by covalent bonding of hydrophilic polymer [27]. Firstly, a new capillary was

Table 1
The primer sequences of five microsatellite loci

Microsatellite locus	Primer sequences	Repetitive sequences	Size (bp)	Related gene
BAT-26	5′-FAM-TGACTACTTTTGACTTCAGCC 5′-AACCATTCAACATTTTTAACCC	...$(A)_n$...	119	hMSH2
D17S261	5′-HEX-AGGGATACTATTCAGCCCGAGGTG 5′-ACTGCCACTCCTTGCCCCATTC	...$(CA)_n$...	128	p53
D3S1283	5′-TET-GGCAGTACCACCTGTAGAAATG 5′-GAGTAACAGAGGCATCGTGTATTC	...$(CA)_n$...	149	hMLH1
D2S123	5′-FAM-AAACAGGATGCCTGCCTTTA 5′-GGACTTTCCACCTATGGGAC	...$(CA)_n$...	210	hMSH2
D3S1611	5′-HEX-CCCCAAGGCTGCACTT 5′-AGCTGAGACTACAGGCATTTG	...$(CA)_n$...	263	hMLH1

rinsed with 0.2 mol/L NaOH, deionized water and methanol, respectively. Then the capillary was filled and left standing for 1 h with 1:1 (v/v) 3-methacryloxypropyltrimethoxysilan and methanol solution, and then for another hour with 3.4% acrylamide solution (10 mL 3.4% acrylamide solution containing 6 μL TEMED and 60 μL 10% APS). At last, the capillary was rinsed with deionized water for 20 min. The detection window was formed by stripping 6 mm of polyimide coating off the capillary with a razor blade, and the effective length is 36 cm.

2.5. Capillary electrophoresis analysis

Half microliter of each PCR products was mixed with 1 μL of TAMRA-labeled GeneScan 500 size standard (Applied Biosystems) and 12 μL of water. Each sample was denatured by heating to 95 °C for 3 min, then snap cooled in ice water. Capillary electrophoresis analysis was performed on an ABI 310 Genetic Analyzer using the homemade kit (a 50 μm × 47 cm coated capillary and 6% SLPA with urea) or the commercial kit. Samples were injected at 15 kV for 5 s and separation was performed at 15 kV and 40–70 °C. Data were collected with data collection software and analyzed by Gene-Scan 3.1 software. GeneScan-500 standard fragments in the size range of 100–300 bp were used for the calculation of relative sizes of microsatellite alleles by Local Southern Method.

3. Results and discussion

3.1. Effect of Urea on microsatellite analysis

Microsatellite analysis was carried out under denaturing conditions, and urea was usually added into the matrix and buffer as an additional denaturant. As shown in Table 2, with increasing quantity of urea the resolution of the neighboring peak pairs of the microsatellite loci was improved. The reason perhaps is that the hydrogen bonding between DNA and urea increases the mass of the DNA fragment. Moreover, urea could increase the solution viscosity and is helpful to separation. The fragment size of microsatellite loci could be calculated in comparison with GeneScan-500 size standard using the local southern method. It was found that the size of allelic fragments reduced with the addition of urea. The reason is that urea can eliminate the secondary structure of DNA resulting from hydrogen bonding.

Table 2
Resolution and size estimates for the analysis of five microsatellite loci at three different concentrations of urea

Locus	0 mol/L urea		4 mol/L urea		8 mol/L urea	
	R	Size (bp)	*R*	Size (bp)	*R*	Size (bp)
BAT-26	0.46	126.15	0.85	120.42	1.18	119.27
D17S261	2.01	132.49	2.42	128.32	2.83	127.63
D3S1283	1.82	153.71	2.33	150.86	2.75	149.09
D2S123	1.05	215.18	1.57	211.32	1.95	209.56
D3S1611	–	269.80	–	265.27	–	262.87

R: the resolution of the neighboring peak pairs of the microsatellite locus.

As we have known, for microsatellite analysis on the ABI 310 CE system, a commercial POP-4 kit is usually used. Fig. 1 shows the electropherogams of five microsatellite loci in the homemade kit (including 6% LPA with 8 mol/L urea matrix and neutral coated capillary: 47 cm × 50 μm i.d.) and commercial POP-4 kit. It was found that under the same conditions, homemade kit exhibited higher peak resolution and shorter analysis time than commercial POP-4 kit.

3.2. Effect of temperature on microsatellite analysis

Temperature is another key factor for microatellite analysis. The resolution of the neighboring peak pairs of the microsatel-

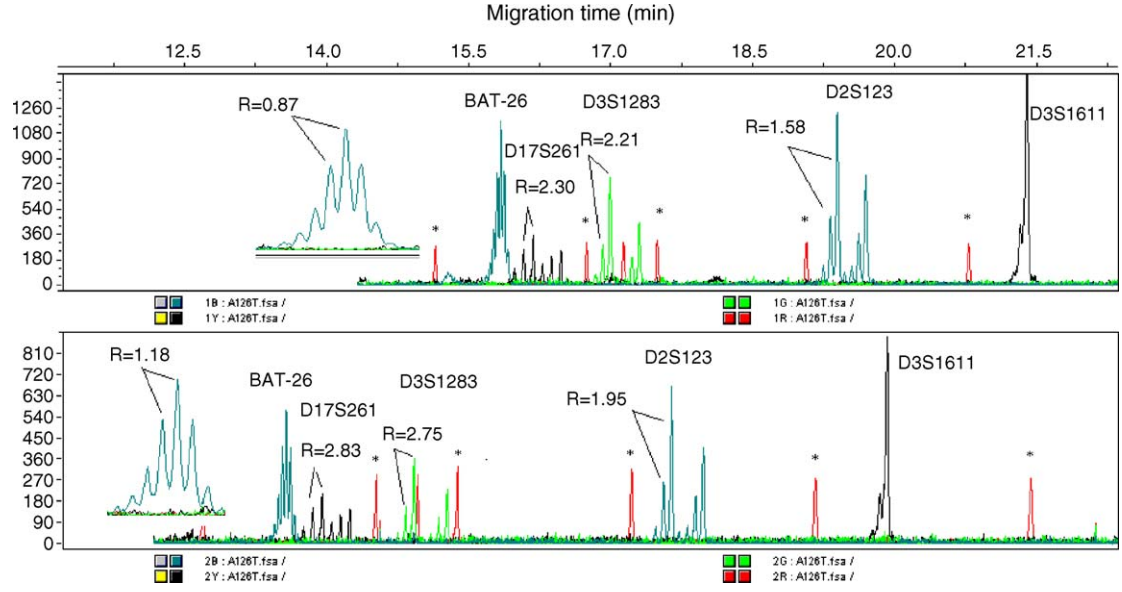

Fig. 1. Electropherograms of five microsatellite loci using the homemade kit and POP-4 kit: (up) POP-4 kit, (down) homemade kit. Electrophoretic conditions: injection, 15 kV × 5 s; temperature, 60 °C; voltage, −15 kV; LIF detection. The peaks with * are GeneScan 500 size standard. The part of BAT-26 was amplified. *R* is the resolution of the neighboring peak pair.

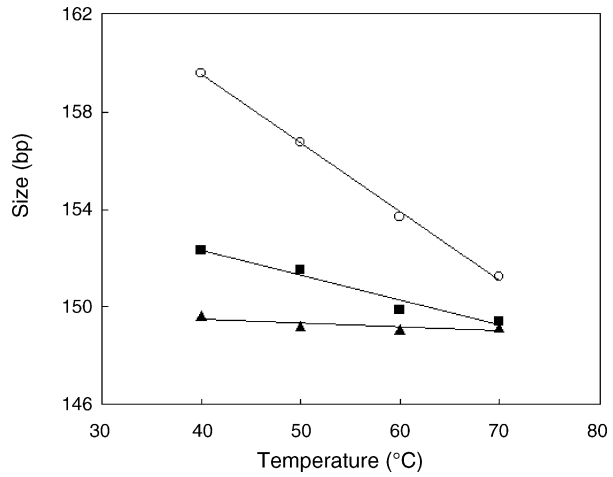

Fig. 2. Temperature vs. estimated size of D3S1283 locus at three different urea concentrations: (◯) without urea; (■) 4 mol/L urea; (▲) 8 mol/L urea.

lite loci was improved with increasing temperature from 40 to 70 °C, which attributes that elevated temperature is helpful to DNA denaturing [28]. When the resolution is satisfactory, 60 °C should be selected because of the shortened separation time. In addition, the response of the size estimate of the microsatellite loci with respect to temperature was also investigated. Fig. 2 illustrated the differences of the allelic size of D3S1283 locus in temperature range from 40 to 70 °C. It was obvious that the size of allele fragment of D3S1283 locus reduced with increasing temperature. Furthermore, the slopes of the linear relationship between estimated size and temperature were −0.280 base/°C without urea, −0.104 base/°C at 4 mol/L urea and −0.016 base/°C at 8 mol/L urea, respectively, which illustrated that the effect of temperature on the size estimate is less under denatured conditions than non-denature conditions. The temperature effect on the other four loci was similar to the D3S1283 locus (data not shown).

3.3. Detection of loss of heterozygosity

Loss of heterozygosity (LOH) was assigned if the allelic ratios of microsatellite locus in tumor tissue showed at least a 50% reduction compared with that in matched normal tissue [29]. Based on CE method, the allelic ratios are calculated as the peak height ratios of the microsatellite alleles, which offer a reliable method for the quantitative assessment of LOH. To estimate the degree of LOH, reduced ratios are calculated as the following formula:

$$\left\{1 - \frac{T_1/T_2}{N_1/N_2}\right\} \times 100\% \quad (1)$$

where T_1 and N_1 are the peak heights of the smaller alleles in tumor tissue and in normal tissue, respectively, T_2 and N_2 are the peak heights of the larger alleles in tumor tissue and in normal tissue, respectively. It was found that the optimal conditions for LOH analysis are the same as those for microsatellite analysis.

Finally, the selected CE conditions included 6% SLPA with 8 mol/L urea as sieving medium, 60 °C as separation temperature. Under these conditions, the precision (standard deviation) of allele size and allelic ratio ranged from 0.09 to 0.21 base and 1.2 to 3.0%, respectively.

3.4. Clinical applications

The five microsallite loci were analyzed in 52 sporadic gastric cancers using the developed method. Fig. 3 showed a typical

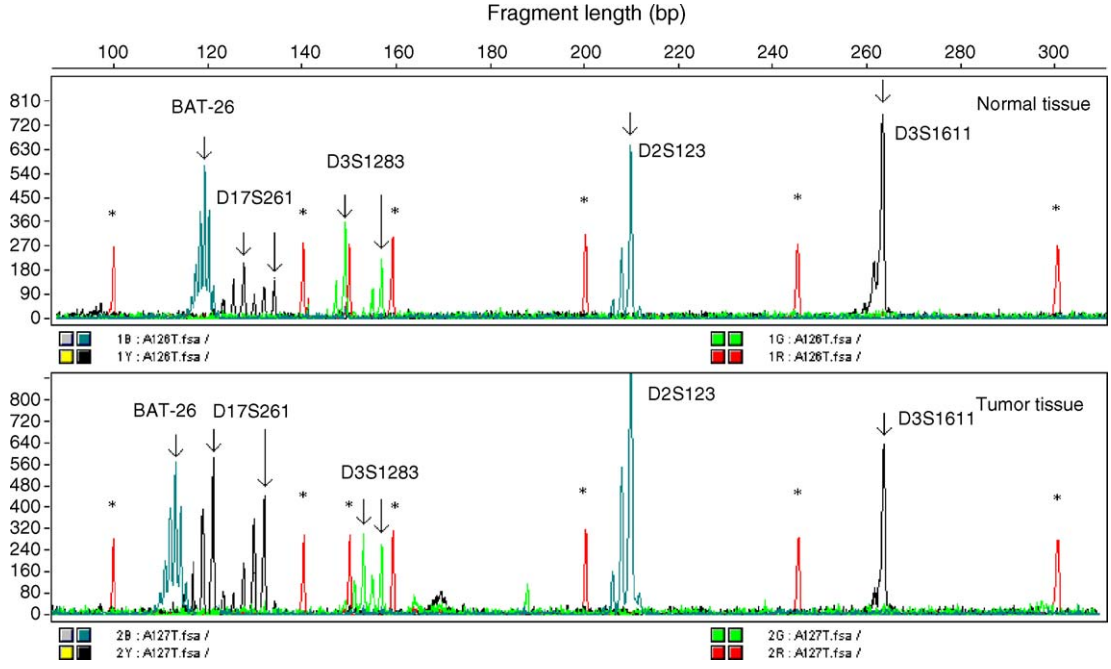

Fig. 3. Electropherograms of five microsatellite loci in the normal tissue and tumor tissue of No. 17 cancer patient. Electrophoretic conditions: neutral coated capillary, 47 cm × 50 μm i.d.; injection, 15 kV × 5 s; sieving matrix, 6% SLPA with 8 mol/L urea; temperature, 60 °C; voltage, −15 kV; LIF detection. Arrowheads indicate the predominant peaks for each allele at five loci. The peaks with * are GeneScan 500 size standard (100, 139, 150, 160, 200, 250 and 300 bases).

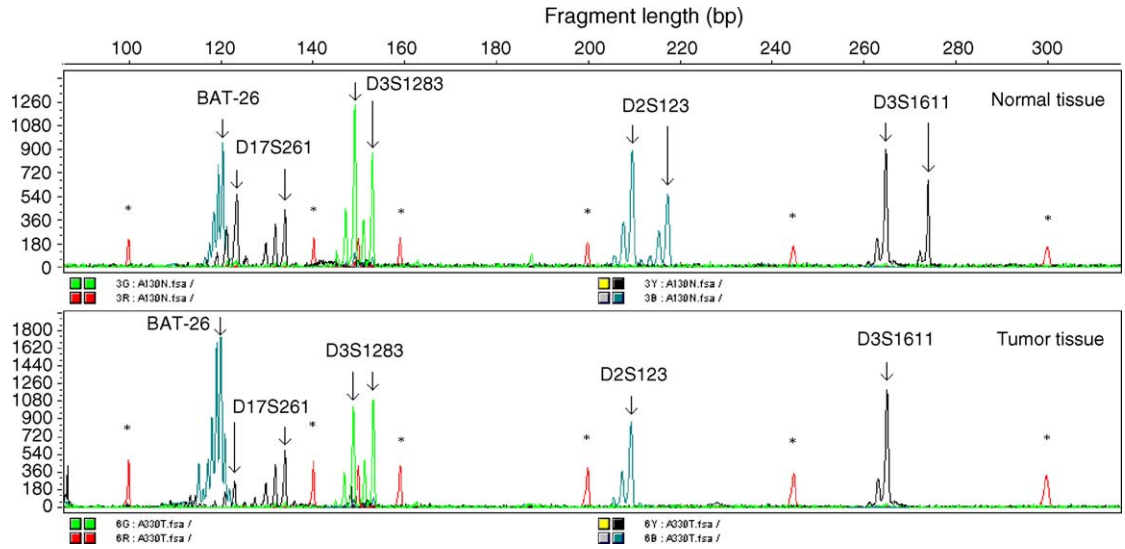

Fig. 4. Electropherograms of five microsatellite loci in the normal tissue and tumor tissue of No. 36 cancer patient. Others are the same as in Fig. 3.

electropherogram of microsatellite instability. Arrowheads indicate the predominant peaks for each alleles at five loci. The peaks with * are the GS 500 size standard (100, 139, 150, 160, 200, 250 and 300 bases). In the top panel, the germline alleles from the normal tissue are seen. The sizes of the predominant peaks at five loci are BAT-26 (119 bases), D17S261 (128 and 134 bases), D3S1283 (149 and 157 bases), D2S123 (209 bases) and D3S1611 (263 bases). The bottom panel is the electropherogram for the matched tumor tissue. The sizes of the predominant peaks at five loci are BAT-26 (112 bases), D17S261 (122 and 132 bases), D3S1283 (153 and 157 bases), D2S123 (209 bases) and D3S1611 (263 bases). The results showed that the three loci, BAT-26, D17S261 and D3S1283, display the shift.

When the sizes of the microsatellite alleles in tumor tissue were the same as those in normal tissue, loss of heterozygosity should be detected. A typical example of LOH was shown in Fig. 4. The allelic ratios of D17S261 locus in the normal tissue and tumor tissue were 1.23 and 0.41, respectively. The smaller allele reduced 67% (>50%) in the tumor tissue compared to that in the normal tissue calculated according to the formula (1), which implicated LOH at D17S261 locus. On the contrary, there was no LOH in D3S1283 locus because the smaller allele of D3S1283 locus reduced only 34% (<50%). In addition, LOH is obvious because the larger alleles of D2S123 and D3S1611 loci were completely lost in the tumor tissue.

MSI and LOH, at least one locus were observed in 15 of 52 (28.8%) patients. Only MSI (shift) was detected at the BAT-26 locus, whereas D3S1611 locus found only LOH. The other three loci had the two types of shift and LOH. The relationships of MSI and LOH phenotype to several clinicopathological variables were shown in Table 3. MSI and LOH appeared not to be significantly associated with gender, age, tumor position, and tumor stage. However, a more than two-fold increase of MSI and LOH was seen in males and over 61 years old patients. A statistically significant association was detected only between MSI and LOH and tumor-differentiated level ($p < 0.05$). Therefore, gastric cancers of poorly differentiated level showed increased risk of having MSI and LOH in comparison with those of well-differentiated level.

Table 3
Relationship between MSI and LOH and the pathology of gastric cancers

Groups	MSI and LOH		OR[a]	p[b]
	−	+		
Gender				
Female	17	4	1.0	0.199
Male	20	11	2.3	
Age				
⩽60	18	4	1.0	0.146
⩾61	19	11	2.6	
Tumor position				
Antrum	20	7	1.0	0.629
Cardia	17	8	1.3	
Differentiated level				
Well	25	5	1.0	0.024
Poorly	12	10	4.2	
Tumor stage				
Early	13	5	1.0	0.902
Advanced	24	10	1.1	

[a] OR, odds ratio.
[b] $p < 0.05$ was considered to be statistically significant.

4. Conclusions

Here, the homemade kit was first applied for microsatellite analysis on the ABI 310 capillary genetic analyzer. Its separation capability is better than the commercial kit and the cost is lower. MSI and LOH, at least one locus were observed in 15 of 52 (28.8%) sporadic gastric cancers. Moreover, it is found that a statistically significant association between MSI and LOH and tumor-differentiated level. Our results offer a low-cost, high throughput and high sensitive method for clinical microsatellite analysis.

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