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ORIGINAL PAPER

Genotyping of single nucleotide polymorphism in *MDM2* genes by universal fluorescence primer PCR and capillary electrophoresis

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Abstract Single nucleotide polymorphism (SNP) 309 in the promoter region of the murine double minute 2 (*MDM2*) gene plays an important role in human tumorigenesis. We established a simple and effective CE method for SNP detection in the *MDM2* gene. We designed one universal fluorescence-based nonhuman-sequence primer and one fragment-oriented primer, which were combined in one tube, and proceeded with the polymerase chain reaction (PCR). The amplicons were analyzed by capillary electrophoresis using single-strand conformation polymorphism method. PCR fragments generated from this two-in-one PCR displayed either T/T or G/G homozygosity or T/G heterozygosity. A total of 304 samples were blindly genotyped using this developed method, which included the DNA from 138 healthy volunteers, 43 chronic myeloid leukemia (CML) patients, and 123 colorectal cancer (CRC) patients. The results were confirmed by DNA sequencing and showed good agreement. The SSCP-CE method was feasible for SNP screening of *MDM2* in large populations.

Keywords Universal fluorescence primer PCR · CE · *MDM2* gene

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Introduction

The murine double minute 2 (*MDM2*) gene encodes a negative regulator of the *p53* gene, which is activated within the cellular stress pathway and has been proven to play an important role in the suppression of tumor formation [1, 2]. A single nucleotide polymorphism (SNP) in the promoter of the *MDM2* gene, SNP309 (T to G transition at position 309 in the first intron), increases the binding affinity of the transcriptional activator Sp1 and results in high levels of MDM2 protein. Overexpression of MDM2 protein attenuates the *p53* gene pathway and accelerates tumorigenesis [3]. Therefore, the relationship between SNP309 in the *MDM2* gene and tumor formation have become an important research issue in recent years. Zhang et al. evaluated the association with lung cancer [4]; Walsh et al. demonstrated its effect on sporadic endometrial

cancer [5]; Talseth et al. investigated its influence on hereditary nonpolyposis colorectal cancer [6].

A fluorescence-labeled artificial nonhuman sequence was first designed as a universal primer in 2004 [7], and was applied to detect mutations in long QT syndrome [8]. In order to shorten the total PCR procedure and decrease reagent consumption, the two-in-one PCR included fragment-oriented primer and universal fluorescence-based nonhuman-sequence primer in one tube. The amplicons were analyzed by SSCP-CE to detect mutations in familial hypercholesterolemia [7]. Even though it was only applied on a few occasions [7, 8], this technique holds great potential for SNP genotyping. We modified this amplification, designed our primers, and optimized the PCR conditions.

A number of analytical methods have been used to screen for this mutation, such as restriction fragment length polymorphism (RFLP)–gel electrophoresis [1, 4, 5, 9] and DNA sequencing [1, 10–12], but these are time-consuming and expensive. It is necessary to establish an effective method of detecting the SNP in the *MDM2* gene for clinical screening. Capillary electrophoresis (CE) is a powerful separation technique for mutation detection which can be combined with RFLP-CE [13, 14], affinity CE (ACE) [15, 16], heteroduplex analysis–CE (HA-CE) [17, 18], and single-strand conformation polymorphism–CE (SSCP-CE) [19–24]. Among these, SSCP is the most popular technique used for mutation detection because of its simplicity, feasibility and low cost. SSCP relies on the thermal denaturation of PCR products, followed by snap cooling to form single-stranded DNA complexes. Variations due to mutations can modify the shapes of the single-stranded complexes and alter their electrophoretic mobilities [25]. We tried to optimize one SSCP-CE to detect subtle differences in the SNP genotyping of the *MDM2* gene.

We tried to use one universal fluorescence-based nonhuman-sequence primer and forward and reverse fragment-oriented primers, and we optimized the PCR process and the CE conditions for the detection of the SNP309 mutation in the *MDM2* gene.

Materials and methods

Genomic DNA preparation

DNA samples were extracted from 43 chronic myeloid leukemia (CML) patients, 123 colorectal cancer (CRC) patients, and 138 healthy volunteers. DNA was collected from peripheral blood, and purified using a genomic DNA purification kit (Fermentas, Hanover, MD, USA) according to the instructed protocol.

PCR procedures

PCR products were generated by mixing 50–100 ng of genomic DNA with 200 μM dNTP (Takara, Shiga, Japan), 1× PCR buffer (Takara), 1 unit of Takara Taq™ (Takara) and 0.20 μM universal fluorescence-labeled nonhuman-sequence primer (5′-FAM GTGAC GTACT AGCAA CG-3′), 0.04 μM forward fragment-oriented primer (5′-CTAAG TGACG TACTA GCAAC GCTTT GCGGA GGTTT TGTTG GAC-3′), and 0.20 μM reverse fragment-oriented primer (5′-GAGTC AACCT GCCCA CTGAA-3′) (MD Bio, Taipei, Taiwan) to a final volume of 25 μL. PCR reactions were performed in a Px2 thermal cycler (Thermo Electron Corporation, Waltham, MA, USA) using the fragment-oriented amplification and universal fluorescence-tag amplification in one tube. The first amplification was performed with an initial denaturing step at 95 °C for 10 min, followed by three cycles of denaturing at 95 °C for 45 s, and annealing and extension were both done at 60 °C for 1.5 min. The second amplification was performed with 25 cycles of denaturing at 95 °C for 45 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min. Finally, an extension step was realized at 72 °C for 10 min. Prior to CE analysis, the PCR products (284 bp) were diluted fivefold with water, then denatured at 95 °C for 5 min, and then rapidly cooled on ice for 5 min.

DNA sequencing

PCR products were purified using the Qiagen (Venlo, The Netherlands) QIAquick™ PCR purification protocol. Sequencing reactions were performed using the CEQ2000 Dye Terminator Cycle Sequencing Quick Start Kit (Beckman, Fullerton, CA, USA) and separated on a Beckman CEQ 2000XL.

SSCP-CE analysis

This study was performed on a Beckman P/ACE MDQ equipped with a liquid-cooling device and a laser-induced fluorescence detector. Fluorescence detection was achieved with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. SSCP-CE was performed in a 100-μm I.D. DB-17 capillary (J&W Scientific, Folsom, CA, USA) with a 30-cm effective length (total length, 40.2 cm). The sieving matrix was 1×TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) (Amresco, Solon, OH, USA) containing 1.5% (w/v) HEC (wt: 250,000) (Fluka, Seelze, Germany). Additional polymers were evaluated for the separation, including hydroxypropylcellulose (HPC; MW 80,000) and hydroxypropylmethylcellulose (HPMC; MW 90,000). Capillary conditioning was performed in duplicate 10-min

pressure rinses with MeOH and double-distilled water. After filling the capillary with sieving matrix, samples were introduced by electrokinetic injection (−10 kV, 30 s). The separation voltage was set at −10 kV (anode at detector), and the temperature was kept at 25 °C.

Results and discussion

In this study, PCR included fragment-oriented amplification and universal fluorescence-tag amplification. A forward fragment-oriented primer was designed with a 5′-nonhuman-sequence extended end, which would be used to hybridize the universal fluorescence-based primer. A few cycles were performed to amplify the target fragment. Subsequent PCR cycles used one universal fluorescence-based primer. This universal primer incorporated with the nonhuman sequence, adapting the fluorescence tag, and this was used for sensitive detection. This universal primer could then be used in other PCR amplifications.

The single base T → G of SNP309 in the *MDM2* promoter region results in three genotypes: T/T homozygous, T/G heterozygous and G/G homozygous. RFLP–gel electrophoresis was the method most commonly used to distinguish these genotypes [3, 5]. This method provided results that were consistent with DNA sequencing data, but the incubation step was time-consuming and not suitable for high-throughput screening. In this study, a universal fluorescence-tag primer and two-in-one PCR were investigated for target DNA amplification, and one CE method was also optimized for accurate and reproducible SNP monitoring. Several parameters were evaluated, including primer design, PCR conditions, and separation conditions.

Universal primer and parameters of two-in-one PCR

Aydin et al. designed two universal fluorescence-based primers (FAM-labeled and NED-labeled), and needed two detection systems [7]. Actually, a single universal primer can provide sufficient detection sensitivity for the *MDM2* gene. Optimizing the PCR conditions and separation conditions should yield satisfactory amplification and differentiation. For the PCR conditions, parameters such as the concentration of primers and number of cycles affect the yield of amplicons. In this study, we tried to find the optimal PCR conditions needed to get enough signal intensity. Therefore, the concentration ratio of the universal FAM-labeled primer to the forward fragment-oriented primer (0.2 μM/0.04 μM, 0.12 μM/0.12 μM, or 0.04 μM/0.2 μM) was investigated. The highest intensity was obtained when a ratio of 0.2 μM/0.04 μM was used. The number of cycles of fragment-oriented amplification determined the amount of DNA template for further universal amplification. Six cycles produced more PCR amplicons. However, nonspecific peaks can appear in this case, causing some interference. Therefore, three cycles were chosen for the first amplification in this study.

CE parameters

Polymer selection and optimization

SSCP-CE analysis requires a nondenaturing sieving matrix. Several kinds of replaceable polymer solutions were investigated for SNP detection, including linear polyacrylamide (LPA) [26–28], cellulose derivatives [29, 30], and commercial polymer kits [31–35]. Because a high viscosity makes filling difficult, low-viscosity replaceable polymers were found to be the best choices. LPA is often used for DNA analysis in CE because of its high resolution. However, LPA is toxic and an environmental pollutant [36]. The commercial polymer kits were more expensive than common polymers. Therefore, considering the factors of cost, preparation and pollution, cellulose derivatives were chosen for subsequent investigations.

This study evaluated several cellulose derivatives that were easy to prepare and nontoxic as sieving matrices to determine their effects on heterozygous DNA separation; these cellulose derivatives included HEC, HPC and HPMC. 1×TBE buffer was used when evaluating the polymers, along with a separation temperature of 25 °C based on preliminary experimental results. Table 1 lists the three cellulose derivatives, their concentrations, migration times and the resolutions between the wild-type and mutant DNA fragments achieved under each set of conditions. When 3% HPC was selected as the polymer, the peaks of the T/G

Table 1 Effect of the polymer on the migration time and the resolution in SSCP-CE analysis

Polymer	Migration time (min)	Resolution
HPC 3.0%	7.43	0
HPC 4.0%	8.52	1.49
HPC 5.0%	10.65	1.89
HPMC 0.5%	8.04	0
HPMC 1.0%	10.68	1.01
HPMC 1.5%	13.65	0.65
HEC 0.5%	7.43	0
HEC 1.0%	8.52	1.47
HEC 1.5%	10.65	2.14
HEC 2.0%	13.03	1.08

SSCP-CE conditions: 1×TBE buffer containing polymer; applied voltage, −10 kV(detector at anode side); DB-17 capillary, 30 cm (effective length) × 100 μm id; injection 30 s, −10 kV; temperature, 25 °C

heterozygous fragments could not be resolved. When the HPC level was 4.0% and 5.0%, the T/G heterozygote was successfully resolved. However, the peak shapes appeared asymmetric and broad. On the other hand, HPMC was found to be an unsuitable polymer for analyzing T/G heterozygotes in the *MDM2* gene due to its high viscosity and low resolution. Finally, the effect of the HEC level (0.5, 1.0, 1.5, and 2.0% w/v) on the genotyping of T/G heterozygous fragments was tested. The electrophoretic mobility of the PCR fragments was inversely proportional to the HEC level [37]. As shown in Fig. 1a, migration time and resolution both increased as the HEC concentration increased from 0.5% to 1.5%. When 0.5% HEC was used, the peaks of the T/G heterozygous fragments could not be resolved. When the HEC level was increased to 2.0%, the resolution of the T/G heterozygote worsened. Considering the resolution, peak shape, and ease of preparation, 1.5% HEC was chosen for subsequent separations.

Effect of temperature

The ideal separation temperature for SSCP varied among the individual PCR products because of their different sequences. Altering the separation temperature can change the buffer viscosity, DNA conformation, sieving effects, and their interactions. Among these, the separation temperature strongly affects the conformation of single-stranded DNA [38]. At higher temperatures, single-stranded DNA fragments exhibit less-folded structures, and similar migration times. At lower temperatures, single-stranded DNA can, in theory, maintain its conformation. In this study, the effects of four lower temperatures (20, 25, 30, and 35 °C) on the resolution were checked, and the results are shown in Fig. 1b. At 35 °C and 20 °C, the peaks of the T/G heterozygous fragments could not be resolved. At 30 °C, nonspecific peaks that interfered with the peaks of the T/G heterozygous fragments caused incorrect genotype identification. 25 °C was found to provide the best resolution, and was therefore chosen for subsequent separations.

Effect of separation voltage

The separation voltage affects the production of Joule heat, the peak shapes and the migration speed [39]. We tried voltages between −5 and −15 kV. When −15 kV was used, the separation time dropped to seven minutes and the peak shape was sharper than at −5 and −10 kV, but the resolution was insufficient to identify T/G heterozygotes (Rs=1.13). On the other hand, applying −5 kV prolonged the analysis to twenty minutes, but the peak width was >0.2 min. Considering the separation time, peak sharpness and resolution, −10 kV was chosen as the separation voltage. The peak width was 0.1 min and the resolution was 2.14.

Consequently, the optimal SSCP-CE conditions were found to be a 1×TBE buffer solution containing 1.5% (w/v) HEC, a separation temperature of 25 °C, and a separation voltage of −10 kV. The electropherograms of T/T homozygous, T/G heterozygous and G/G homozygous genotypes obtained using

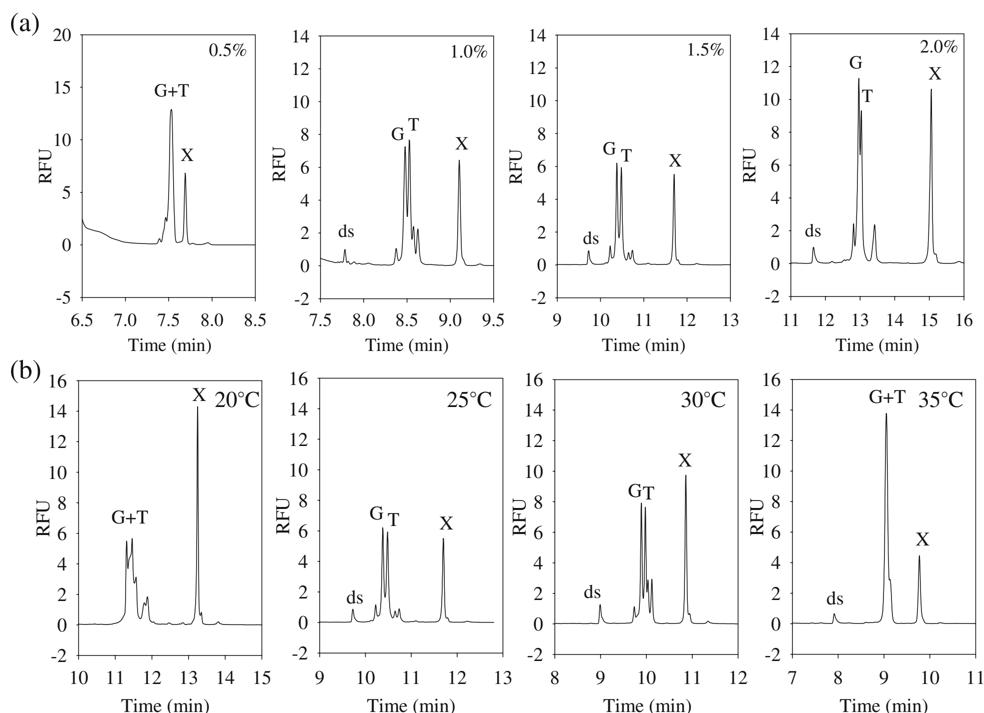

Fig. 1 **a** Effect of HEC concentration on the SSCP-CE separation of T/G heterozygous fragments at 25 °C. **b** Effect of separation temperature on the SSCP-CE separation of T/G heterozygous fragments in 1.5% (w/v) HEC buffer. CE conditions: 1 × TBE buffer containing HEC; applied voltage, −10 kV (detector at anode side); DB-17 capillary, 30 cm (effective length) × 100 μm I.D.; injection, 30 s, −10 kV. *Peak X*: primer–ssDNA constructs

these conditions are shown in Fig. 2a, and their DNA sequencing is shown in Fig. 2b. This SSCP-CE method was able to distinguish these genotypes. To determine the intra-day precision of the electrophoresis, five replicate separations were performed under optimized conditions. The RSD of the migration time was found to be 0.95%, validating the reproducibility of the electrophoretic separation.

Stability of the DNA conformation in different sample mediums

In SSCP analysis, PCR products are always diluted severalfold in a suitable sample medium to avoid fluorescence detector saturation and to decrease the salt concentration in the sample medium, which could affect DNA intake [40]. In this study, PCR products were diluted fivefold in several kinds of sample medium, including H_2O, formamide and NaOH. As shown in Fig. 3, H_2O was found to be the best medium. It was able to maintain the denatured conformation for up to 8 h at room temperature, whereas formamide and NaOH were able to maintain the conformation for less than an hour. For automatic and high-throughput CE analysis, it is necessary to use an optimized matrix. Considering the stability of the DNA conformation, H_2O was chosen as the sample medium for SSCP-CE analysis.

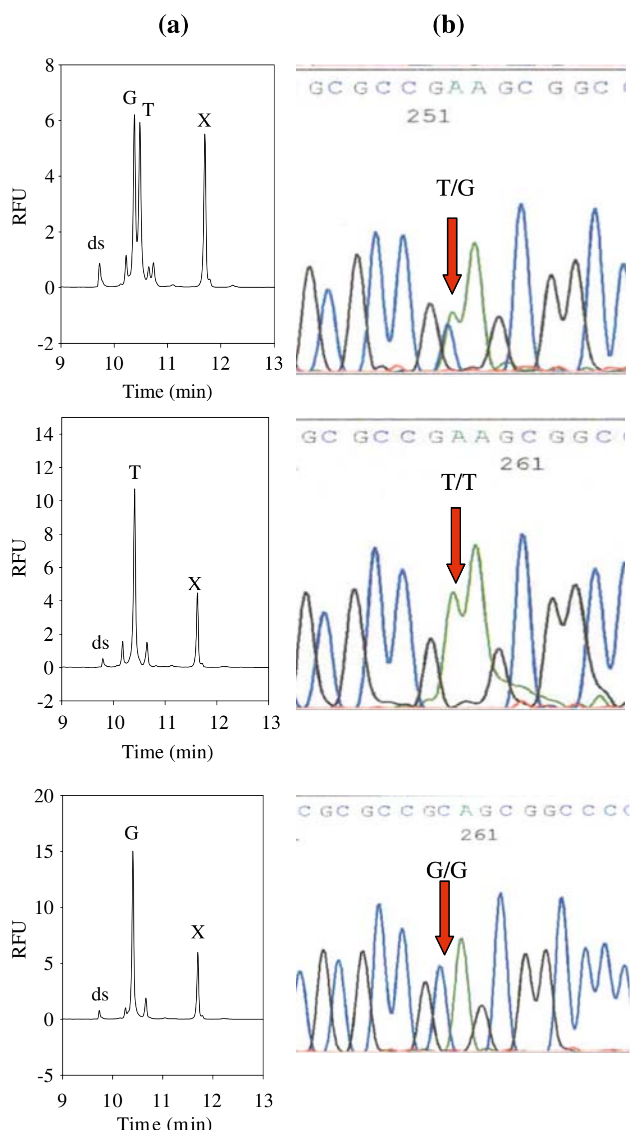

Fig. 2 Analysis of the T/G heterozygous, T/T homozygous, and G/G homozygous genotypes of the *MDM2* gene. **a** Electropherograms performed by SSCP-CE analysis using 1.5% (w/v) HEC; other conditions were as in Fig. 1. **b** Results of DNA sequencing. *Peak X*: primer–ssDNA constructs

Applications

We applied the method that we had established in order to explore the genotypes of SNP309 in *MDM2* genes of CML and CRC patients, as well as healthy volunteers. DNA samples were extracted from 138 healthy volunteers, 43 CML patients, and 123 CRC patients. A total of 304 samples were analyzed using the method developed in this study. The genotypes of the healthy volunteers included 29 T/T homozygotes, 83 T/G heterozygotes, and 26 G/G homozygotes. The homozygous determinations were identified by spiking G/G or T/T homozygous fragments for additional separation. The genotypes of the 43 CML patients included 20 T/G heterozygotes, 6 T/T homozygotes and 17 G/G homozygotes. The genotypes of the 123 CRC patients included 66 T/G heterozygotes, 27 T/T homozygotes and 30 G/G homozygotes. The results are summarized in Table 1. All of the results were confirmed by DNA sequencing, and showed good agreement (data not shown).

Genotype frequencies were tested via the Hardy–Weinberg equilibrium, using the χ^2 test to evaluate the genotype distributions in these three populations (healthy, CML and CRC). The results showed that the three populations were all in Hardy–Weinberg equilibrium (p=0.06, 0.99 and 0.71, respectively), and this meant that all samples were selected at random. The association between genotypes and the risk of developing either CML or CRC was estimated by odd ratios (ORs) and 95% confidence intervals (CIs), and calculated by unconditional logistic regression models. A two-sided p value of less than 0.05 was considered statistically significant. All statistical methods were performed with JMP 6 (SAS Institute Inc., Cary, NC, USA). As shown in Table 2, the G/G homozygote was more frequently observed among the CML than the healthy controls. The G/G homozygote had an elevated OR of 3.16 and a 95% CI from 1.13 to 9.87 compared to the T/T homozygote, and an elevated OR of 2.81 and a 95% CI from 1.33 to 5.94 compared to the T/T and T/G. Therefore, we found that the G/G homozygote was a genetic variant that may influence

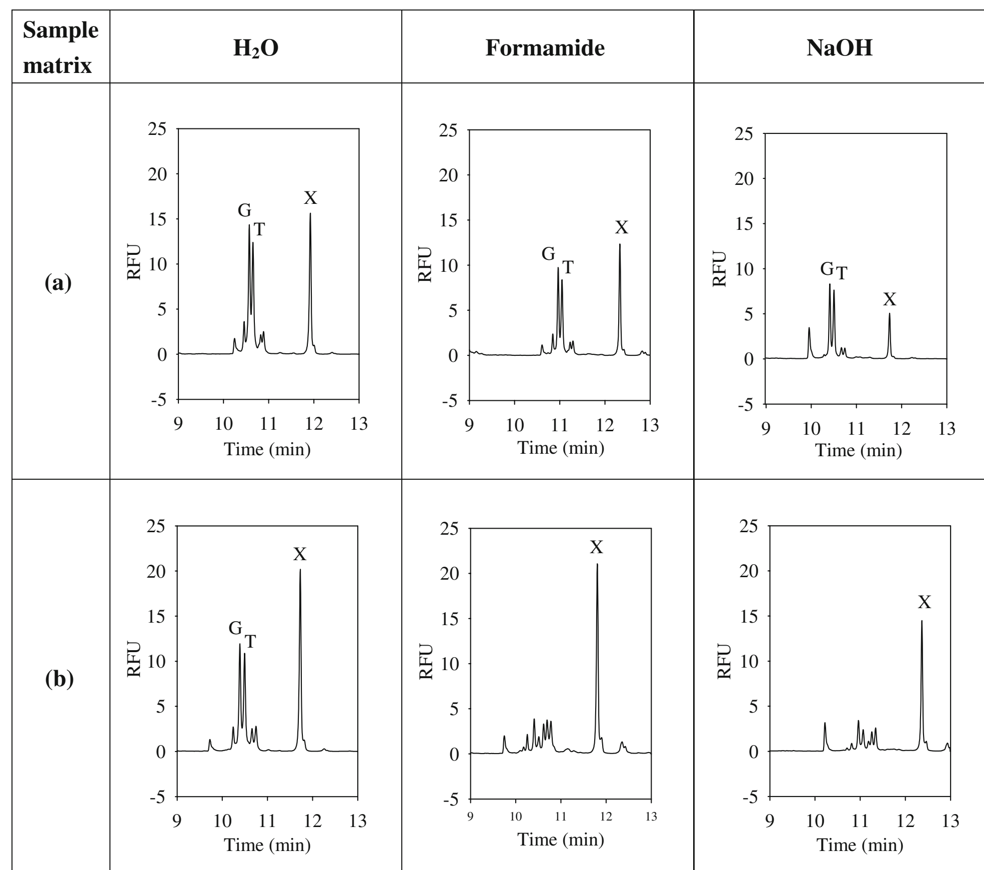

Fig. 3 Effects of the sample medium and stability on the SSCP-CE separation of T/G heterozygous fragments in 1.5% (w/v) HEC buffer. Other conditions were as in Fig. 1. **a** 5 min; **b** 1 h at room temperature after denaturing at 95 °C for 5 min and snap-chilling on ice for 5 min. *Peak X*: primer–ssDNA constructs

CML susceptibility, and subjects who were G/G homozygous had a 2.81-fold increased risk of developing CML compared to those who were T/T homozygous or T/G heterozygous. However, the genotype distributions for *MDM2* polymorphism in the healthy and CRC populations were not significantly different (*p* value greater than 0.05). We observed that the G/G homozygote had an OR value of 1.23, and a 95% CI from 0.59 to 2.61 compared to the T/T homozygote, and an OR of 1.39 and a 95% CI from 0.77 to 2.53 compared to the T/T homozygote and T/G heterozygote. The findings suggest that there were no significant associations between *MDM2* SNP309 variant genotypes and CRC risk.

Conclusions

We used a universal fluorescence-based nonhuman-sequence primer and a two-in-one PCR to amplify target fragments. An optimized SSCP-CE method was used to detect the SNP of the *MDM2* gene. This study showed that the universal primer was cost-effective, and that two-in-one PCR was

Table 2 Associations between the SNP309 genotypes of the *MDM2* gene and the risk of CML or CRC

Genotypes	Healthy	CML	CRC	CML versus healthy OR (95% CI)	*p* value	CRC versus healthy OR (95% CI)	*p* value
T/T	29 (21)	6 (14)	27 (22)	1.00		1.00	
T/G	83 (60)	20 (47)	66 (54)	1.16 (0.45–3.43)	0.76	0.85 (0.46–1.58)	0.61
G/G	26 (19)	17 (39)	30 (24)	3.16 (1.13–9.87)	0.04	1.23 (0.59–2.61)	0.57
Total	138	43	123				
At risk allele							
T/T+T/G	112 (81)	26 (60)	93 (76)	1.00		1.00	
G/G	26 (19)	17 (40)	30 (24)	2.81 (1.33–5.94)	0.01	1.39 (0.77–2.53)	0.28
T/T	29 (21)	6 (14)	27 (22)	1.00		1.00	
G/G+T/G	109 (79)	37 (86)	96 (78)	1.64 (0.67–4.65)	0.31	0.94 (0.52–1.71)	0.85

Values expressed as *n* (%)

OR, odds ratio; *CI*, confidence interval

labor-saving. This SSCP-CE was found to be rapid and reproducible. Its applicability was demonstrated by analyzing 304 subjects of healthy volunteers, CML and CRC patients. The genotyping results were confirmed by DNA sequencing. This method is a feasible and useful tool for mutation screening in the *MDM2* gene.

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