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RESEARCH ARTICLE

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The application of asymmetric PCR-SSCP in gene mutation detecting

Abstract The advantages and disadvantages of asymmetric PCR-SSCP and the traditional PCR-SSCP were compared in this study. The mutations in 3′UTR of *Smad*4 gene of Luxi cattle and the Holstein cow were analyzed by asymmetric PCR-SSCP and one insert "T" mutation and one G/A mutation in this region were found. The G/A mutation created a *Hha*I restriction enzyme digestion position and the frequencies studied by asymmetric PCR-SSCP and *Hha*I-RFLP in 116 Luxi cattle and 75 Holstein cows were all the same. The asymmetric PCR-SSCP had fewer, clearer and more stabile bands than traditional PCR-SSCP. This indicates that the asymmetric PCR-SSCP is suited for mutation detection.

Keywords asymmetric PCR-SSCP, PCR-RFLP, mutation, *Smad*4

1 Introduction

The mutation detecting technology is a major field in molecular biology and has great importance in molecular diagnosis and animal breeding. Detecting known and unknown mutations is a major task in gene mutation detection (Butler et al., 2001). With the rapid development of molecular biology technology, more and more new technologies, such as DNA chip (Ho-Pun-Cheung et al., 2006; Kim et al., 2004; Vernet and Tran, 2005) and DHPLC (Lilleberg, 2003; Ribas et al., 2001) are used in this field. These new technologies can improve the efficiency and accuracy in mutation detection, but many special instruments and software are required which limits its generalization in many fields. The PCR-SSCP analysis is rapid and low-cost compared with other mutation detecting technologies and it has become a major method in detecting unknown mutation. The theory of PCR-SSCP is to denature dsDNA (PCR product) using a denaturing agent at high temperature. The denatured ssDNA can refold to a specific second structure under rapidly cooling. The mutation can change the second structure of ssDNA and the mobility in polyacrylamide gel which may induce the formation of different bands between wild type DNA and mutation DNA. Therefore, the wild type animal and gene mutation animal can be discriminated by PCR-SSCP.

Because the second structure of ssDNA is vulnerable and many physical and chemical factors can break this structure to form another specific second structure, different SSCP results, even from the same samples, may appear under different PCR-SSCP conditions. The bands in some PCR-SSCP analyses are near and fuzzy and this may increase the difficulty in judging gene types by PCR-SSCP. In recent years, many new PCR-SSCP technologies have been developed. The asymmetry PCR-SSCP is one of these new PCR-SSCP technologies. This study analyzed the mutations of *Smad*4 gene 3′UTR in Luxi cattle and Holstein cow using asymmetry PCR-SSCP and compared the characteristics of PCR-SSCP and asymmetry PCR-SSCP in mutation detection.

2 Materials and methods

2.1 Materials

116 Luxi cattle and 75 Holstein cows were included in this study and genome DNA was isolated from blood leukocytes. The DNA was stored at −80°C until use.

Taq DNA polymerase, dNTPs mixture and DNA gel extraction kit were purchased from the Tiangen Biotech (Beijing) Corporation. *Hha*I restriction enzyme was purchased from the Takara Corporation (Dalian).

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Two pairs of primers were designed by Primer 5.0 software according to cattle *Smad*4 gene 3′UTR. The first pair of primers (M1) had forward: 5′-ACCAGACAAAGG-AGTATCGT-3′ and reverse: 5′-TTAGC TTAGCATTG-CCACCTGT-3′ and the predicted product length was 259 bp. The second pair of primers (M2) had forward: 5′-TGTGCCATGTGGGTGAGTTA-3′ and reverse: 5′-AGG CTCCTTAGTAACCAACC-3′ and the predicted product length was 345 bp. Primers were synthesized by the SBS Genetech corporation.

2.2 Methods

2.2.1 Asymmetry PCR

Asymmetry PCR was divided into two stages. In the first stage, a pair of primers at the same dosage was used to clone the dsDNA and in the second stage, the dsDNA produced in the first stage was added into the PCR reaction system as a template. One of a pair of primers was used to clone the ssDNA. The PCR reaction mixture in the first stage contained 0.4 μg of genomic DNA, 40 $\mu mol \cdot L^{-1}$ per dNTP, 0.2 $\mu mol \cdot L^{-1}$ per primer, 0.6 $mmol \cdot L^{-1}$ of $MgCl_2$ and 0.4 U of *Taq* polymerase in 20 μL final volume. The conditions for PCR amplification were: 95°C for 5 min; followed by 33 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, with final extension at 72°C for 10 min. The PCR reaction mixture in the second stage contained 0.4 μg of genomic DNA, 40 $\mu mol \cdot L^{-1}$ per dNTP, 0.2 μL of primer in a pair, 0.6 $mmol \cdot L^{-1}$ of $MgCl_2$ and 0.4 U of *Taq* polymerase in 20 μL final volume. The conditions for PCR amplification in the second stage were the same as in the first stage. The reaction system and conditions in the second pair of primers were the same as in the first pair of primers except that the melt temperature of the second pair of primers was 57°C.

2.2.2 Polyacrylamide gel electrophoresis

Three microlitre per asymmetry PCR product was mixed with 1 μL 6 × loading buffer and then was loaded on acrylamide isacrylamide (29:1, 12%) gels. Electrophoresis was performed using 100 V at room temperature in 1 × TBE buffer and gels were silver-stained after electrophoresis.

2.2.3 Restriction fragment length polymorphism analysis

A total of 10 μL reaction system including 0.3 μL of 10 $U \cdot \mu L^{-1}$ *Hha*I restriction enzyme, 1 μL of 10 × buffer, 4 μL of PCR product and 4.7 μL of distilled water. The mixture was put into an incubator at 37°C for 8–10 h and was separated on acrylamide isacrylamide (29:1, 10%) gels.

3 Results

3.1 Comparison of PCR-SSCP with asymmetry PCR-SSCP

The bands in PCR-SSCP were often more and fuzzier than those of asymmetry PCR-SSCP. Figures 1 and 2 show the results of PCR-SSCP and asymmetry PCR-SSCP of same samples, respectively. We can find lane 1, lane 3 and lane 6 were very different from lane 2, lane 4 and lane 5, and lane 3 was also different from lane 1 and lane 6 in Fig. 1. Figure 2 shows the results of asymmetry PCR-SSCP of the same samples in the same order. We can find lane 1, lane 3, and lane 6 were AB gene type and lane 2, lane 4 and lane 5 were AA gene type in Fig. 2. The results of *Hha*I-RFLP were the same as in asymmetry PCR-SSCP. This indicated that we can judge the gene type easily by asymmetry PCR-SSCP compared with PCR-SSCP.

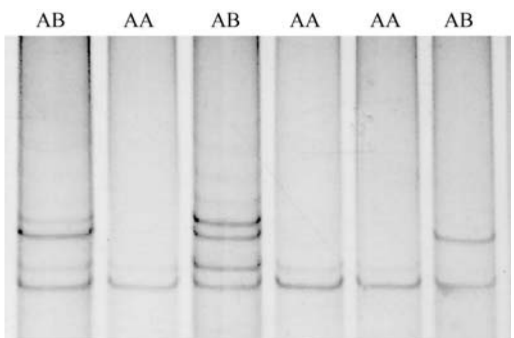

Fig. 1 PCR-SSCP analysis of M1 primers PCR product

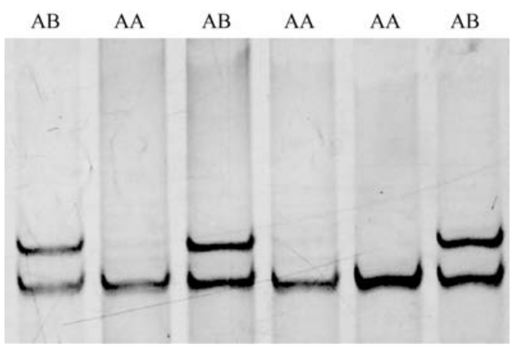

Fig. 2 Asymmetric PCR-SSCP analysis of M1 primer PCR product

The asymmetry PCR-SSCP was more stabile than PCR-SSCP. The bands in PCR-SSCP changed in different SSCP analysis of the same samples, but this phenomenon did not happen in asymmetry PCR-SSCP.

3.2 The application of asymmetry PCR-SSCP in mutation detecting

One insert “T” mutation and one G/A transitions mutation were found by asymmetry PCR-SSCP. The gene type of each sample could be judged by asymmetry PCR-SSCP easily (Figs. 2 and 3). The G/A transitions mutation created a *Hha*I-RFLP site, which divided the 259-bp PCR product into two fragments: one fragment was 143 bp and the other was 116 bp. Figure 4 shows the results of *Hha*I-RFLP.

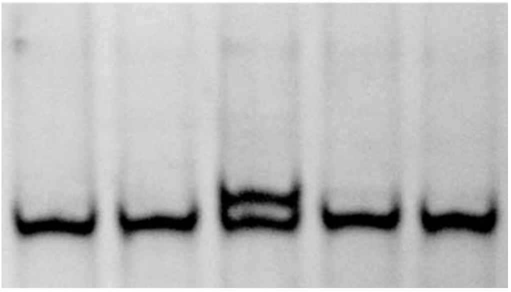

Fig. 3 Asymmetric PCR-SSCP analysis of M2 primer PCR product

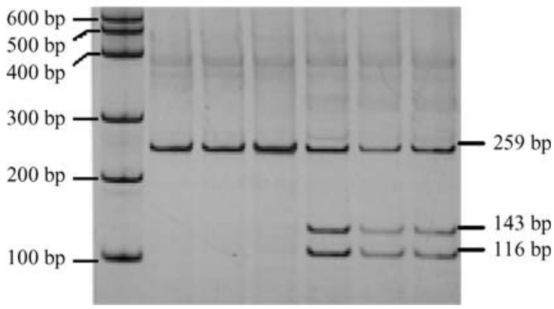

Fig. 4 The *Hha*I-RFLP analysis of M1 primer PCR product

3.3 Comparison of asymmetry PCR-SSCP and *Hha*I-RFLP

The G/A transitions mutation in *Smad*4 gene 3′UTR was analyzed by asymmetry PCR-SSCP and *Hha*I-RFLP in 116 Luxi cattle and 75 Holstein cows, respectively. 108 AA gene type samples and 8 AB gene type samples were found in 116 Luxi cattle and 71 AA gene type samples and 4 AB gene type samples were found in 75 Holstein cows by asymmetry PCR-SSCP. No BB gene type sample was found in these two groups by asymmetry PCR-SSCP (Table 1). The results of *Hha*I-RFLP were completely consistent with the results of asymmetry PCR-SSCP from the same samples. This indicated the asymmetry PCR-SSCP could be used in judging samples gene type for its high discrimination and reproducibility.

Table 1 Comparison of asymmetric PCR-SSCP and *Hha*I-RFLP in gene typing

method	Luxi cattle			Holstein cows			total
	AA	AB	BB	AA	AB	BB	
asymmetric PCR-SSCP	108	8	0	71	4	0	191
*Hha*I-RFLP	108	8	0	71	4	0	191

4 Discussion

4.1 The comparison of asymmetry PCR-SSCP and normal PCR-SSCP

Many factors, such as room temperature, voltage and concentration of polyacrylamide gel can affect the result of PCR-SSCP. The bands in different PCR-SSCP analysis of the same samples may be different, especially when more and fuzzier bands appear. In recent years, many new technologies have been applied in PCR-SSCP analysis to increase the stability and reproducibility. Asymmetry PCR-SSCP is one of these new technologies. The asymmetry PCR-SSCP has more reproducibility under permanent room temperature even at high voltage of electrophoresis (Kiyama and Fujita, 1996). This may shorten the time of asymmetry PCR-SSCP analysis compared with the normal PCR-SSCP analysis. The ratio of ssDNA/dsDNA is usually higher by asymmetry PCR than by denaturation, and the conformation of ssDNA in asymmetry PCR-SSCP is usually more stable than in normal PCR-SSCP. This may be one of the reasons that the asymmetry PCR-SSCP has stronger resistance to variable environment temperature. Voltage also has an effect on PCR-SSCP by raising the temperature of polyacrylamide gel. If the conformation of ssDNA is not strong enough to resist the change of environment temperature, the bands of SSCP will change simultaneously.

If the concentration of PCR product is very high, denaturation of dsDNA will be difficult and incomplete. On the other hand, the denatured ssDNA may anneal during electrophoresis in PCR-SSCP analysis. These two factors may affect the results of PCR-SSCP, but have no effect on asymmetry PCR-SSCP because only one kind of ssDNA is amplified in asymmetry PCR (Lazaro and Estivill, 1992). The residual primers may anneal with ssDNA and interfere with the conformation of ssDNA, and afterwards, may have an effect on the results of PCR-SSCP (Isabelle, 1993). This disadvantage can be overcome in asymmetry PCR-SSCP which may completely consume the primer and the residual primer cannot anneal with ssDNA because it has the same nucleotide sequence as ssDNA.

4.2 The application of asymmetry PCR-SSCP in mutation detecting

Mutation sometimes can only change the bands of one kind of ssDNA on the pair of dsDNA. In our study, the G/A mutation only affected the mobility of the plus ssDNA in the PCR product of the first pair of primers, while the insert “T” mutation only changed the mobility of the negative ssDNA in the PCR product of the second pair of primers. This characteristic of asymmetry PCR-SSCP is greatly useful in gene typing because we can only use one primer of a pair to judge the sample gene types. The results of asymmetry PCR-SSCP were also identical with *Hha*I-RFLP in mutation detection in this study. Asymmetry PCR-SSCP was applied in detecting mutations on neurofibromatosis type I (Lazaro and Estivill, 1992). 86 mutations in mouse β-globin gene were found using asymmetry PCR-SSCP (Glavac and Dean, 1993). The nucleotides around the mutation would affect the sensitivity of PCR-SSCP. A/G mutation occurred in the G/C rich region would increase the mobility of the band (s), while G/A mutation which occurred in the G/C rich region would decrease the mobility of the band(s). In addition, asymmetry PCR-SSCP was also applied in detecting mutations in one sequence, such as sheep OLA-DRB1 gene (Bhide and Mikula, 2005).

With the development of molecular biology, more and more new technologies will be applied in PCR-SSCP. In recent years, some researchers have labeled the primers with fluorescent material to increase the sensitivity (Makino et al., 2000; Boutin et al., 1997) or have used capillary electrophoresis (Kozlowski and Krzyzosiak, 2004; Nishimura and Tsuhako, 2000) instead of normal polyacrylamide gel electrophoresis in PCR-SSCP analysis. These new PCR-SSCP technologies will be widely used in the future for their automation and high sensitivity.

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