Chinese Chemical Letters Vol.17, N0.4, pp 499-501, 2006
http://www.imm.ac.cn/journal/ccl.html

A Novel Real-time Fluorescence Mutant-allele-specific Amplification Method for Rapid Single Nucleotide Polymorphism Analysis

De Bin ZHU, Da XING*, Xian LI, Lan ZHANG

MOE Key Laboratory of Laser Life Science and Institute of Laser Life Science, South China Normal University, Guangzhou 510631

Abstract: Current methods for single nucleotide polymorphism (SNP) analysis are time-consuming and complicated. We aimed at development of one-step real-time fluorescence mutant-allele-specific amplification (MASA) method for rapid SNP analysis. The method is a marriage of two technologies: MASA primers for target DNA and a double-stranded DNA-selective fluorescent dye, SYBR Green I. Genotypes are separated according to the different threshold cycles of the wild-type and mutant primers. K-*ras* oncogene was used as a target to validate the feasibility of the method. The experimental results showed that the different genotypes can be clearly discriminated by the assay. The real-time fluorescence MASA method will have an enormous potential for fast and reliable SNP analysis due to its simplicity and low cost.

Keywords: Mutant-allele-specific amplification, single nucleotide polymorphism analysis, SYBR Green I, K-*ras* oncogene.

Single nucleotide polymorphism (SNP) analysis is very important in cancer research and treatment[1]. Current methods for SNP analysis, such as single-strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), oligonucleotide ligation assay (OLA), *etc.* are usually multi-stage, tedious and laborious. These procedures involve handling of large numbers of test tubes, time-consuming blots and hybridizations. Furthermore, they all require use of radioactive isotopes or other hazardous materials[2-4]. Thus, a rapid and simple method for genotyping is expected.

We developed the SNP analysis method using K-*ras* oncogene as target, which is often activated by point mutation in human malignancy. The codon 12 is the major "hot spots" for its activation[5-7].

Experimental

Human embryo kidney (HEK) cell line 293 was purchased from the Center of Experimental Animal Sun Yat-sen University. Human colorectal adenocarcinoma cell line SW480, which harbors a homozygous K-*ras* codon 12 mutation (G12V), was purchased from China Center for Type Culture Collection. Primers were synthesized by

* E-mail: xingda@scnu.edu.cn

Shanghai Sangon Biological Engineering & Technology services Co., Ltd. (SSBE). The wild-type (WT) primer was 5′-tgg tag ttg gag ctg g-3′. The mutant (MT) primer was 5′-tgg tag ttg gag ctg t-3′. The common reverse primer was 5’-ttg ttg gat cat at-tcgt-3’.

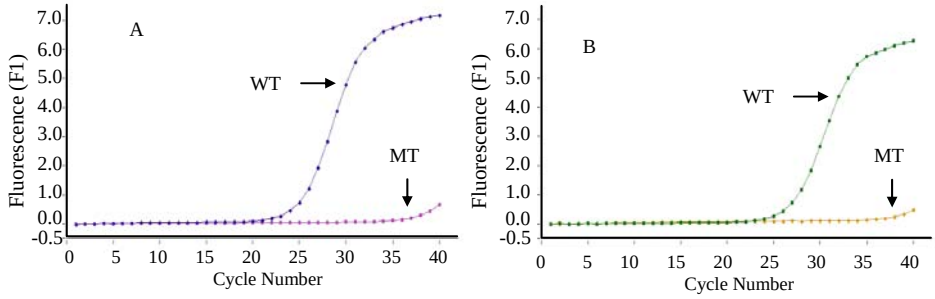
Genome DNA was extracted according to the protocol of UNIQ-10 column genome DNA extraction kit (SSBE). MASA was performed with the Roche LightCycler System (Roche Diagnostics). The fluorescence emissions by the double-stranded DNA-selective fluorescent dye SYBR Green I during the reaction were monitored by the instrument (LightCycler). The amplification protocol consisted of 95 °C for 10 min for initial denaturation, 45 cycles of 10 s at 95 °C for denaturation, 5 s at 56 °C for primer annealing, and 10 s at 72 °C for extension. When the value of the emitted fluorescence exceeded the threshold calculated by the instrument, the samples were deemed positive at any given cycle. The threshold cycle (C_t) is defined as the cycle at which PCR amplification reached a significant value (usually 10 times the standard deviation of the baseline). The C_t values showed linear correlation with the relative DNA copy numbers.

Results and Discussion

Figure 1 is the real-time fluorescence amplification curves of wild-type (A) and mutant (B) homozygous K-*ras* oncogene. For the sample with wild-type homozygote (HEK293 DNA), the MT primer showed no reaction, whereas the WT primer generated amplicon until approximately 36 cycles (**Figure 1** A). On the contrary, for the sample with GTT mutant homozygote (SW480 DNA), the WT primer showed no reaction whereas the MT primer generated amplicon until approximately 36 cycles (**Figure 1** B). **Figure 1** clearly showed that each specific profile can distinguish between wild-type and mutant homozygous K-*ras* oncogene.

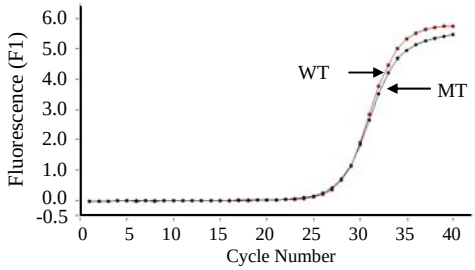
The heterozygous K-*ras* sample was prepared by mixing the SW480 and HEK293 DNA with the ratio of 1:1. **Figure 2** shows the real-time fluorescence amplification curve of heterozygous K-*ras* oncogene. In the heterozygous model, both reactions proceed due to equal proportion of mutant and wild-type alleles.

Figure 1 Real-time fluorescence amplification curves of wild-type and mutant homozygous K-*ras.*



(A) K-*ras* genotyping using HEK293 DNA (wild-type) as template. C_{tWT}=25.14, C_{tMT}>36.00.
(B) K-*ras* genotyping using SW480 DNA (GGT mutant) as template. C_{tMT}=27.00, C_{tWT}>36.00. WT: WT primer, MT: MT primer.

Figure 2 The real-time fluorescence amplification curve of heterozygous K-*ras*.



K-*ras* genotyping using the 1:1 DNA mixture of SW480 and HEK293 as template. C_{tWT}=27.72, C_{tMT}=27.74. WT: WT primer, MT: MT primer.

In this study, we describe a closed-tube method for genotyping entire amplicons that uses the dsDNA dye SYBR Green I in combination with MASA techniques. Genotypes are separated according to the different threshold cycles of the wild-type and mutant primers. This assay is rapid, simple, inexpensive, and reproducible. It does not require restriction enzyme cleavage or purification of PCR products. Furthermore, the assay does not include electrophoresis, which is often used in traditional methods, and is therefore highly specific, unaffected by the formation of spurious PCR amplification products. In contrast to traditional assays, which often depend on analysis of ethidium bromide-stained gels, the results are more accurate. The interpretation in this study is based on the difference between C_t values generated using wild-type and mutant primers. The evaluation does not need specialized laboratory personnel, because it is not visual and the results can be interpreted by computer.

In conclution, we have developed a rapid real-time fluorescence MASA method to discriminate different genotype of K-*ras* oncogene. This approach will have an enormous potential for fast and reliable SNP analysis due to its simplicity and low cost.

Acknowledgments

This research is supported by the National Natural Science Foundation of China (60378043; 30470494), and the Natural Science Foundation of Guangdong Province (015012; 04010394).

References

1. H. C. Erichsen, S. J. Chanock, *Brit. J. Cancer*, **2004**, *90*(4), 747.
2. D. Zhu, D. Xing, X. Shen, *et al.*, *Biosens. Bioelectron.*, **2004**, *20*(3), 448.
3. D. Zhu, D. Xing, X. Shen, *et al.*, *Biochem. Biophys. Res. Commun.*, **2004**, *324*(2), 964.
4. D. Zhu, D. Xing, X. Shen, *et al.*, *Chem. J. Chin. Univ.*, **2005**, *26*(7), 1248.
5. S. Rodenhuis. *Semin. Cancer Biol.*, **1992**, *3*(4), 241.
6. T. Minamoto, M. Mai, Z. Ronai, *Cancer Detect. Prev,*, **2000**, *24*(1), 1.
7. C. A. Ellis, and G. Clark, *Cell Signal.*, **2000**, *12*(7), 425.

Received 24 August, 2005