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T85C polymorphisms of the dihydropyrimidine dehydrogenase gene detected in gastric cancer tissues by high-resolution melting curve analysis

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Background/aims: *Dihydropyrimidine dehydrogenase is a key enzyme acting on the metabolic pathway of medications for gastric cancer. High-resolution melting curve technology, which was developed recently, can distinguish the wild-type dihydropyrimidine dehydrogenase gene from multiple polymorphisms by fluorescent quantitative polymerase chain reaction products in a direct and effective manner.* ***Materials and Methods:*** *T85C polymorphisms of dihydropyrimidine dehydrogenase in the peripheral blood of 112 Chinese gastric cancer patients were detected by real-time polymerase chain reaction combined with high-resolution melting curve technology. Primer design, along with the reaction system and conditions, was optimized based on the GenBank sequence.* ***Results:*** *Seventy nine cases of wild-type (TT, [70.5%]), 29 cases of heterozygous (TC, [25.9%]), and 4 cases of homozygous mutant (CC, [3.6%]) were observed. The result was completely consistent with the results of the sequencing.* ***Conclusions:*** *Real-time polymerase chain reaction combined with high-resolution melting curve technology is a rapid, simple, reliable, direct-viewing, and convenient method for the detection and screening of polymorphisms.*

Key words: Polymorphism, dihydropyrimidine dehydrogenase, T85C, high-resolution melting, gastric cancer

Mide kanseri dokularında yüksek çözünürlüklü erime eğrisi analizi ile dihidroprimidin dehidrogenaz geninde T85C polimorfizmi tespit edilmiştir

Giriş ve Amaç: *Dihidroprimidin dehidrogenaz mide kanserinde kullanılan tedavilerin metabolizması için anahtar role sahip bir enzimdir. Yeni geliştirilen, yüksek çözünürlüklü erime eğrisi teknolojisi, doğal tip dihidroprimidin dehidrogenaz geni ile çoklu polimorfizmini, flöresan ile kantifiye zincirleme polimeraz reaksiyonu kullanarak doğrudan ve etkin bir şekilde ayırabilir.* ***Gereç ve Yöntem:*** *Dihidroprimidin dehidrogenaz T85C polimorfizmi 112 Çinli mide kanseri hastasının periferik kanında gerçek zamanlı zincirleme polimeraz reaksiyonu ve yüksek çözünürlüklü erime eğrisi teknolojisi kullanılarak tespit edildi. Primer üretimi, reaksiyon sistemi ve koşulları GenBank sekansı dikkate alınarak optimize edildi.* ***Bulgular:*** *Doğal tip 79 vakada (TT; %70.5), heterozigot 29 vakada (TC; %25,9) ve homozigot mutasyon 4 vakada (cc; %3,6) tespit edildi. Sonuçların sekans analizi ile tam uyumlu olduğu görüldü.* ***Sonuç:*** *Gerçek zamanlı zincirleme polimeraz reaksiyonu ile yüksek çözünürlüklü erime eğrisi teknolojisinin kombine edilmesi polimorfizmlerin tespit edilmesinde hızlı, kolay, güvenilir, doğrudan gözlemlenebilir bir yöntemdir.*

Anahtar kelimeler: Polimorfizm, dihidroprimidin dehidrogenaz, T85C, yüksek çözünürlüklü erime, mide kanseri

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INTRODUCTION

There are around one million newly diagnosed gastric cancer patients in the world annually, and many of these are Chinese (1,2). There is also a wide spectrum of opinions on the pathogenic hereditary factors of gastric cancer. Chemotherapy is an important therapeutic option for gastric cancer. Dihydropyrimidine dehydrogenase (DPYD) is a key enzyme acting on the metabolic pathway of medications for gastric cancer. Accordingly, the content of DPYD can affect the metabolic rate of medicine and influence the toxicity and efficacy of chemotherapy (3-5). Currently, methods for testing enzyme activity rely mainly on the use of high-performance liquid chromatography (HPLC) methods combined with radioactive labeling and mRNA expression. However, this complicated and long process makes it less attractive (6-9). High-resolution melting (HRM) curve technology, which was developed recently, is an analytic method based on fluorescent quantitative polymerase chain reaction (PCR) using a saturated dye method. It can distinguish the wild-type DPYD gene from multiple polymorphisms by PCR products in a direct and effective manner. The current study was undertaken to use the technique to determine the distribution of T85C polymorphisms of DPYD and to demonstrate the value of the method in the analysis of gene polymorphisms.

MATERIALS AND METHODS

Participants

Peripheral blood samples of 112 gastric cancer patients were obtained in our hospital during September 2009 - November 2010 after acquiring their written informed consent. The protocol was approved by the Institutional Ethics Committee of The First Affiliated Hospital, College of Medicine, Zhejiang University. There were 63 males and 49 females aged 25-60 years. Further information, including pathologic characteristics, surgical procedures, and chemotherapy regimens, are not shown in this paper. DNA from the 112 samples was isolated with the QIAamp DNA Mini Kit (Qiagen, Germany).

Reagents and Equipment

EvaGreen dye, PCR buffer, DNA polymerase, dNTPs, and $MgCl_2$ were purchased from Huirui Biotechnology Co., Ltd., Shanghai, China. The competent *Escherichia coli* JM109 and DNA Gel Extraction kit were purchased from Takara (Dalian, China). The pGEM-T vector, pGEM-T reagent (A3600), and plasmid mini kit, Wizard® Plus Minipreps DNA Purification System (A7100), were purchased from Promega (Wisconsin, USA). We used a Rotor-Gene 6000TM (Corbett Research, Mortlake, New South Wales, Australia) to perform PCR and HRM analysis.

Design and Synthesis of Primers

Using Primer 5.0 software, PCR primer pairs were designed upstream and downstream to the locus for DPYD (10). Because of the fluorescent dye method, any secondary structure produced in the PCR reaction could have had a significant effect on the assessment of results, even inducing incorrect results. Therefore, it was important to avoid primer-dimers and hairpin structures when primer pairs were chosen and optimized. Thus, we attempted to make the Tm of the forward and reverse primers as close as possible for being suitable for a reaction. The primer pairs were as follows: F: 5′-CCT GGC TTT AAA TCC TCG AAC A-3′R: 5′-AGG ATT TCT TTT CCA ATG TTT C -3′. The length of the PCR product was 91 bp, and the sequence of primers was synthesized and purified by Huirui Biotechnology Co., Ltd., Shanghai, China.

Construction of Standard Plasmids

A restriction-free method for inserting target genes into plasmids was used (11,12). Briefly, PCR segments containing wild-type and homozygous mutant genes were inserted into separate pGEM-T vectors. The primers were as follows: F1: 5′-ACT CGA GAC TGT AGG CAC TG -3′, R1: 5′-AAG AGT CGT GTG CTT GAT GT -3′. The amplified fragment length was 223 bp. From a blood sample chosen at random, genomic DNA was extracted as a template for PCR amplification. The conditions of amplification were as follows: The PCR reaction system included 1xPCR reaction buffer, 0.5 µmol/L each of F1 and R1, 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTPs, 1U DNA polymerase, and 50 ng genome DNA template; deionized water was added to make 50 µL. PCR reaction conditions were: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 20 s, annealing at 55°C for 20 s, elongation at 72°C for 20 s, 35 cycles, and elongation at 72°C for 5 min, and storage at 4°C. After purification by gel electrophoresis, amplified products were ligated to the pGEM-T vector to transform it to *E. coli* JM109. After culturing the bacteria overnight at 37°C, positive-transformed bacteria containing the PCR segment were screened

and stored in glycerin at -80°C. Plasmid DNA was extracted by using the Promega Wizard® Plus Minipreps DNA Purification System (A7100) according to the manufacturer's instructions. The PCR segments for insertion were determined by direct sequencing. Next, standard plasmid with another locus type was built by mutagenesis amplification of an overlapping fixed-point (11,12). A standard plasmid with another locus type was sequenced for verification. Wild-type and mutant plasmid DNA were extracted for PCR amplification or stored at -20°C for future applications.

Real-time PCR Amplification and HRM Curve Analysis

In the current study, we optimized the concentrations of primer pairs, $MgCl_2$, and fluorescent dye to obtain an appropriate amplification reaction system. For the real-time PCR reaction system, the final concentration of PCR reaction: 1xPCR reaction buffer, dNTPs 200 µM, forward and reverse primers 0.5 µM, MgCl2 3.5 mmol/L, 1xEva Green dye, 1U DNA polymerase, and 50 ng genome DNA template; deionized water was added to make 20 µL. Reaction conditions were: pre-denaturation at 94°C for 2 min, denaturation at 94°C for 15 s, and annealing at 60°C for 45 s. There were a total of 45 cycles, and the melting-curve generation reaction was performed at 75°C for 30 s. To generate the melting curve, the temperature was increased by 0.1°C/s up to 90°C. The experimental results were analyzed with high-resolution melting curves using the attached software (RotoGene Q, Qiagen, Germany).

Sensitivity and Reproducibility Experiments

The initial concentration of recombinant plasmid DNA was calculated based on the plasmid concentration calculation method (11,12). Recombinant plasmid DNA was serially diluted up to 10-fold with wild-type plasmid; then, through the real-time PCR and HRM analysis, the detection limit was evaluated. Meanwhile, high and low concentrations of plasmid DNA were chosen to study reproducibility. From numerous experiments, variation coefficients between Tm values obtained from each repeated template reaction with various concentrations were calculated to evaluate reproducibility of the assay.

Detection and Verification of Clinical Samples

Based on the plasmid standards, the genomic DNA of samples was amplified by PCR using the reaction conditions described above. PCR-amplified products were gel-extracted and sequenced in both sides with the F1 and R1 primers. The sequencing results were compared with the results obtained from real-time PCR integrated with an HRM curve method to verify accuracy and reliability.

RESULTS

Specific Evaluation Results

The results showed that the primers selected in this experiment can effectively amplify the DPYD gene containing the T85C locus without generation of primer-dimers and other non-specific secondary structures; regardless of the sample (wild-type, homozygous mutant, or heterozygous), the template amplifications were obtained. Based on the HRM curve results, the three types (wild-type, mutant, and heterozygous) could be clearly identified (Figure 1a). The T85C locus was mutated from T to C, which increased the GC%, resulting in a different unwinding time of the PCR segment. The results

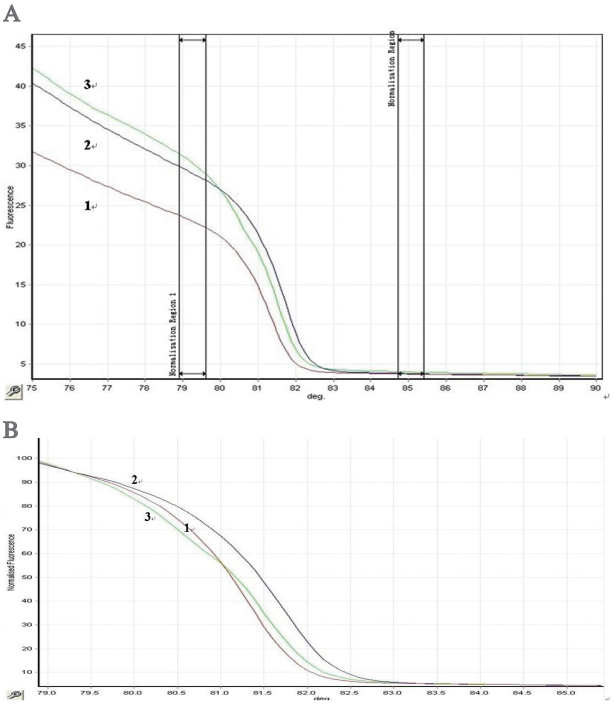

Figure 1. HRM curve results of the three DPYD gene amplification segments.

HRM analyses were performed based on fluorescent quantitative PCR using a saturated dye as described in Materials and Methods.

A: Fluorescent signals of melting curves from three different segments.

B: HRM curve showing high-resolution melting curve analysis results of samples in Figure A.

Notes: 1: Wild-type had a Tm of 81.30; 2: homozygous-mutant type had a Tm of 81.68; and 3: heterozygous type had a Tm of 81.45.

indicated that the Tm values corresponding to the three types of DNA templates were 81.30 (wild-type), 81.68 (homozygous mutant), and 81.45 (heterozygous) (Figure 1b). Furthermore, three types of melting curves of different shapes were observed that could be useful as a specific means of detecting alterations at the T85C locus. The PCR products of mutated templates (T→C) were increased by temperature after analyzing the melting curve, and then the sharp of the HRM curve was changed according to the increase in GC%. The unwinding speed of double-stranded DNA was slower than that of PCR products from the wild-type template. The above results demonstrated that real-time PCR amplification combined with the HRM curve can distinguish polymorphisms of the DPYD T85C locus effectively and accurately.

Construction Results of Standard Plasmid

Based on the above reaction conditions, samples were amplified as PCR segments with a length of 223 bp. PCR products were inserted into the pGEM-T vector for determining the mutant type of the DPYD T85C locus by direct sequencing. Standard plasmid corresponding to another locus type was constructed by using a mutagenesis amplification method with an overlapping fixed-point based on the sequencing results, and the sequences determined in wild-type and mutant plasmids were aligned using Genedoc software. Figure 2 showed the partial sequencing results of the wild- and mutant-type plasmid. Two kinds of standard plasmids were constructed successfully and could be used for reference standards in the analysis by real-time PCR and HRM curves.

Sensitivity of Real-Time PCR Combining HRM Curve Method

In this current work, wild-type plasmid was selected to evaluate the sensitivity of the method. The extracted standard plasmid was determined for initial concentration, and then serially diluted 10-fold into standards ranging from 10^0-10^5 copies/μL. The results showed that there was no positive amplified signal for the plasmid with 10^0 copies/μL. The remaining five gradients produced positive signals with linear relationships (Figure 3). Therefore, the detection limit was 10 copies/μL. At the same time, the standard curve equation was generated with 0.996 of R^2. The copy number of the target gene in unknown samples was accurately calculated using this equation (Figure 4). (Figure 5A,5B) shows the HRM results of a series of

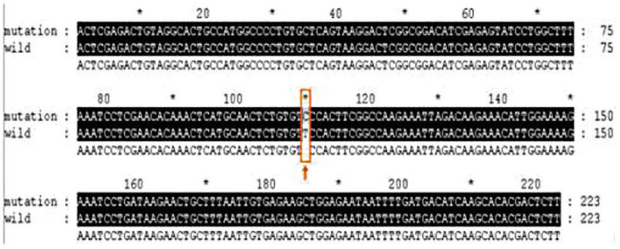

Figure 2. Comparison of sequences of standard plasmids.
Notes: wild: wild-type plasmid; mutation: homozygous-mutant plasmid; Arrow indicates T85C.

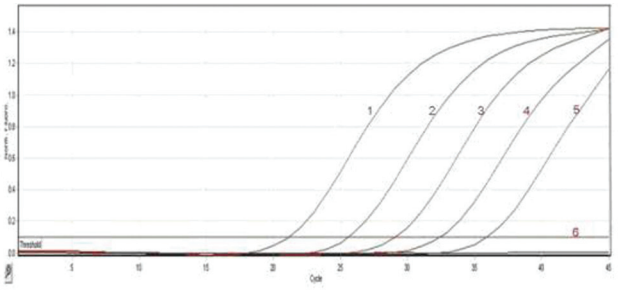

Figure 3. Fluorescent quantitative PCR outcomes of standard plasmid. HRM analyses were performed based on fluorescent quantitative PCR as described in Materials and Methods.
Notes: 1-6: 10^5-10^0 copies/μL. There was no positive amplified signal for the plasmid with 10^0 copies/μL. The remaining five gradients produced positive signals with linear relationships.

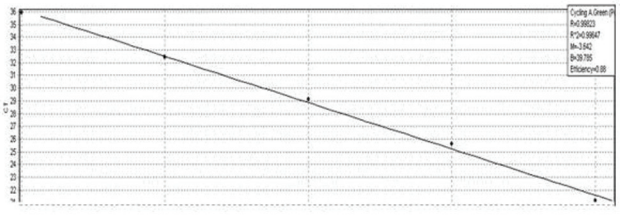

Figure 4. Standard curve, HRM analyses were performed based on fluorescent quantitative PCR with a set of serially diluted standards as described in Materials and Methods.
Notes: y=-3.642×lgx+39.79; y: corresponding CT value; x: CFU value of *E. coli* (The copy number of the target gene in unknown samples was accurately calculated using this equation).

standard plasmids. In spite of the different template concentrations at the beginning of the reaction, the curves were all similar in shape because all the amplification segments were wild-type.

Method Reproducibility

Two concentrations of wild-type plasmid standards, 10^5 copies/μL and 10 copies/μL, were randomly selected to perform reproducibility studies, done in triplicate for each template concentration. Tm values of the results were analyzed statistically to calculate the variation coefficient. The variation coefficients of the two templates correspon-

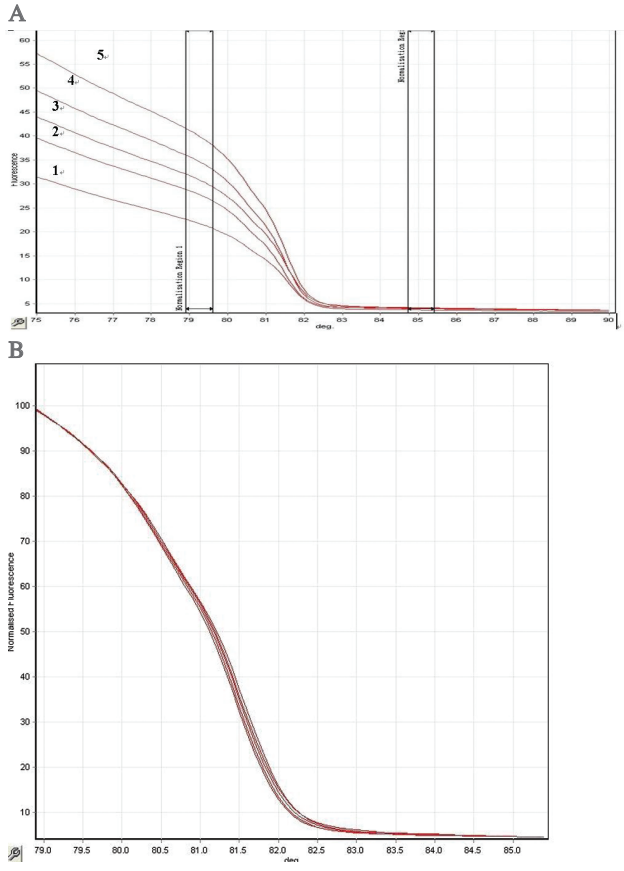

Figure 5. Melting curve results.
HRM analyses were performed based on fluorescent quantitative PCR as described in Materials and Methods.
A: Melting curve showing the linear relationships of different template concentrations at the beginning of the reaction.
B: HRM curve results showing similar curve morphology after high-resolution melting analysis.
Notes: 1-5: 10^1-10^5 copies/μL.

ding to the Tm values were shown to be 0.983% and 0.992%, respectively. The above results indicate that the method was highly reproducible, stable, and suitable for clinical applications.

Testing Result of Clinical Specimens

Table 1 indicates that there were 79 cases of wild-type (70.5%), 29 cases of heterozygous (25.9%), and 4 cases of mutant (3.6%) loci. The result was in exact accordance with the result of the sequencing method, with a 100% accuracy rate.

DISCUSSION

The susceptibility of individuals to many diseases and the variability of drug metabolism between individuals have been shown to be related to genetic polymorphisms (13,14). Currently, finding the cause of the disease and formulating the right therapeutic schedule based on individual differences are rapidly developing trends in medicine.

DPYD T85C polymorphism results in a non-polar amino acid cysteine at the 29th position substituted for the basic amino acid, arginine. This might result in a difference in enzyme activity. The most common genotypes are wild-type (TT), heterozygous (TC), and homozygous mutant (CC). Research has shown that the TC genotype is a high-risk factor for gastric cancer (15,16). In addition, in Chinese patients with gastrointestinal cancer who received chemotherapy, the incidence rate of nausea and vomiting in TC patients increased significantly when compared to the TT genotype (17). Because of insufficient data, the distribution frequency of the DPYD T85C TC genotype in eastern and western populations has been reported to vary from 4.3% to 33.8% (15,18-20). A larger sample size and higher performance methods may be required to detect differences in these populations. Conventional genotyping methods such as single-strand conformational polymorphism (SSCP) analysis require a separation step, which can take hours to perform, and which increases the risk of contamination (21). HRM is the only method that can be performed in the same container that is used for PCR amplification. Such a closed-tube method requires no processing or automation and is immediately available after scanning for genotyping (22). Additionally, genotyping by HRM requires only PCR primers and high-resolution dye, whereas hydrolysis single nucleotide polymorphism (SNP) analysis (TaqMan) requires more expensive labeled probes (23). Therefore, HRM is a less-expensive method, providing precise and powerful capabilities.

This present study is the first to describe the use of real-time PCR combined with HRM curve technology for analysis of DPYD polymorphism. Be-

Table 1. Comparison of results of fluorescent quantitative PCR-HRM and direct sequencing for the detection of T85C polymorphisms

Gene	Fluorescent quantitative PCR-HRM (n)			Direct sequencing (n)		
Locus	Wild type	Heterozygous	Homozygous mutant	Wild type	Heterozygous	Homozygous mutant
T85C	79	29	4	79	29	4

cause only a total of 112 gastric cancer patients were selected as experimental subjects, we propose further sample testing and clinical observation in the future to provide the basis for individual analysis of the etiology of gastric cancer and tailored therapeutic decision-making.

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