

# Association of *IL1B* polymorphisms with gastric cancer in a Chinese population

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## Abstract

**Objectives:** To investigate the association between *IL1B* polymorphisms and risk of gastric cancer in a Chinese population, seven SNPs in the *IL1B* gene were selected for this study.

**Methods:** A multiplex genotyping method, which is based on adapter ligation and allele-specific amplification, was established to type seven SNPs on the *IL1B* gene simultaneously. One hundred and forty-one non-cancer outpatients and 97 patients with gastric cancer were genotyped, and the relation between *IL1B* polymorphisms and gastric cancer was statistically analyzed.

**Results:** The seven SNPs were successfully typed and the results were consistent with those obtained by both Sanger's sequencing and PCR-RFLP. Handling with statistical analysis, we observed significantly different genotype frequencies of 0794C>T ( $\chi^2=6.11$ ,  $P=0.05$ ), 1274C>T ( $\chi^2=6.86$ ,  $P=0.03$ ) and 2143T>C ( $\chi^2=6.86$ ,  $P=0.03$ ) between patients and controls.

**Conclusion:** ALM-ASA is a potential method for multiplex SNP typing with a low consumption of genomic DNA. SNPs 0794C>T, 1274C>T, and 2143T>C are associated with gastric cancer.

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**Keywords:** Risk association; Gastric cancer; Polymorphisms; *IL1B*; ALM-ASA

## Introduction

Stomach cancer was the fourth most common cancer worldwide with an estimated 934,000 new cases in 2002 (8.6% of new cancer cases), and is the second most common cause of death from cancer (about 700,000 deaths annually). Almost two-thirds of the cases occurred in developing countries and 42% in China alone [1]. Up to now, the aetiology of gastric cancer is still unclear, although multiple factors are thought to play a role in gastric carcinogenesis, including diet, tobacco smoking and alcohol consumption, infectious agents such as *Helicobacter pylori*, and genetic factors [2–4]. Recent studies suggest that genetic events such

as mutations in the *IL1B* gene may contribute to the aetiology of gastric cancer [4–10]. However, most of the studies were focused on three loci, 0794C>T, 1274C>T and 5277C>T. To better understand the risk associated with gastric cancer, seven SNPs (0794C>T, 1274C>T, 2143T>C, 2766T>del, 3298G>A, 5200G>A, and 5277C>T) in a Chinese population were used for the investigation in this report. Usually the genomic sample amount is very limited, but a large amount of genomic DNA is required to type multiple SNPs if a conventional SNP typing method, e.g., polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) [10–12] or polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) [5], is used. Although 4-plex SNPs could be simultaneously typed by multiplex polymerase chain reaction–restriction fragment length polymorphism (multiplex PCR/RFLP) assay, the throughput is relatively low [13]. In this report, we proposed an improved multiplex SNP typing method [14,15] to type the

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seven SNPs in the *IL1B* gene simultaneously with the consumption of a small amount of genomic DNA.

## Materials and methods

### Principle

The method is based on adapter ligation-mediated allele-specific amplification (“ALM-ASA”) (see Fig. 1). To decrease the consumption of genomic sample, a pre-amplification step is first carried out to produce a long fragment containing all SNPs of interest. Secondly, the pre-amplified DNA fragment is digested by a restriction endonuclease to form sticky ends. Thirdly, the adapter is ligated to either end of the digested fragment. The adapter is designed as a “Y” shape to prevent the extension reaction from the 3′-ends of the ligated fragments. Fourthly, allele-specific amplification is performed with allele-specific primers and a universal primer. To type a biallelic SNP, generally, two allele-specific primers are required. For  $n$ -plex SNP typing,  $n+1$  primers (a universal primer and  $n$  allele-specific primers) are needed in each of two reaction tubes (a wild-type-specific tube and a mutant-specific tube). The amplification only occurs when the 3′-end of the allele-specific primer matches the target sequence. Finally, the allele-specific

amplification products are separated by electrophoresis, e.g., agarose gel electrophoresis, polyacrylamide gel electrophoresis or chip-based electrophoresis. The genotype of each SNP is obtained by comparing the electrophoresis profiles from each of the two tubes.

### Study subjects

A total of 238 unrelated Mainland Chinese outpatients (141 non-cancer and 97 patients with gastric cancer), examined at the Jinling Hospital in Nanjing, participated in the study after giving written informed consent. They were all born and raised in the city of Nanjing and were of Han nationality. In the control group, the average age was  $62.0 \pm 16.5$  years (range, 18–90 years) and the male:female ratio was 1.9:1. In the gastric cancer group, the average age was  $63.3 \pm 12.4$  years (range, 21–85 years) and the male:female ratio was 3.0:1. Genomic DNA was extracted according to the standard phenol/chloroform method from whole blood collected in tubes containing anticoagulant EDTA. The purity and concentration of the extracted DNA were determined by UV–VIS Spectrophotometer (Naka Instruments Co., Ltd., Tokyo, Japan). The extracted DNA was stored at 4 °C in TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.0).

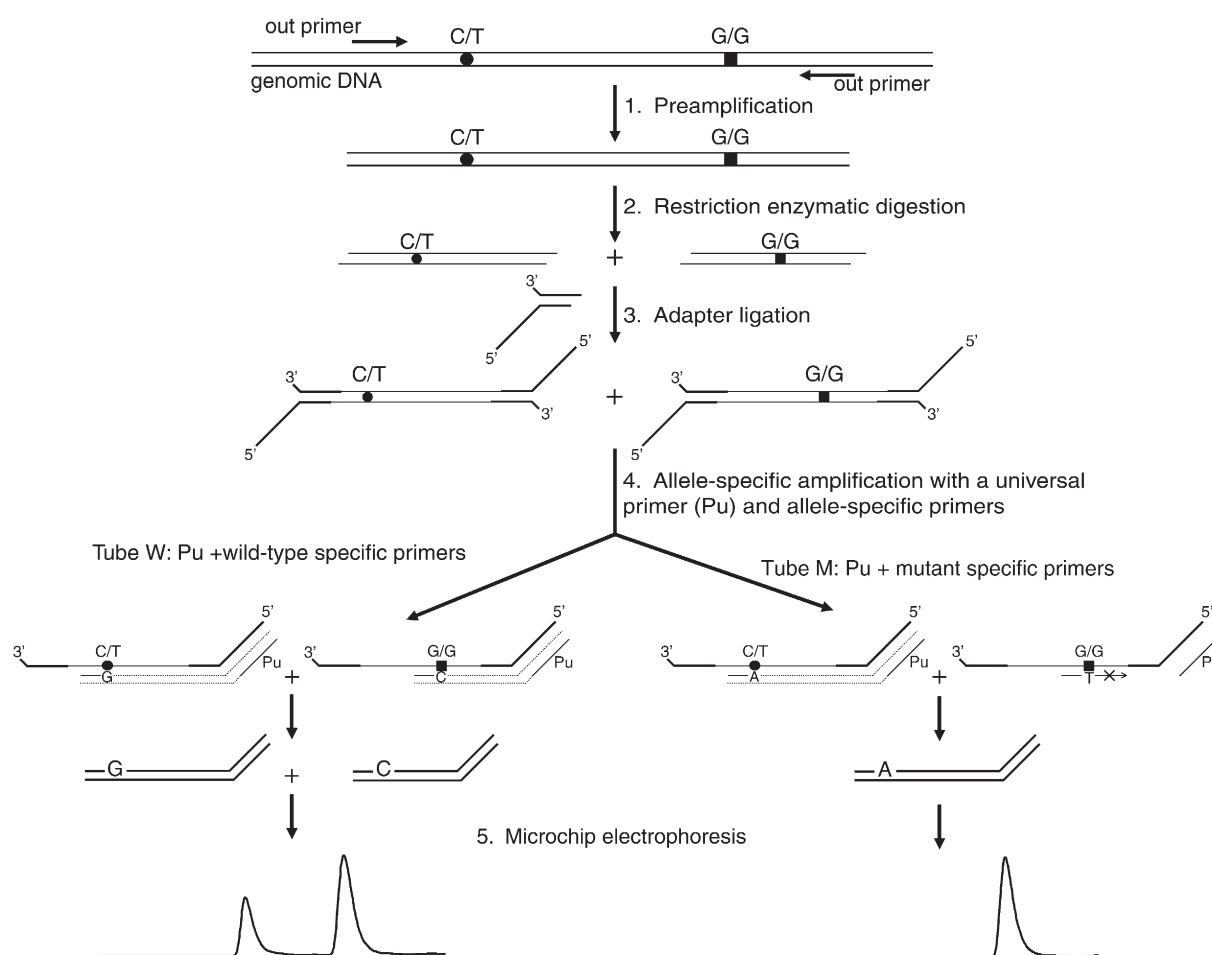


Fig. 1. The schematic of SNP genotyping by ALM-ASA. Two SNPs with the genotypes CT and GG were used as an example for the illustration.

## Genotyping

Pre-amplification of a 4610-bp *IL1B* gene fragment containing the SNPs of interest was carried out using primer pair of 5'-ACCCTGCATACCGTATGTTCTCTGC-3' and 5'-GTA-CAGGTGCATCGTGACATAAGC-3' in 10 µL of mixture containing 1× LA PCR reaction buffer II, 2.5 mmol/L MgCl<sub>2</sub>, 0.4 mmol/L of each dNTP, 0.4 µmol/L each primer, 0.5 U of LA Taq™ DNA polymerase (TaKaRa Biotech., Dalian, China), and 10–20 ng of genomic DNA. Thermal cycling was performed in PTC-225 Thermal Cycler (MJ Research, Inc., Massachusetts, USA) with an initial denaturation of 5 min at 94 °C, followed by 25 cycles of 1 min at 94 °C, 30 s at 60 °C, and 5 min at 68 °C. Final extension was 10 min at 68 °C. Then, 0.5 µL of pre-amplified PCR products (about 100–500 ng) was digested with 5 U of *Mbo*I restriction enzyme (TaKaRa Biotech., Dalian, China) for 2 h at 37 °C in 5 µL of mixture. After heat inactivation for 15 min at 70 °C, 1 µL of the digested DNA was incubated with 1.0 µmol/L adapter for 2 h at 16 °C in 5 µL of mixture containing 5 U of T4 DNA ligase (TaKaRa Biotech., Dalian, China). The adapter was prepared by annealing oligonucleotides of 5'-CCCCACTTCTTGTCTCTCAT-CAGGCGCATCACTCG-3' and 5'-pGATCCGAGT-GATGCGCTAAG-3' for 5 min at 92 °C, and then slowly cooled down to room temperature. The ligation was terminated by heating at 70 °C for 15 min. PCR on each adapter-ligated fragment was carried out in two separate tubes in 10 µL of mixture consisting of 10 mmol/L Tris–HCl (pH 8.0), 50 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L of each dNTP, 0.5 µL of adapter-ligated template solution, 1.0 µmol/L universal primer (5'-CCCCACTTCTTGTCTCTCAT-3'), 2.0 µmol/L allele-specific primer mixture (see Table 1), and 0.4 U of Taq DNA polymerase (TaKaRa Biotech., Dalian, China). The PCR cycling was carried out as follows: 94 °C for 3 min, followed

by 30 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min. Final extension was completed at 72 °C for 7 min. Finally, allele-specific PCR products were analyzed by microchip electrophoresis on Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Waldbronn, Germany) with commercially available DNA 1000 assay sizing reagent kits. All the oligonucleotides were synthesized and purified by Invitrogen Inc. (Shanghai, China).

## Statistical analysis

Hardy–Weinberg equilibrium of alleles at individual loci was assessed by  $\chi^2$  statistics. Haplotype frequencies for pairs of alleles were estimated using the Estimating Haplotype frequencies software program (<http://linkage.rockefeller.edu/software/eh>). Odds ratios with Cornfield 95% confidence intervals and unconditional logistic regression models controlling for the effects of possible confounders were computed using STATA version 8.0 software (STATA Corp., College Station, TX). Allele and genotype frequencies were calculated from two groups, and differences between patients and controls were tested by Fisher's Exact test and  $\chi^2$  test (SPSS V11.5).

## Results

### Development of ALM-ASA

To check the specificity of allele-specific primers, each of single-locus allele-specific amplifications was carried out separately using the universal primer and an allele-specific primer. The wild-type template was used to validate the specificity of mutant-specific primer and the mutant template was used to validate the specificity of wild-type-specific primer. Because the templates with del/del genotype at 2766 locus or

Table 1  
Sequences and locations of allele-specific primers used in this study

| SNP <sup>a</sup> | Primer | Primer sequence (5'→3') <sup>b</sup> | Position <sup>c</sup> | Tm (°C) <sup>d</sup> | Amplicon length (bp) | Concentration in 7-plex PCR (µmol/l) |
|------------------|--------|--------------------------------------|-----------------------|----------------------|----------------------|--------------------------------------|
| 0794C>T          | 0794C  | TGCAATTGACAGAGAGCTGCC                | 0774 to 0794          | 55.6                 | 1005                 | 0.111                                |
|                  | 0794T  | TGCAATTGACAGAGAGCTGCT                |                       | 55.6                 |                      |                                      |
| 1274C>T          | 1274C  | CTACTTCTGCTTTTGAAAGCC                | 1254 to 1274          | 53.7                 | 525                  | 0.111                                |
|                  | 1274T  | CTACTTCTGCTTTTGAAAGCT                |                       | 51.7                 |                      |                                      |
| 2143T>C          | 2143T  | AGGCAAATGGGGAGAAAACAT                | 2123 to 2143          | 55.6                 | 164                  | 0.444                                |
|                  | 2143C  | AGGCAAATGGGGAGAAAACAC                |                       | 57.6                 |                      |                                      |
| 2766T>del        | 2766T  | TTCCTACTATGAAATGGGC <del>AA</del>    | 2766 to 2786          | 51.7                 | 576                  | 0.888                                |
|                  | 2766d  | TTCCTACTATGAAATGGGC <del>AC</del>    |                       | 53.7                 | 575                  |                                      |
| 3298G>A          | 3298G  | CCAGAGAAAAGTGGCTGC <del>ATC</del>    | 3298 to 3318          | 57.6                 | 268                  | 0.222                                |
|                  | 3298A  | CCAGAGAAAAGTGGCTGC <del>ATT</del>    |                       | 57.6                 |                      |                                      |
| 5200G>A          | 5200G  | GCAGGCCTCGTGAGGCGA <del>TGC</del>    | 5200 to 5220          | 65.4                 | 357                  | 0.185                                |
|                  | 5200A  | GCAGGCCTCGTGAGGCGA <del>TGT</del>    |                       | 63.4                 |                      |                                      |
| 5277C>T          | 5277C  | GCCTCGTTATCCCATGTGA <del>CG</del>    | 5277 to 5297          | 59.5                 | 434                  | 0.037                                |
|                  | 5277T  | GCCTCGTTATCCCATGTGA <del>CA</del>    |                       | 57.6                 |                      |                                      |

<sup>a</sup> The refSNP IDs of 0794C>T, 1274C>T, 2143T>C, 2766T>del, 3298G>A, 5200G>A, and 5277C>T are rs16944, rs1143627, rs1143629, rs3917354, rs3917356, rs1143633, and rs1143634, respectively.

<sup>b</sup> The underlined letters represent the artificially mismatched base.

<sup>c</sup> Nucleotide positions according to GenBank accession number: AY137079.

<sup>d</sup> Calculated according to the software of Primer Premier Version 5.0 (<http://www.premierbiosoft.com/>).

TT genotype at 5277 locus were not observed in this study, “mock” templates, generated artificially by PCR [16], were used to test the specificity of the allele-specific primers. The specificity is perfect if no amplicon is observed by amplifying a wild-type template with a mutant-specific primer, and a mutant template with wild-type-specific primer. The result showed that only one pair of allele-specific primers (1274C and 1274T) was highly specific. While by introducing an artificial mismatch base at the third position from the 3'-terminus of the primer [17], the specificity of other 6 pairs of primers (specific to wild type and mutant respectively) was improved significantly.

Once the cycling conditions have been optimized for each reaction, the wild-type-specific primers and the mutant-specific primers were combined separately into multiplex mixtures. Each allele-specific primer corresponded to a PCR product with a specific length, which was used to identify the polymorphic locus. For typical multiplex PCR reactions, one of the problems is the impaired amplification efficiency of allele-specific products. Even if equimolar allele-specific primers were employed, the amplification efficiency might change at a different locus (Fig. 2A), which is primarily due to the different annealing efficiency of allele-specific primers [18,19]. Using multiplex PCR guidelines, we optimized the cycling conditions and reaction components to generate the best amplification of all products. The relative amplicon yields were increased or decreased by adjusting primer concentrations, thus generating more balanced multiplex reactions (Fig. 2B). By trying various concentrations of dNTP and MgCl<sub>2</sub> for 7-plex PCR, the optimum concentrations of dNTP and MgCl<sub>2</sub> were determined as 0.2 mmol/L and 1.5 mmol/L, respectively, which are similar to the concentrations suggested in the protocol of a PCR reagent kit. Therefore, an even amplification could be easily achieved by only adjusting the proportion of the allele-specific primers.

### *The effects of different artificial mismatch base on the specificity of amplification*

To investigate the effect of the species of an artificial mismatch base on the specificity of amplification, various artificial bases (A, T and G) were introduced into the third position from the 3'-end of the C allele-specific primer 0794C. As shown in Fig. 3(a, b and c), non-specific amplification was not observed by amplifying the sample of 0794TT genotype with the C allele-specific primer containing any types of artificial mismatch bases; while there was non-specific amplification shown as peak 7 in Fig. 3(d) when the conventional 0794C allele-specific primer without an artificial mismatch base was used. Therefore, the specificity was improved significantly by introducing an artificial mismatch base into the 3'-end region of the allele-specific primer. To simplify the primer design, the artificial mismatch base same as the target at the corresponding position was introduced in the allele-specific primer.

### *Competitive amplification of allele-specific primers sharing the same template*

Fig. 2A indicated that the amplification efficiency of allele-specific primers sharing the same template was different. This suggested that competitive amplification took place between primers sharing the same template. To investigate the strength of the competitive amplification, multiplex PCR using wild-type-specific primers 5200G and 5277C with the concentration ratio of 10/1, 5/1, 2/1, 1/1, 1/2, 1/5 and 1/10 (primer 5200G/5277C) was performed. The results (Fig. 4A) showed that the amplification efficiency of primer 5277C was much stronger than that of 5200G. No amplicon from the primer 5200G was observed if the same concentration of two allele-specific primers was used, and the concentration of primer 5200G (1.0

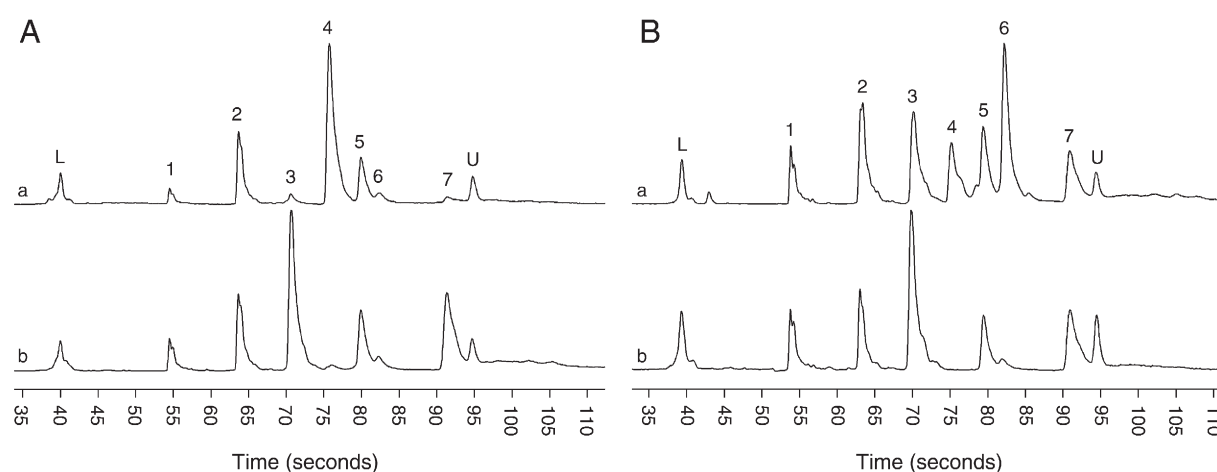


Fig. 2. Electrophoretograms of 7-plex PCR with different concentration of allele-specific primers. (A) Electrophoretograms of 7-plex PCR with equimolar allele-specific primers. Peaks 1–7 represent the allele-specific products of 2143, 3298, 5200, 5277, 1274, 2766 and 0794 loci respectively; peak L represents the lower internal standard with the length of 15 bp; peak U represents the upper internal standard with the length of 1500 bp. (B) Electrophoretograms of 7-plex PCR with optimized concentration (listed in Table 1). The assignment of all peaks is the same as that in panel A. Electrophoretograms a and b are the electrophoretograms from products by wild-type-specific and mutant-specific primers respectively. The genotypes are TC, GA, GA, CC, CT, TT and CT at 2143, 3298, 5200, 5277, 1274, 2766 and 0794 loci respectively.

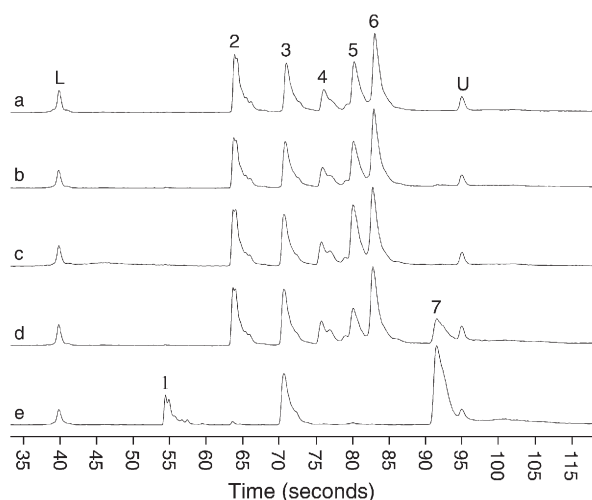


Fig. 3. The effects of different artificial mismatch base on the specificity of amplification. (a–c) Different artificial mismatch bases (A, T, G, respectively) are introduced at the third position from the 3'-end of allele-specific primers for typing allele C at 0794. (d) No artificial mismatch base is introduced in allele-specific primer for typing allele C at 0794. (e) The genotyping result of 7-plex PCR with all mutant-specific primers. The assignment of all peaks is the same as that in Fig. 2A. The genotypes are CC, GG, GA, CC, CC, TT and TT at 2143, 3298, 5200, 5277, 1274, 2766 and 0794 loci respectively.

mmol/L) should be 5 times higher than that of primer 5277C (0.2 mmol/L) to get an even amplification (Fig. 4Ac); but a clear amplification band was observed in a single-plex PCR with the low concentration of primer 5277C (0.05 mmol/L). The biased amplification was also observed in multiplex PCR with mutant-specific primers 5200A and 5277T. Therefore, it is necessary to optimize the primer concentration in multiplex PCR to get an even amplification.

As multiple primers in a single PCR may cause non-specific amplification, a template, which could be amplified by all wild-type-specific primers, was used for multiplex PCR by six of seven wild-type-specific primers, and seven 6-plex PCRs were totally performed. Results in Fig. 4B indicated that there was no non-specific product interfering with the SNP identification in the multiplex reaction mixture. Multiplex PCRs using six of seven mutant-specific primers were also carried out, and we came to the same conclusion. As the chip-based electrophoresis can give a precise length of an amplicon, mispriming product is not a problem if the size of the product is different from other allele-specific amplicons.

#### Genotypes

The typing results of five typical genomic samples are shown in Fig. 5. Based on the peaks in a pair of electrophoretograms, it

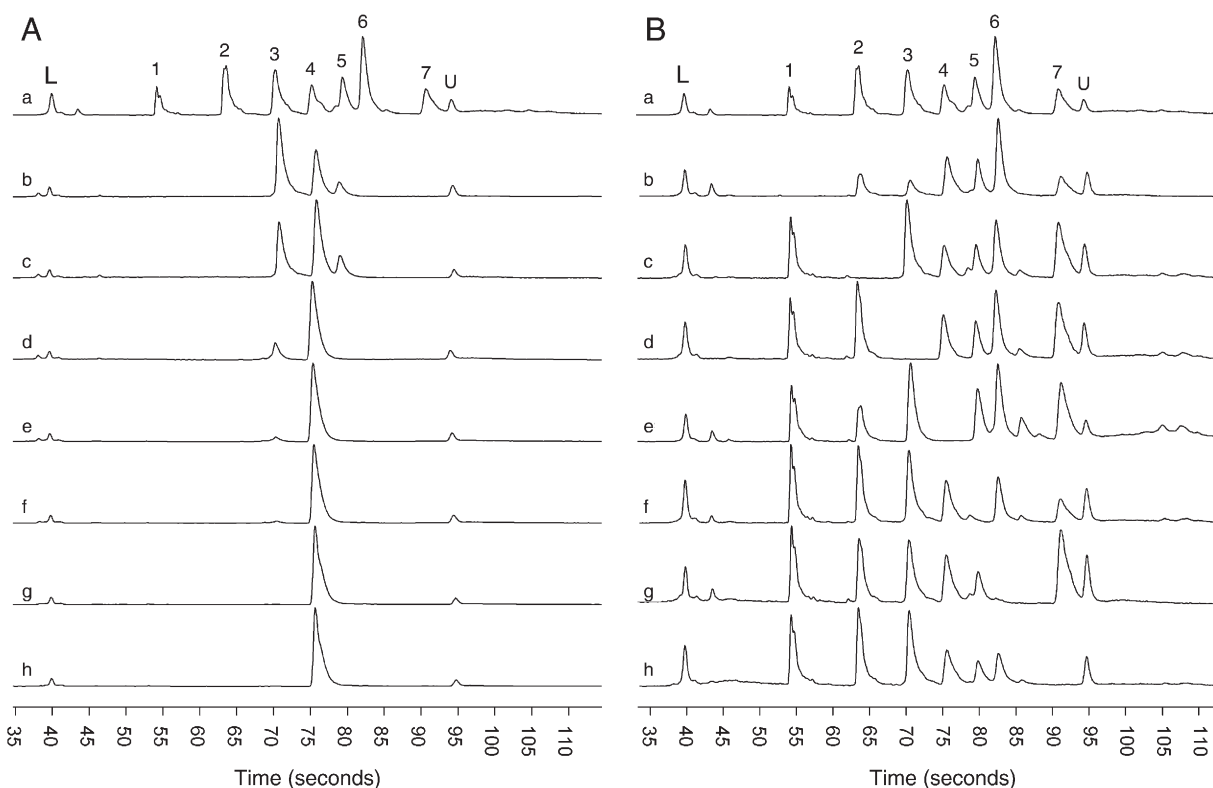


Fig. 4. (A) Competitive amplification of allele-specific primers sharing the same template. The concentration ratios of primer 5200G to 5277C were 10/1, 5/1, 2/1, 1/1, 1/2, 1/5 and 1/10 for electrophoretograms b, c, d, e, f, g, and h, respectively. Electrophoretogram a was from a standard sample with all seven allele-specific peaks. (B) Multiplex PCR by six of seven allele-specific primers. Electrophoretogram a was from a standard sample with all seven allele-specific peaks. Electrophoretograms b, c, d, e, f, g and h were from 6-plex amplicons without the addition of primer 2143T, 3298G, 5200G, 5277C, 1274C, 2766T and 0794C, respectively. The assignment of all peaks is the same as that in Fig. 2A.

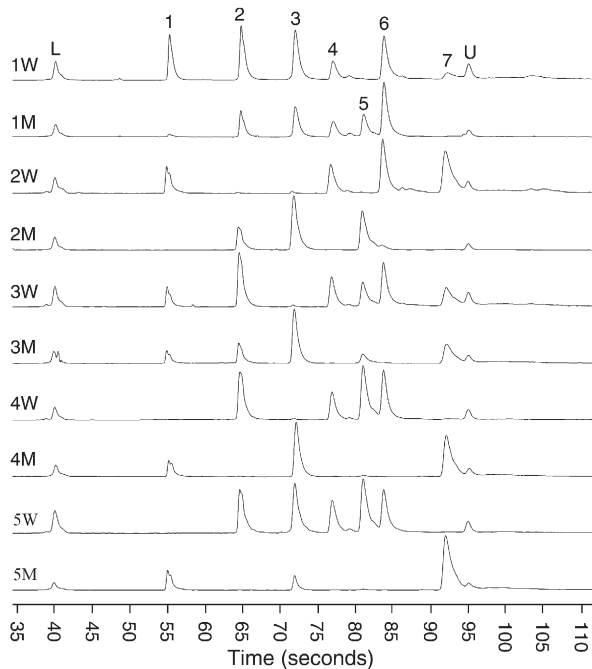


Fig. 5. The typical typing results of seven SNPs for 5 individual genome samples by microchip electrophoresis. Electrophoretograms 1W, 2W, 3W, 4W and 5W were from the wild-type-specific amplicons; electrophoretograms 1M, 2M, 3M, 4M and 5M were from the mutant-specific amplicons. The assignment of all peaks is same as that in Fig. 2A. The genotypes of samples 1, 2, 3, 4 and 5 were TT, TT, TC, CC and CC at 2143 locus, GA, AA, GA, GG and GG at 3298 locus, GA, AA, AA, AA and GA at 5200 locus, CT, CC, CC, CC and CC at 5277 locus, TT, TT, TC, CC and CC at 1274 locus, Tdel, TT, TT, TT and TT at 2766 locus, and CC, CC, CT, TT and TT at 0794 locus, respectively.

is easy to determine the genotypes of each SNP. To evaluate the reliability of the method developed in this report, 15 typical genomic samples were selected and re-genotyped by PCR-RFLP. For further confirmation, each possible genotype of the seven SNPs was detected by Sanger's sequencing method. The typing results were completely consistent with those obtained by ALM-ASA.

Frequency distribution of age and sex in gastric cancer patients and healthy controls is presented in Table 2. There were no statistically significant differences between patients and controls. In both control and patient population, the alleles at the individual loci of *IL1B* were in Hardy–Weinberg equilibrium, with non-significant  $\chi^2$  values (Table 3). Near-complete linkage disequilibrium was observed among polymorphisms at 0794, 1274, 2143, and 3298 loci, with the haplotype of either C-T-T-A or T-C-C-G, which has the frequency of 96.0% in the control groups ( $\chi^2=688.8$ ) and 92.2% in the patient groups ( $\chi^2=479.4$ ).

Based on the typing results (Table 3), the association of the seven SNPs with the risk of gastric cancer in Chinese subjects was investigated. Comparison of genotype frequencies of 0794C>T ( $\chi^2=6.11$ ,  $P=0.05$ ; Table 3), 1274C>T ( $\chi^2=6.86$ ,  $P=0.03$ ; Table 3) and 2143T>C ( $\chi^2=6.86$ ,  $P=0.03$ ; Table 3) showed a significant difference between gastric cancer patients and controls. No significant difference in genotype frequencies of 2766T>del ( $\chi^2=0$ ,  $P=1.00$ ; Table 3), 3298G>A ( $\chi^2=2.38$ ,

$P=0.31$ ; Table 3), 5200G>A ( $\chi^2=0.99$ ,  $P=0.61$ ; Table 3), and 5277C>T ( $\chi^2=0.82$ ,  $P=0.36$ ; Table 3) was observed between patients and controls. Moreover, we observed a significantly increased frequency of the 1274TT genotype ( $\chi^2=5.40$ ,  $P=0.02$ ; Table 3) and significantly decreased frequencies of the 0794CT genotype ( $\chi^2=5.93$ ,  $P=0.02$ ; Table 3), the 2143CC genotype ( $\chi^2=5.40$ ,  $P=0.02$ ; Table 3), and the 2143TC genotype ( $\chi^2=5.64$ ,  $P=0.02$ ; Table 3) in patients compared with controls. A near significantly decreased frequency of the 0794TT genotype ( $\chi^2=3.36$ ,  $P=0.07$ ; Table 3) was observed in patients compared with controls. In addition, we have looked for differences in allele frequency between patients and controls. Significant differences in allele frequencies of 1274T allele ( $\chi^2=4.46$ ,  $P=0.04$ ; Table 3) and 2143T allele ( $\chi^2=4.46$ ,  $P=0.04$ ; Table 3) were observed. There was no statistically significant difference in allele frequencies of 0794C>T ( $\chi^2=2.65$ ,  $P=0.10$ ; Table 3), 2766T>del ( $\chi^2=0$ ,  $P=1.00$ ; Table 3), 3298G>A ( $\chi^2=1.59$ ,  $P=0.21$ ; Table 3), 5200G>A ( $\chi^2=0.70$ ,  $P=0.40$ ; Table 3) and 5277C>T ( $\chi^2=0.81$ ,  $P=0.37$ ; Table 3) between patients and controls.

In comparison with the 1274C homozygotes, the 1274T homozygotes were associated with a significantly increased risk of gastric cancer with an OR of 2.51 ( $\chi^2=5.40$ ,  $P=0.02$ ; Table 3). Carriers of 0794T or 2143C seem to be protected against gastric cancer at ORs of 0.45 ( $\chi^2=5.98$ ,  $P=0.01$ ; Table 3) and 0.43 ( $\chi^2=6.65$ ,  $P=0.01$ ; Table 3) respectively, but with no significant difference in the risk of gastric cancer between the homozygotes and heterozygotes of these two SNPs (Table 3). No association with gastric cancer was found for SNPs 2766T>del, 3298G>A, 5200G>A and 5277C>T. When age and sex were included in a logistic model, the estimated effects of *IL1B* genotypes were similar in subgroups of gastric cancer patients defined by age (categorized as <50, 50–59, 60–69 and >69) and sex (Table 3).

## Discussion

In this study, we used an adapter to ligate either end of the digested fragment to reduce the number of primers in multiplex PCR. As every PCR shared a common primer specific to the adapter, only  $n+1$  primers were employed for  $n$ -plex PCR. Therefore, the preferential amplification of certain targets was

Table 2  
Frequency distribution analysis of age and sex in gastric cancer patients and healthy controls

|                    | Controls ( <i>n</i> =141) <sup>a</sup> | Patients ( <i>n</i> =97) <sup>a</sup> | <i>P</i> -value <sup>b</sup> |
|--------------------|----------------------------------------|---------------------------------------|------------------------------|
| <i>Age (years)</i> |                                        |                                       |                              |
| <50                | 21 (14.9%)                             | 11 (7.8%)                             | 0.06                         |
| 50–59              | 19 (13.5%)                             | 19 (13.5%)                            |                              |
| 60–69              | 75 (53.2%)                             | 38 (27.0%)                            |                              |
| >69                | 26 (18.4%)                             | 29 (20.6%)                            |                              |
| <i>Sex</i>         |                                        |                                       |                              |
| Male               | 93 (66.0%)                             | 73 (75.3%)                            | 0.13                         |
| Female             | 48 (34.0%)                             | 24 (24.7%)                            |                              |

<sup>a</sup> Data are expressed as the number of subjects (percentage of each genotype).

<sup>b</sup> Two-sided  $\chi^2$  test.

Table 3

Frequency distribution of *IL1B* polymorphisms between patients and controls and its association with risk of gastric cancer

| SNP       | Genotype     | Patients (n=97) <sup>a</sup> | Controls (n=141) <sup>a</sup> | ORc (95%CI) <sup>b</sup> | ORa (95%CI) <sup>b</sup> |
|-----------|--------------|------------------------------|-------------------------------|--------------------------|--------------------------|
| 0794C>T   | C/C          | 27 (19.1%)                   | 21 (14.9%)                    | 1.00                     | 1.00                     |
|           | C/T          | 46 (32.6%)                   | 82 (58.2%)                    | 0.44 (0.22–0.86)         | 0.47 (0.24–0.94)         |
|           | T/T          | 24 (17.0%)                   | 38 (27.0%)                    | 0.49 (0.23–1.06)         | 0.52 (0.24–1.13)         |
|           | T carriers   | 70 (49.6%)                   | 120 (85.1%)                   | 0.45 (0.24–0.86)         | 0.49 (0.25–0.93)         |
|           | T allele     | 94 (48.5%)                   | 158 (56.0%)                   |                          |                          |
| 1274C>T   | C/C          | 21 (14.9%)                   | 39 (27.7%)                    | 1.00                     | 1.00                     |
|           | C/T          | 49 (34.8%)                   | 82 (58.2%)                    | 1.11 (0.59–2.10)         | 1.07 (0.56–2.05)         |
|           | T/T          | 27 (19.1%)                   | 20 (14.2%)                    | 2.51 (1.14–5.50)         | 2.42 (1.08–5.42)         |
|           | T carriers   | 76 (53.9%)                   | 102 (72.3%)                   | 1.38 (0.75–2.54)         | 1.35 (0.73–2.51)         |
|           | T allele     | 103 (53.1%)                  | 122 (43.3%)                   |                          |                          |
| 2143T>C   | T/T          | 27 (19.1%)                   | 20 (14.2%)                    | 1.00                     | 1.00                     |
|           | C/T          | 49 (34.8%)                   | 82 (58.2%)                    | 0.44 (0.22–0.87)         | 0.48 (0.24–0.95)         |
|           | C/C          | 21 (14.9%)                   | 39 (27.7%)                    | 0.40 (0.18–0.87)         | 0.42 (0.19–0.94)         |
|           | C carriers   | 70 (49.6%)                   | 121 (85.8%)                   | 0.43 (0.22–0.82)         | 0.46 (0.24–0.89)         |
|           | C allele     | 91 (46.9%)                   | 160 (56.7%)                   |                          |                          |
| 2766T>del | T/T          | 93 (66.0%)                   | 134 (95.0%)                   | 1.00                     | 1.00                     |
|           | T/del        | 4 (2.8%)                     | 7 (5.0%)                      | 0.82 (0.23–2.89)         | 0.82 (0.23–2.90)         |
|           | del/del      | 0 (0.0%)                     | 0 (0.0%)                      | 0.00                     |                          |
|           | del carriers | 4 (2.8%)                     | 7 (5.0%)                      | 0.82 (0.23–2.89)         | 0.82 (0.23–2.90)         |
|           | del allele   | 4 (2.8%)                     | 7 (5.0%)                      |                          |                          |
| 3298G>A   | G/G          | 22 (15.6%)                   | 38 (27.0%)                    | 1.00                     | 1.00                     |
|           | G/A          | 54 (38.3%)                   | 83 (58.9%)                    | 1.12 (0.60–2.10)         | 1.09 (0.58–2.06)         |
|           | A/A          | 21 (14.9%)                   | 20 (14.2%)                    | 1.81 (0.81–4.06)         | 1.67 (0.72–3.89)         |
|           | A carriers   | 75 (53.2%)                   | 103 (73.0%)                   | 1.26 (0.69–2.30)         | 1.21 (0.66–2.23)         |
|           | A allele     | 96 (49.5%)                   | 123 (43.6%)                   |                          |                          |
| 5200G>A   | G/G          | 6 (4.3%)                     | 13 (9.2%)                     | 1.00                     | 1.00                     |
|           | G/A          | 53 (37.6%)                   | 79 (56.0%)                    | 1.78 (0.60–5.27)         | 1.75 (0.58–5.25)         |
|           | A/A          | 38 (27.0%)                   | 49 (34.8%)                    | 2.02 (0.66–6.15)         | 1.91 (0.61–5.98)         |
|           | A carriers   | 91 (64.5%)                   | 128 (90.8%)                   | 1.87 (0.64–5.42)         | 1.80 (0.61–5.25)         |
|           | A allele     | 129 (66.5%)                  | 177 (62.8%)                   |                          |                          |
| 5277C>T   | C/C          | 92 (65.2%)                   | 138 (97.9%)                   | 1.00                     | 0.00                     |
|           | C/T          | 5 (3.5%)                     | 3 (2.1%)                      | 2.50 (0.58–10.7)         | 2.21 (0.51–9.59)         |
|           | T/T          | 0 (0.0%)                     | 0 (0.0%)                      | 0.00                     | 1.00                     |
|           | T carriers   | 5 (3.5%)                     | 3 (2.1%)                      | 2.50 (0.58–10.7)         | 2.21 (0.51–9.59)         |
|           | T allele     | 5 (3.5%)                     | 3 (2.1%)                      |                          |                          |

<sup>a</sup> Data are expressed as the number of subjects (percentage of each genotype).<sup>b</sup> Obtained from unconditional logistic regression models; ORc, crude OR; ORa, adjusted OR with adjustment for age and sex.

greatly minimized [18,20]. In addition, to suppress the non-specific amplification in multiplex PCR, the adapter was specially designed as a “Y” shape to prevent the extension of templates. The extension reaction of adapter-specific primer only occurred when the 3'-end of an allele-specific primer was complementary to the base in the target; thus undesired products in PCR mixtures were greatly reduced. Furthermore, the possibility of mispriming by primers used in our study is minimized greatly by the pre-amplification of a DNA fragment containing all SNPs of interest. We used the Premier Primer 5 for checking the possible mispriming by the universal primer specific to the adapter (third primer) and allele-specific primers. If mispriming occurs, we can change the sequence of the third primer or employ the other strand of the allele-specific primer as there are two possible sequences of an allele-specific primer for a given SNP.

In ALM-ASA, the universal primer is adapter-specific and is common for all SNPs. A pair of allele-specific primers is complementary to the target sequence and is designed to differ by a 3'-terminal nucleotide, which is specific to one of the alleles of an SNP. Therefore, the location of an allele-specific

primer is fixed, and only the length of an allele-specific primer can be regulated to get a similar melting temperature. A uniform allele-specific amplification can be realized by adjusting the concentration of each primer. Our results also showed that the switching performance of 3'-end in an allele-specific primer is not enough for an accurate SNP typing. To block the non-specific amplification, an artificial mismatch base was introduced in the 3'-terminal region, which significantly improved the specificity of ALM-ASA.

Our study demonstrates several features of the ALM-ASA system, including a relatively low consumption of genomic DNA (usually 10–20 ng), an easy and low-cost set up (simple condition optimization), and a relatively high multiplexing capability (simultaneous genotyping of multiple SNPs in one reaction). Although four steps were required for detection, all of these steps can be operated in parallel by automated sample handling devices. The throughput can be facilitated by adapting the ALM-ASA system to 384-well plate or microplate array diagonal gel electrophoresis (MADGE) [21].

Interleukin (IL)-1 $\beta$  is an important prototypic pro-inflammatory cytokine and a strong inhibitor of gastric acid secretion

that has been implicated in the pathogenesis of gastric cancer. In this study, the genotype frequencies of 0794C>T, 1274C>T and 2143T>C were found to be significantly different in gastric cancer patients compared with controls (Table 3). The risk of developing gastric cancer with 1274TT genotype was significantly increased. Individuals with the TT genotype at 1274 locus, making a TATA box, are strongly associated with gastric cancer.

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