Journal of Chromatography A, 1218 (2011) 2114–2120

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Genotyping of two single nucleotide polymorphisms in 5,10-methylenetetrahydrofolate reductase by multiplex polymerase chain reaction and capillary electrophoresis

Hui-Ling Cheng[a], Shyh-Shin Chiou[b], Yu-Mei Liao[b], Yen-Ling Chen[c], Shou-Mei Wu[a,d,*]

[a] School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Department of Pediatric, Kaohsiung Medical University, Kaohsiung, Taiwan
[c] Department of Fragrance and Cosmetic Science, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan
[d] Center of Excellence for Environmental Medicine, Kaohsiung Medial University, Kaohsiung, Taiwan

ARTICLE INFO

Article history:
Available online 8 September 2010

Keywords:
Acute lymphoblastic leukemia
Capillary electrophoresis
5,10-Methylenetetrahydrofolate reductase
Genotyping

ABSTRACT

Two single nucleotide polymorphisms (SNPs) of 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene, A1298C and C677T, were widely considered to be related with various neoplasia disorders. We established a simple and effective capillary electrophoresis (CE) method for detection of two SNPs in *MTHFR* gene simultaneously. DNA samples were amplified by multiplex PCR with universal fluorescence-labeled primer and analyzed by single-strand conformation polymorphism (SSCP)-CE method. The CE method was performed using 1.5% hydroxyethyl cellulose in 1× TBE buffer containing 1 M urea. The PCR products after SSCP procedure were electrokinetically injected at −10 kV, 30 s. Separation voltage was −6 kV and the temperature was set at 20 °C. The optimal SSCP-CE method was applied to detect two polymorphisms in *MTHFR* gene of acute lymphoblastic leukemia (ALL) and attention-deficit/hyperactivity disorder (ADHD) patients. Genotyping results were evaluated in terms of relationships between outcomes for ADHD patients after ALL chemotherapy and ALL disease. The SSCP-CE method and multiplex PCR with universal fluorescence primer were used as the fast technique for screening two SNPs in *MTHFR* gene, A1298C and C677T. The genotyping data were coincident with DNA sequencing. This SSCP-CE method was found feasible for detecting mutation of *MTHFR* gene in populations.

1. Introduction

Methotrexate (MTX) is an anti-folate drug that inhibits folate metabolism enzyme to induce folate depletion in tumors and normal cells [1]. The 5,10-methylenetetrahydrofolate reductase (MTHFR) irreversibly catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in the folic acid cycle, thus playing a central role in folate metabolism pathway to balance DNA synthesis and methylation [2–6]. Two SNPs in *MTHFR*, A1298C and C677T, are associated with catalytic activity of MTHFR enzyme. The A1298C decreased enzyme activity and C677T also reduced activity of MTHFR, increased homocysteine level, and influenced distribution of folate. Since two polymorphisms might regulate therapeutic response after treatment with the anti-folate chemotherapeutic agents through differences in enzyme activity [4], SNPs of *MTHFR* were reported to increase the risk of some diseases such as neural tube defects, schizophrenia, bipolar disorder, depression, neoplasia disorders, and acute lymphoblastic leukemia (ALL) [7–12].

ALL is the more frequent type of cancer in the childhood. Performance of treatment of ALL with intensive chemotherapeutic agents has improved impressively, to realize curing rates as high as 80%, over the past 50 years [13–15]. MTX is extensively used for chemotherapy of ALL but it enhances the risk of growth retardation, neurotoxicity or cognitive impairment [16–18]. Patients after intensive MTX treatment suffered neurocognitive impairment and attention problems were common among children [19]. In 2008, Krull et al. provided preliminary data to imply strong relationships between two common *MTHFR* polymorphisms, A1298C and C677T, and attention-deficit/hyperactive disorder (ADHD) in patients who survived after ALL treatment [15].

The most commonly employed method for screening *MTHFR* polymorphisms used restriction enzyme to digest specific sites and formed different lengths for separation; it was known as restriction fragment length polymorphism (RFLP)-gel electrophoresis [20]. Another method used two-color fluorescence cross-correlation measurements for detecting single polymorphism, C677T, which needed two excitation lasers to excite two different fluorescent

* Corresponding author at: School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, 100 Shih Chen 1st Rd., Kaohsiung 807, Taiwan.
Tel.: +886 7 3121101x2164; fax: +88673210683.
E-mail address: shmewu@kmu.edu.tw (S.-M. Wu).

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doi:10.1016/j.chroma.2010.08.080

Table 1
Primers used for *MTHFR* gene.

Primer	Concentration (μM)	Sequence	DNA length (bp)
Universal fluorescence primer	2.00	FAM[a]-**GTGACGTACTAGCAACG**	
1298-F[b]	0.05	ATAA**GTGACGTACTAGCAACG**GCAA TTCCTCTTCCCCTGCCTTT	215
1298-R[b]	1.00	CCACTCCAGCATCACTCACTTT	
677-F	0.10	ATAA**GTGACGTACTAGCAACG**TGTC ATCCCTATTGGCAGGT	259
677-R	2.00	GGAGCTTATGGGCTCTCCTG	

[a] FAM: 6-carboxy-fluorescein.
[b] F: forward primer; R: reverse primer.

primers and detected *MTHFR* polymorphisms by two separated channels [21]. One study also reported restriction enzymes to digest specific sites of C677T, and then separated them in capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection [22]. One paper showed that two SNPs could be simultaneously screened using primers labeled with different dyes and restriction digestion coupled with CE platform [23]. Heteroduplex generator technology has also been used to detect *MTHFR* gene for hyperhomocysteinemia-associated pathologies [24]. High-resolution amplicon melting technology was also employed to identify *MTHFR* variants depending on diverse melting curves of heterozygous and homozygous genotypes [25].

The existing methods needed digestion or different dyes to separate the SNPs, while we have established one CE method for mutation detection. CE is a powerful analytical separation method that is superior to gel electrophoresis. The single-strand conformation polymorphism (SSCP) procedure was used to denature PCR products after amplification; PCR products were snapped on ice to form single-strand DNA (ssDNA) complexes. For this reason, the mutant changed the shape and electrophoretic mobility, and differed from the normal type [26]. SSCP is a popular method for genotyping to detect variations even with one base-pair difference based on different conformation and electrophoretic mobility of ssDNA [27–31]. In order to simplify labeling and amplifying process, multiplex PCR was performed using universal fluorescence and some specific primers. The universal fluorescence primer contains a fluorescence dye at 5′ end for enhancing sensitivity and a strand of non-human sequences [32]. In this study, we tried to establish one CE method, and applied to clinical evaluation.

2. Materials and methods

2.1. DNA extraction

DNA samples were collected from 130 ALL patients after chemotherapy at a hospital (Kaohsiung, Taiwan). Of these 58 were ADHD patients. The genomic DNA samples were extracted from blood specimens and purified by genomic DNA purification kits (Fermentas, Hanover, MD, USA) according to the instructed protocol. DNA samples were measured by spectrophotometer and absorbance ratio of 260 nm/280 nm was determined around 1.8–2.0. DNA concentration was diluted to 50 μg/mL by water before amplification.

2.2. PCR procedures

In this study, multiplex PCR was used with universal fluorescence primer to simultaneously amplify two SNPs in *MTHFR* gene. The universal part of the primers is non-human sequence fragment (GTGACGTACTAGCAACG) [32,33]. We added ATAA fragment in front of the non-human sequence fragment as this could improve annealing of the primers during PCR procedures. PCR products were generated by mixing 50–100 ng of genomic DNA with 200 μM dNTP, 1× PCR buffer and 1 unit of Takara Taq (Takara, Shiga, Japan) in each of universal fluorescence primers and other specific primers (1298-F, 1298-R, 677-F, and 677-R) in a final volume of 25 μL. The sequences and concentrations of the primers in this reaction are shown in Table 1. PCR reactions were performed in a Px2 thermal cycler (Thermo Electron Corporation, Waltham, USA). The first amplification was performed with initial denaturing step at 95 °C for 10 min, followed by three cycles of denaturing at 95 °C for 50 s. Both annealing and extension were done at 60 °C for 90 s. The second amplification was performed by 25 cycles of denaturing at 95 °C for 50 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. Finally, an extension step was set at 72 °C for 10 min.

2.3. SSCP-CE method

The experiment was performed on the Beckman ProteomeLab™ PA800 system (Fullerton, CA, USA) equipped with a LIF detector. The excitation wavelength was set at 488 nm and emission wavelength at 520 nm. DB-17 capillaries (J & W Scientific, Folsom, CA, USA) (100-μm I.D., 30-cm effective length and 40 cm total length) were employed for separation. The sieving matrix was composed of 1.5% (w/v) hydroxyethyl cellulose (HEC; MW 250000) (Fluka, Seelze, Germany) and 1 M urea (Merck, Darmstadt, Germany) in 1× TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA) (Amresco, Solon, OH, USA). Capillary conditioning was performed with methanol and double-distilled water by pressure, and was repeated twice. After the capillary was filled with sieving matrix, the sample was introduced by electrokinetic injection (−10 kV, 30 s). Separation voltage was set at −6 kV (anode at detector end) and temperature was kept at 20 °C. Prior to electrophoresis, PCR products were diluted 10-fold with water and then denatured at 95 °C for 3 min and immediately cooled on ice for 3 min.

Table 2
Effect of polymers on resolutions of heterozygous fragments.

Polymers	Resolution[a]	
	A1298C, A/C	C677T, C/T
HEC		
0.5%	0.67	0.00
1.0%	0.00	0.64
1.5%	0.53	0.68
2.0%	0.51	0.00
HPMC		
0.5%	0.00	0.48
1.0%	0.00	0.65
1.5%	0.00	0.60
1.8%	0.00	0.00
HPC		
2.0%	0.00	0.00
4.0%	0.00	0.00
6.0%	0.00	0.00

CE conditions: the polymers in 1× TBE buffer containing 1 M urea; DB-17 capillary, 30-cm effective length × 100-μm I.D.; sample injection, −10 kV, 30 s; separation voltage, −6 kV, temperature, 20 °C.
[a] Resolution $= 2 \times (t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 represent the migration time of two peaks of interest and w_1 and w_2 are peak widths measured at the baseline between tangents drawn to the peak sides.

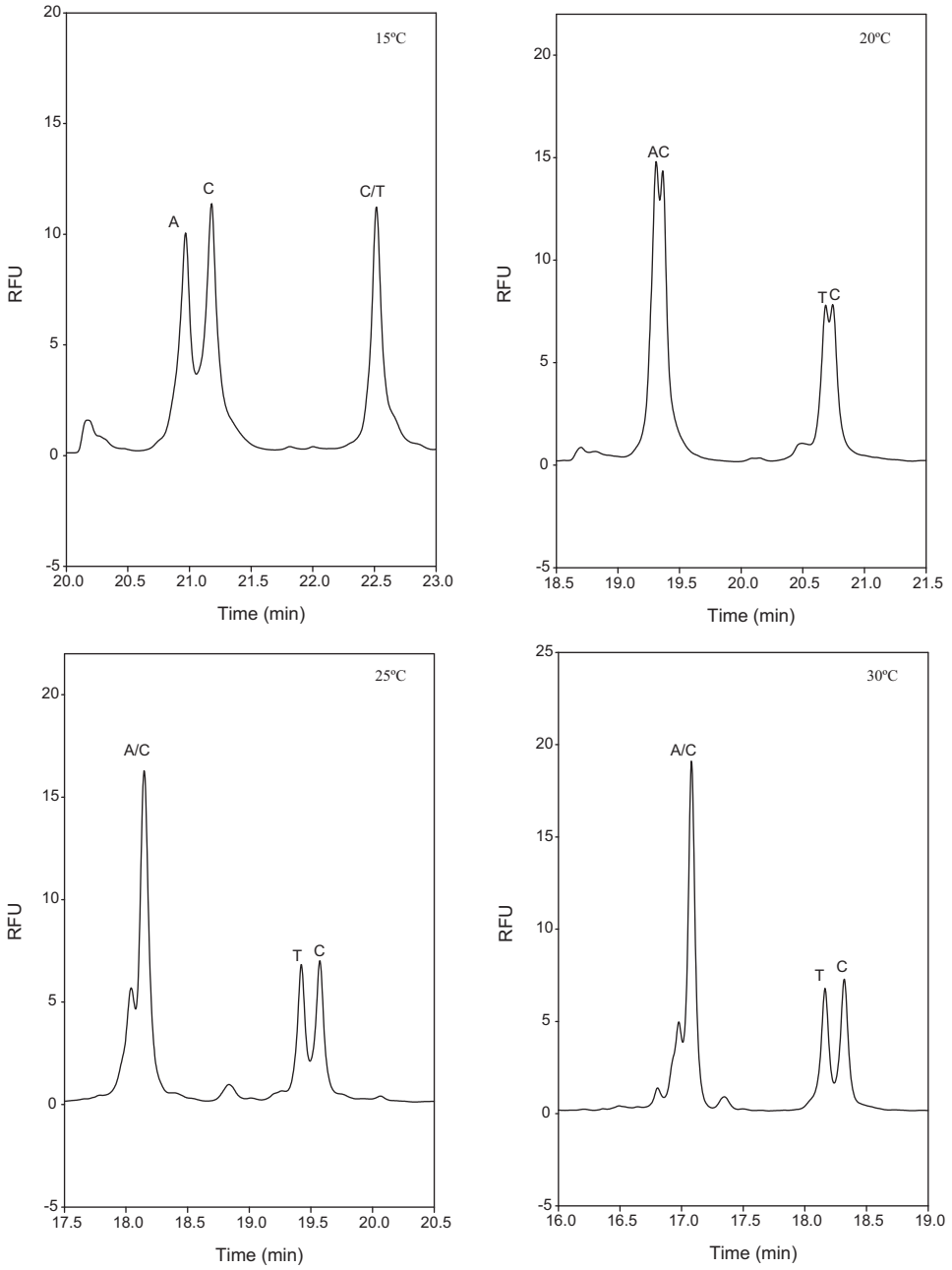

Fig. 1. Effect of temperature on SSCP-CE separation for A1298C and C677T heterozygous fragments. CE conditions: 1.5% HEC in 1× TBE buffer containing 1 M urea; DB-17 capillary, 30-cm effective length × 100-μm I.D.; sample injection, −10 kV, 30 s; separation voltage, −6 kV. Length of PCR products: 215 bp of A1298C; 259 bp of C677T.

3. Results and discussion

We established a simple, fast and high-throughput method to screen two SNPs, A1298C and C677T in *MTHFR* gene. Multiplex PCR was performed by one universal fluorescence primer and specified primers. SSCP-CE could replace conventional gel electrophoresis to be potential genotyping technique due to its high resolution [34–36]. We also applied this SSCP-CE method to evaluate the relationship between *MTHFR* and ADHD.

3.1. Polymer selection

Use of appropriate polymers provided the sieving mediums for resolution of SNPs [35]. Sieving mediums in CE were replaceable polymer solutions, including linear polyacrylamide (LPA), cellulose derivatives, and commercial kits. Low-viscosity replaceable polymers are the best selection because high-viscosity polymers are difficult to fill into the capillary. LPA has good DNA-sieving ability [27], but it is toxic and environmentally polluting [31]. As to the commercial kits, they are more expensive, and limit experiment conditions. We tested some polymers and chose cellulose derivatives for further investigation in this study.

Cellulose derivatives, including HEC (MW 250000; entanglement threshold concentration; Φ^* 0.79%) [37], hydroxypropyl cellulose (HPC) (MW 80000; Φ^* 0.65%) [38], and hydroxypropylmethyl cellulose (HPMC) (MW 90000; Φ^* 0.50%) [39], were tested as sieving mediums. 1× TBE was used for preparing polymers. We tested different compositions of HEC (0.5%, 1.0%, 1.5% and 2.0%, w/v), HPC (2.0%, 4.0%, and 6.0%, w/v), and HPMC (0.5%, 1.0%, 1.5% and 1.8%, w/v). As Table 2 shows, A1298C and C677T heterozygous fragments could not be resolved in HPC levels which might be related to sieving structures and viscosity. The HPMC polymer

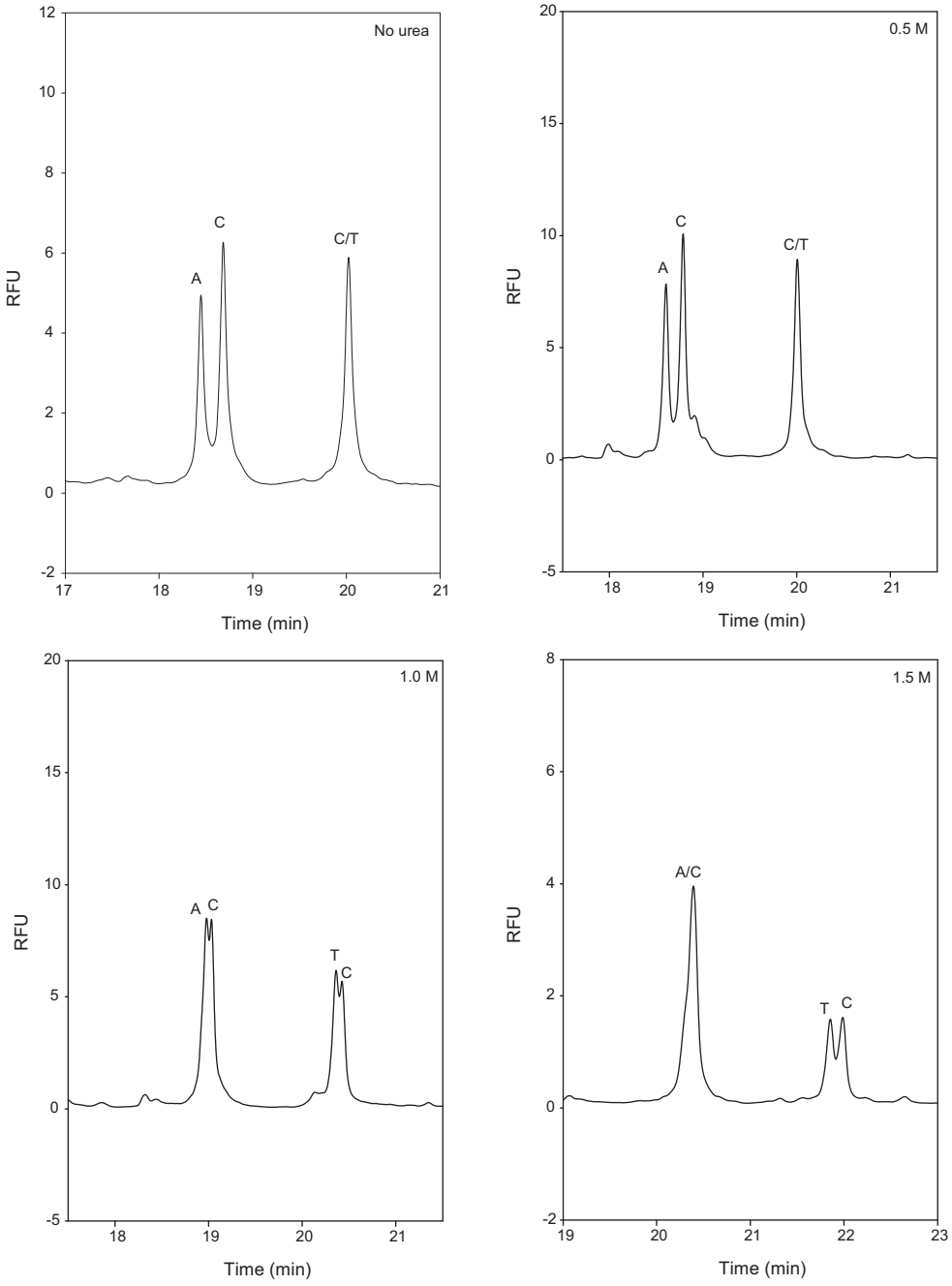

Fig. 2. Effect of urea on the separation. CE conditions as Fig. 1.

was excluded owing to the poor resolution of heterozygous nature of A1298C. HEC showed better resolution and was chosen as the sieving medium. As shown in Table 2, the C677T heterozygous type could not be resolved as 0.5% and 2.0% HEC was used. Only at 1.5% HEC, the two polymorphisms, A1298C and C677T heterozygous, could be resolved. Hence, 1.5% HEC was used as the sieving medium.

3.2. Effect of temperature

Temperature could affect the structures and viscosity of the sieving medium, DNA conformation and mobility [30,40–42]. The ssDNA fragments showed different folded structures and migration times at different temperatures [27,30,41]. In our study, A1298C and C677T heterozygotes showed no resolution when temperature was higher than 35 °C. The effects of lower temperatures were evaluated between 15 °C and 30 °C (Fig. 1). At 15 °C, peaks of A1298C heterozygote showed better resolution but C677T heterozygote could not be resolved. At 25 °C and 30 °C, peaks of C677T heterozygote could provide better resolution, but not A1298C heterozygous gene. Meanwhile, some nonspecific peaks migrated closely with A1298C gene. The nonspecific peaks should be the incomplete fragments produced in PCR reactions. Therefore, we chose 20 °C for SNP screening.

3.3. Effect of urea

Urea is the common additive used in DNA analysis. The role of urea is to keep DNA fragments in denatured state and unfold the ssDNA fragments [35,43,44]. Addition of urea could change the conformation of ssDNA fragments and increase the viscosity

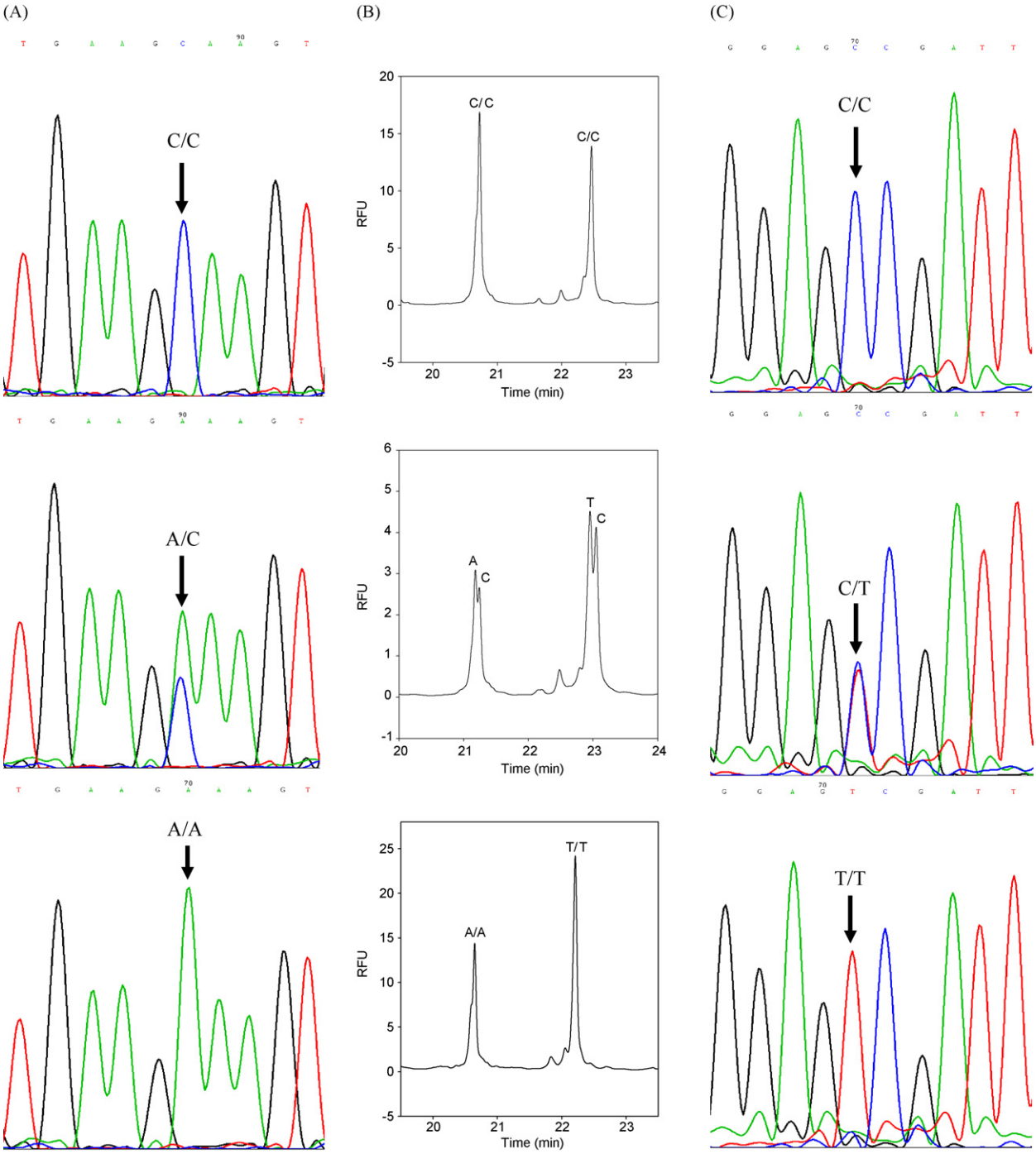

Fig. 3. Analysis of the homozygous and heterozygous genotypes of A1298C and C677T in *MTHFR* gene. (A) Results of DNA sequencing of A1298C. (B) Electropherograms performed by SSCP-CE method using 1.5% HEC containing 1 M urea; other conditions as Fig. 1. (C) Results of DNA sequencing of C677T.

of polymer solution. We optimized and got the better resolution [43–45]. HEC without urea could not resolve both SNPs simultaneously. After adding urea, it improved the resolution efficacy. As Fig. 2 shows, A1298C heterozygous fragment could be completely resolved with less then 0.5 M urea. The resolution of C677T heterozygous fragment was further improved until urea concentration increased above 1 M. The resolution of C677T heterozygote got better but A1298C grew worse, while peak width of C677T heterozygote became broader. There were many factors dominating the mobility such as DNA conformation, buffer components and viscosity. Even the sequence of base pairs would make some difference on migration. In this analysis, the urea additive made different effects on these two DNA fragments. Finally, 1 M urea was used for separation of A1298C and C677T in *MTHFR* gene.

3.4. Effect of separation voltage

The effect of separation voltage was also examined for SSCP analysis. Voltage would affect the migration and peak resolution. Higher voltage might increase Joule heat which enhanced the temperature of inner capillary and affected the rate of mobility and diffusion of ssDNA, and potentially changed the conformation and electrophoretic mobility between mutant and wild-type fragments [27,30,36]. The separation voltage was investigated from −4 kV

Table 3
Association between the genotypes of two polymorphisms in the *MTHFR* gene and the risk of ADHD.

Genotypes	ALL (%)	ADHD (%)	OR[a] (95% CI[b])	*p* value
A1298C				
A/A	46 (64)	36 (62)	1.00	
A/C	21 (29)	18 (31)	1.10 (0.51–2.36)	0.81
C/C	5 (7)	4 (7)	1.02 (0.24–4.13)	0.98
C677T				
C/C	48 (67)	35 (61)	1.00	
C/T	18 (25)	17 (29)	1.30 (0.58–2.87)	0.52
T/T	6 (8)	6 (10)	1.37 (0.40–4.73)	0.61
A1298C–C677T				
A/A–C/C	27 (38)	19 (33)	1.00	
The others	45 (62)	39 (67)	1.23 (0.60–2.57)	0.57

[a] OR, odds ratio.
[b] CI, confidence interval.

to −10 kV. When −10 kV was applied, the migration time was decreased in 14 min and peak shapes were sharper than others. Only C677T heterozygote could be resolved at −10 kV. At −8 kV, A1298C and C677T heterozygotes could be both resolved, but resolutions were not good enough. At −4 kV, analysis time was more than 30 min and the peak of C677T heterozygote could not be resolved. When −6 kV was used, the A1298C and C677T heterozygotes could be both resolved within 21 min and their resolutions were 0.53 and 0.68, respectively. Finally, we chose −6 kV as the separation voltage.

The optimal SSCP-CE conditions included the sieving medium as 1.5% HEC (w/v) containing 1 M urea in 1× TBE buffer, the separation temperature at 20 °C and voltage at −6 kV. The electropherograms of wild- and mutant-types in *MTHFR* gene were obtained by optimal conditions and confirmed by DNA sequencing, as shown in Fig. 3. To determine the repeatability of this method, five replicate separations were performed. The RSD values of A1298C and C677T in migration time were 0.29% and 0.23%, respectively. These results showed good repeatability of this method.

3.5. Application

We utilized the optimized SSCP-CE method to detect two polymorphisms, A1298C and C677T, in *MTHFR* genes of 130 ALL patients. Among them, 58 patients were survivors (post ALL chemotherapy), and were diagnosed as ADHD patients. We tried to use our method to investigate the relationship of their *MTHFR* genes and ADHD. Our concern was whether the mutant type of *MTHFR* contributed some effects on the metabolism of MTX and caused the ADHD disease. DNA samples were extracted from 130 ALL patients. The A1298C genotypes of ALL patients included 46 A/A homozygotes, 21 A/C heterozygotes, and 5 C/C homozygotes. The C677T genotypes of ALL patients contained 48 C/C homozygotes, 18 C/T heterozygotes, and 6 T/T homozygotes. The homozygotes were terminated by spiking A/A and C/C or C/C and T/T homozygous fragments for additional separation. The A1298C genotypes of ADHD patients included 36 A/A homozygotes, 18 A/C heterozygotes, and 4 C/C homozygotes. The C677T genotypes of ADHD patients contained 35 C/C homozygotes, 17 C/T heterozygotes, and 6 T/T homozygotes. All results were confirmed by DNA sequencing and showed good agreement. The results are summarized in Table 3.

Frequencies of genotypes were tested by the Hardy–Weinberg equilibrium and we used the χ^2 test to estimate the distribution of genotypes in ALL and ADHD populations. The *p* values of A1298C and C677T in *MTHFR* genes of ALL patients were 0.51 and 0.12 and values of ADHD patients were 0.71 and 0.25, respectively.

Results of evaluation by the Hardy–Weinberg equilibrium meant all samples selected were random. The association between genotypes and the risk of developing ADHD was estimated by odd ratios (OR) and 95% confidence intervals (CI) and were calculated by unconditional logistic regression models. The *p* values were based on 2-sided tests and less than 0.05 were considered statistically significant. All statistical methods were performed with JMP 6 (SAS Institute Inc., Cary, NC, USA). The results of the reference paper showed that A1298C polymorphism was associated with significantly higher rates of ADHD and the combined genotype of *MTHFR* (A1298C and C677 T combinations) was also related to increased rates of ADHD symptoms [15]. As shown in Table 3, the A/A genotype of A1298C and C/C genotype of C677T were used as controls. The A/C heterozygote of A1298C had an OR value of 1.10 and 95% CI value from 0.51 to 2.36, compared to A/A homozygote; the C/C heterozygote of A1298C had an OR value of 1.02 and 95% CI value from 0.24 to 4.13 compared to A/A homozygote. The C/T heterozygote of C677T had an OR value of 1.30 and 95% CI value from 0.58 to 2.87 compared to C/C homozygote; the T/T heterozygote of C677T had an OR value of 1.37 and 95% CI value from 0.40 to 4.73 compared to C/C homozygote. The genotypes of patients had A/A genotype of A1298C and C/C genotype of C677T as controls of combinations. The OR value of other combinations was 1.23 and 95% CI value from 0.60 to 2.57 compared to controls. However, we found that the results were insignificant for correlation of *MTHFR* and ADHD. This statistical analysis has some weaknesses, including small sample size, and insufficient parameters to adjust numerical values for complete statistical analysis. There were some parameters including patient number, period of MTX treatment, response for MTX, years of survivals after ALL treatment, and age of ADHD diagnosis. We established this analysis and needed more patients to confirm their association between MTX treatment, genotyping and ADHD.

4. Conclusions

We established the multiplex PCR with universal fluorescence primer and SSCP-CE method for analyzing A1298C and C677T polymorphisms. Multiplex PCR with the universal fluorescence primer was cost-effective and labor-saving. The SSCP-CE method was a simple, fast and reproducible technique, and feasible for screening polymorphisms in *MTHFR* genes. The genotyping results were all coincident with DNA sequencing. The SSCP-CE and multiplex PCR with universal fluorescence primer methods could be successfully applied to screen two SNPs in *MTHFR* genes and provide the DNA genotype results for further evaluating the association with risk of various disorders.

Acknowledgements

We gratefully acknowledge the support of the National Science Council of Taiwan and Kaohsiung Medical University in funding this work. We also express our gratitude to the Center of Excellence for Environmental Medicine (KMU-EM-98-1-2) for partial financial support provided for this work.

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