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Methylenetetrahydrofolate reductase (MTHFR) gene polymorphism is associated with abortion in Chinese Holstein cows

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Polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene are associated with abortion, early embryo loss and recurrent spontaneous abortion in human. However, information on the association between MTHFR polymorphism and cow abortion is scarce. In the present study, the effects of MTHFR polymorphism on cow abortion were investigated. The PCR-SSCP and DNA sequencing were applied to detect the polymorphisms in exon 4 and 7 of MTHFR gene in a total of 569 Chinese Holstein cattles. The homocysteine levels during various periods of pregnancy were detected by HPLC. The results show that 8137C/T SNP was identified in exon 7 of MTHFR, but no polymorphism was detected in exon 4. Association analysis revealed that the 8137C/T mutation was significantly associated with cow abortion ($P = 0.0002$). Regarding relative risk of incidence, cows with CT genotype showed greater relative risk than those with CC ($OR = 2.05$, $95\% CI = 1.40$ to 3.00). The blood homocysteine levels of individuals with CT genotype were significantly higher than those with CC genotype during the first 6 months of pregnancy and in non-pregnant cows ($P < 0.05$). In conclusion, MTHFR may be a beneficial candidate gene to control cow abortion.

Key words: Methylenetetrahydrofolate reductase (MTHFR), abortion, Chinese Holstein cattle, SNP.

INTRODUCTION

The abortion rate often exceeds 14% in some cattle herds (Thurmond et al., 1990), which is an important cause of reproductive wastage and economic loss. Abortion could extend calving interval, reduce the number of potential female herd replacements and lifetime milk production. It was reported that the prevalence of pregnancy loss was 14.4% ($n = 3775$) (Bamber et al., 2009). The proportion of aborted cows and abortions per confirmed pregnancy were 5.9 and 10.2%, respectively in Israel (Markusfeld-Nir, 1997). The abortion rate was 7.17% ($n = 4951$) among 10 herds in NingXia Province of China between 2004 and 2007 (Yang, 2008). The annual

cost of a dairy with 600 pregnancies (8% abortion incidence) per year could be as high as \$31,200 (Thurmond et al., 1990).

The 5,10-methylenetetrahydrofolate reductase (MTHFR) is a key enzyme of folate and homocysteine metabolism (Klerk et al., 2002). MTHFR mutation elevated homocysteine level in the blood, and increased risk of abortion and recurrent spontaneous abortion by 2 to 3-fold (Nelen et al., 2000). The hyperhomocysteinaemia was able to result in neural tube defects (Cunha et al., 2002) and recurrent embryo loss (Callejon et al., 2007). It has been reported that the homocysteine is a risk factor of abortion and recurrent spontaneous abortion (Blanco et al., 1998; Nelen et al., 2000). The primary function of folate is that it is an essential nutrient for normal mammalian cell growth and a carrier for single carbon fragments in the conversion of homocysteine to

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methionine and in purine and pyrimidine synthesis (Kim et al., 2006). The enzyme MTHFR catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, the primary form of serum folate (Klerk et al., 2002). MTHFR has been described as being located at the branch point of directing folate metabolites toward remethylation of homocysteine and away from DNA and RNA biosynthesis (Goyette et al., 1998). Deficiency of MTHFR enzyme not only results in deficiency of DNA and protein methylation which is necessary for fetal development, but also increases the concentration of plasma cysteine which would develop into hyperhomocysteinaemia in the end (Blanco et al., 1998; Suormala et al., 2002). The gene coding for MTHFR enzyme is located at the end of the short arm of chromosome 1.p36.3 and includes 11 exons in human (Goyette et al., 1998). The best characterized SNPs that influence homocysteine metabolism are 677C/T in exon 4 and 1298C/T in exon 7 of MTHFR gene in human. The TT homozygote at locus 677 is associated with higher homocysteine levels ($P = 0.01$), especially in individuals with low plasma folate levels (Kim et al., 2006). The C to T mutation at position 1298 leads to an amino acid exchange of glutamine to alanine, and to a lesser extent, influences specific activity of the enzyme than that of the MTHFR 677 polymorphism (van der Put et al., 1998).

For the multiple function of MTHFR, the present study aimed to identify exons 4 and 7 polymorphisms of MTHFR in 569 Chinese Holstein cattle, and to analyze their associations with abortion and homocysteine level.

MATERIALS AND METHODS

Samples and data collection

All procedures involving animals were approved by the Animal Care and Use Committee of Animal Science and Technology College of Huazhong Agricultural University, Hubei, China. A total of 569 Chinese Holstein cows were chosen for this study. Pregnancy diagnosis was conducted by skilled technician at 42 days after AI (Pierson and Ginther, 1984) with a portable ultrasound scanner and a 5 MHz linear-array transrectal transducer (WED-3000, WELLD, Shenzhen, China). Abortion was defined as a 'loss of pregnancy' in the period between 42 and 260 days of gestation according to the Committee on Bovine Reproductive Nomenclature in 1972, including cows with observed abortion and confirmed open, or inseminated in the same lactation after pregnancy (Committee, 1972). Reproduction data of the first parity were collected between November 2008 and November 2010, in order to remove the parity effect on abortion rate. In order to evaluate the relative risk, the populations were divided into cases and controls according to the result of pregnancy diagnosis. A total of 108 plasma samples were collected to detect the blood homocysteine level using HPLC method (Govindaiah et al., 2009). The samples were then divided into pregnant group and non-pregnant group according to PCR-SSCP pattern, and pregnant group were further divided into 8 treatments according to the month of pregnancy. Genomic DNA was extracted from whole blood by the phenol-chloroform protocol (Sambrook and Russell, 2006).

The DNA quality was measured by electrophoresis using a 0.8% agarose gel (Biowest Agarose, Spain).

Primer design

Primers were designed on the basis of bovine genomic sequence (GenBank accession no. NW_001493415) using Premier 5.0 software and synthesized by Shanghai Sangon Company. The primers of exon 4 were: 5'-GAG CCA GCC TCT TTC TAT G-3' (forward) and 5'-TCG GAG CAA GCC TTC ACA-3' (reverse), which generated a 198-base pair (bp) fragment. The primers of exon 7 were: 5'-GGA AGA GCT GCT CAA GAT G-3' (forward) and 5'-TCG GAG CAA GCC TTC ACA-3' (reverse) which generated a 186-bp fragment.

PCR-SSCP analysis

The PCR reaction was carried out in a final volume of 20 μ l containing 20 ng template DNA, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Fermentas, Canada). The PCR reaction was performed in a thermal cycler under the following conditions: denaturation for 5 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58 or 63.5°C respectively, 30 s at 72°C, and a final extension at 72°C for 5 min. Aliquots of 5 μ l PCR products mixed with 10 μ l of formamide and 1 μ l of 6 \times loading buffer, denatured at 98°C for 10 min and quickly chilled on ice for 5 min. Denatured DNA was subjected to 10% polyacrylamide gel electrophoresis (PAGE) (acrylamide: bisacrylamide = 29:1) analysis which was electrophorized with 1 \times TBE at 4°C at constant voltage (120 V) for 12 h after a pre-run at 250 V for 10 min and visualized with silver staining (Sambrook and Russell, 2006). The samples with different SSCP patterns were sequenced to identify the SNPs (ABI-PRISM3730 and Big Dye terminator v3.1, Shanghai Sangon, China).

Statistical analysis

Allele and genotype frequencies of the studied SNPs were determined by direct counting. The chi-square test was used to test differences between abortion rate of different genotypes. In each group, odd ratios (ORs) and their corresponding 95% confidence intervals (CIs) were calculated using an unconditional logistic regression model (SAS, version 8.2), with adjustment for genotypes, sires, seasons and years. A backward elimination strategy was used to obtain a reduced model. Effect modifiers, with a P value greater than 0.05, were removed from the model. Covariants that were significantly associated with spontaneous abortion ($P < 0.05$) were retained in the reduced model (Thurmond et al., 2005; Zhang et al., 2010). The statistical model is:

$$Y_{ijkl} = \mu + G_i + S_j + Y_k + P_l + e_{ijkl}$$

Where, Y_{ijkl} is a vector of observations for abortion traits (0, 1), 1 represents abortion, 0 represents no abortion; μ is the population mean; G_i is fixed effect of genotypes; S_j is fixed effect of sire; Y_k is fixed effect of year; P_l is fixed effect of calving season (winter: from December to next year February; spring: from March to May; summer: from June to August and autumn: from September to November); e_{ijkl} is random residual error.

RESULTS

Polymorphisms of the cow MTHFR gene

No polymorphism was detected in exon 4 of MTHFR in the studied population (Figure 1A). The PCR-SSCP analysis of exon 7 generated two genotypes: 186 with

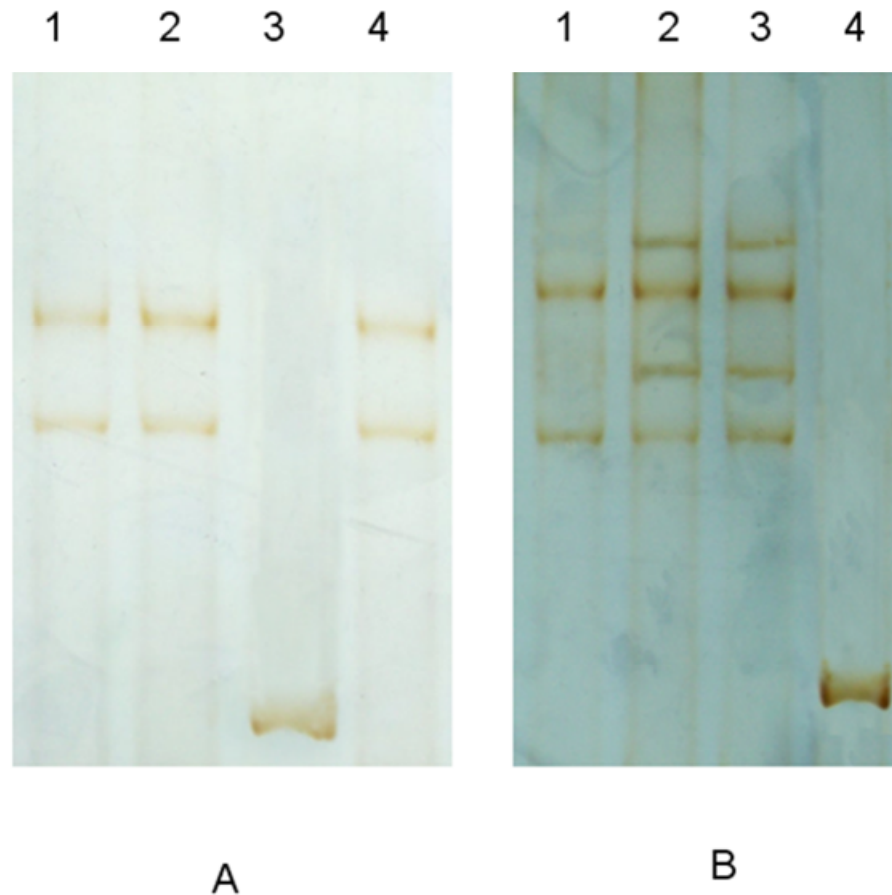


Figure 1. Genotyping of MTHFR gene at loci exon 4 (A) and exon 7 (B) by PCR-SSCP. A: Lanes 1, 2, 4 with two bands have no polymorphism. Lane 3 represents non-denaturing PCR product as the positive control. B: Lane 1 with 2 bands represents genotype CC; lanes 2 and 3 with 4 bands represent genotype CT; lane 4 represents non-denaturing PCR product as the positive control.

heterozygote (CT) genotype and 383 with homozygote CC genotype, no homozygote of TT were found (Figure 1B). The sequences of different genotypes have been submitted to GenBank and the accession numbers for exon 7 are GU247116 to GU247126 (Figure 2). The allelic and genotypic distribution of MTHFR gene exon7 is shown in Table 1. The theoretical genotypic frequencies for CC and CT are 0.673 and 0.327, respectively.

Effects of polymorphisms on relative risk of abortion

Relative risk of abortion between different genotypes was analyzed using the logistic regression analysis, to evaluate the association of relative risk with different genotypes. According to abortion standard after pregnancy, 569 cows were divided into two groups of abortion (106 cows) and non-abortion (463 cows). Cows with CT genotype had significantly greater relative risk (OR = 2.05, 95% CIs:1.40 to 3.00, $P = 0.0002$) than those

with CC (Table 2).

Homocysteine level

Homocysteine levels of CC and CT genotypes in different pregnant periods are shown in Table 3. The homocysteine levels of CT genotype were significantly higher than that of CC in 1 to 6 months of pregnancy and non-pregnant cows ($P < 0.01$). However, there were no significant differences between CT and CC genotype in 7 to 8 months of pregnancy ($P > 0.05$).

DISCUSSION

Polymorphisms of MTHFR

Three genotypes (CC, CT and TT) of MTHFR C667T mutation were found in human (Callejon, 2007; Callejon

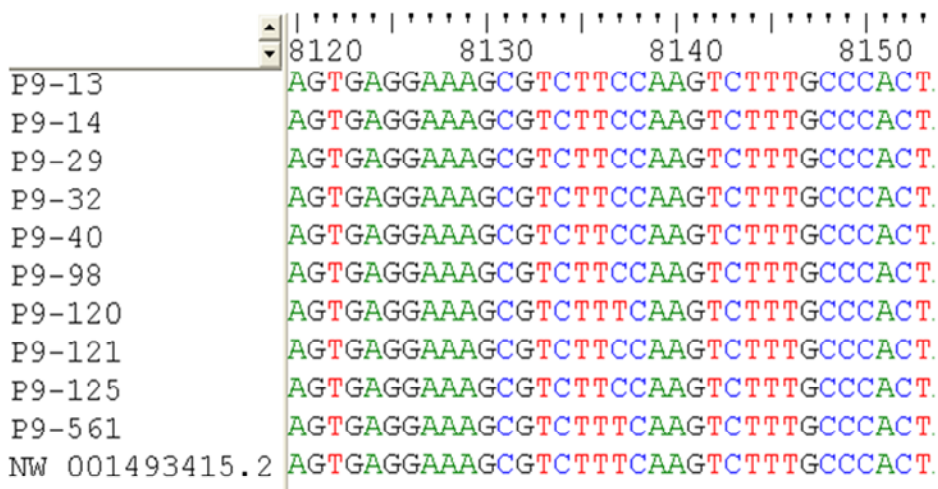
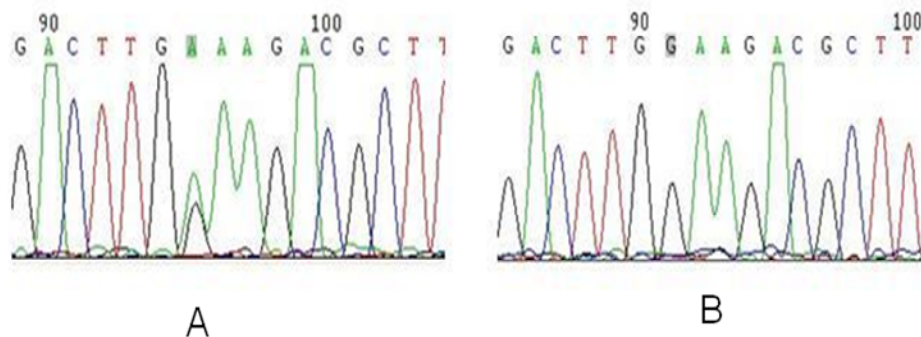


Figure 2. Gene sequencing profile of exon 7. A and B: Shade base represent the situation of the base substitution. Heterozygous (CT) lies in the left map (7th bp in the picture), while homozygous (CC) lies in the right map (7th bp in the picture). Both maps are sequenced using reverse primers. So they are complementary strand. C: A C to T transition at loci 8137 of exon 7. The GenBank accession numbers for exon 7 nucleotide sequences of dairy cows no. 13, 14, 29, 32, 40, 98, 120, 121, 125, 561 and 392 were GU247116 to GU247126, respectively. The position 8137 was accorded to *Bos Taurus* MTHFR sequence NW_001493415 from GenBank database of NCBI by Clustal W multiple alignments.

Table 2. Association of polymorphisms with relative risk of abortion.

Genotype	Abortion (%)	Non-abortion	OR (95% CI)	P value
CC	13.6 (52)	86.4 (331)	1 (reference)	
CT	29.0 (54)	71.0 (132)	2.05 (1.40-3.00)	0.0002

Figures in brackets are the number of samples.

et al., 2007). Interestingly, the TT genotype in exon 7 of MTHFR was not detected in our studied population. This may be caused by natural selection, that is, individuals with the homozygous TT genotype died during the embryo developmental period (Givens and Marley, 2008; Lucock et al., 2008). It is also possible that TT genotype was not found in this study population due to the low allele frequency.

The nucleotide substitution of C to T is a synonymous mutation, which may affect protein function and cause diseases through possible alterations of transcription,

translation, post-translational modification of protein structure (Evans and McLeod, 2003).

Effects of polymorphisms on relative risk of abortion

In our study, the abortion rate of first parity was 18.6% (106 abortions to 569 calving episodes), which was higher than that of Thurmond et al. (1990). Unfortunately, causes of cow abortion are difficult to determine. Any disturbance in the normal physiology of gestation, such

Table 3. Homocysteine levels of CC and CT genotypes in different pregnant periods.

Genotype ($\mu\text{mol/L}$)	Pregnant period								
	Non-pregnant	1st month	2nd month	3rd month	4th month	5th month	6th month	7th month	8th month
CC	11.4 \pm 0.6	10.3 \pm 0.3	24.1 \pm 1.6	20.4 \pm 1.3	16.0 \pm 0.7	17.3 \pm 1.6	11.6 \pm 0.8	9.6 \pm 0.7	12.1 \pm 0.9
CT	19.9 \pm 1.1*	25.9 \pm 1.8*	31.8 \pm 2.1*	25.6 \pm 2.0*	27.1 \pm 1.8*	33.3 \pm 2.3*	29.4 \pm 2.2*	10.5 \pm 0.9	14.5 \pm 1.1

*P < 0.05 in the same column. Data are shown as mean \pm SD.

as infectious agents (brucella abortus, neospora caninum, bovine viral diarrhea virus, leptospirosis, toxoplasma gondii and trypanosoma evansi), toxins, hormonal imbalance, vaccinations, poor nutrition, chromosomal disturbances and physical influences may lead to abortion (Anderson, 2007; Givens and Marley, 2008; Neta et al., 2009; Reiterova et al., 2009; Zhu et al., 2009). The polymorphisms of MTHFR were associated with many human diseases, especially hyperhomocysteinaemia (He et al., 2009), spontaneous abortion (Zetterberg et al., 2003), early spontaneous embryo loss (Callejon et al., 2007), and recurrent pregnancy loss (Govindaiah et al., 2009). Based on the multiple functions of MTHFR, we proposed that the polymorphisms were also associated with unexplained abortion in Chinese Holstein cattle. The present study showed that CT genotype of MTHFR exon 7 had a significant higher relative risk of abortion than that of CC genotype (OR = 2.05). The result was consistent with the report that CC genotype of the C677T polymorphism showed a strong protective effect against abortion (Callejon et al., 2007).

Bovine brucellosis is one of the most important zoonotic diseases worldwide, and is of particular significance in developing countries, Mediterranean countries and Central Asia (Basso Perna et al., 1996; Godfroid and Kasbohrer, 2002; Pappas et al., 2006). Brucellosis is not a causative factor in our study. The herd serologically positive rate for brucellosis was lower than 1% because of the severe intensive serological testing system twice a year and that no positive cow was included in the experiment. Cows were fed the same diet and were in the same herd, so toxin, vaccinations, poor nutrition factors were also excluded in our study.

Homocysteine level

The MTHFR is a key enzyme of folate and homocysteine metabolism, and homocysteine is one of the important risk factors for abortion and recurrent spontaneous abortion (Nelen et al., 2000; Chatzikiyriakidou et al., 2008). It is well recognized that the impact of homocysteine metabolism on pregnancy is associated with the problem of neural tube defects (NTD) (Hague, 2003). Homocysteine also has a direct effect in producing NTD in the avian embryo (Rosenquist et al., 1996). Thus, the genetic polymorphisms associated with mild to moderate

hyperhomocysteinaemia and possible NTD are very relevant.

High homocysteine plasma concentrations in different pregnancy periods for cows with CT genotype had a higher abortion rate, which indicated that the accumulation of homocysteine may be harmful to embryo development and could cause abortion. A meta analysis of six studies in humans showed that high homocysteine concentrations increased the risk of abortion by 40% (OR = 1.4, 95 % CI: 1.0 to 2.0) (Nelen et al., 2000).

In conclusion, the mutation of MTHFR exon 7 might play an important role in unexplained cow abortion and may be considered as a potential candidate gene for abortion, and could be useful in MAS during early life of the calf to reduce the incidence of abortion.

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