

Polymorphisms of Fatty Acid Synthase Gene and their Association with Milk Production Traits in Chinese Holstein Cows

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ABSTRACT

The multifunctional enzyme fatty acid synthase (FASN) catalyzed the second step of the de novo biosynthesis of long-chain fatty acids in mammals. In this research, we investigated the variants in ketoacyl reductase (KR) and thioesterase (TE) domains in FASN by using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). As a result, two single nucleotide polymorphisms (SNPs) were detected and genotyped in 407 Chinese Holstein cows. The non-synonymous g.16024G>A, g.17924G>A in KR and TE domains respectively resulted in a non-conservative substitution of alanine by threonine. g.17924G>A was associated with milk fat percentage, and the milk fat percentage of homozygous genotype AA was significantly higher than the other genotypes. The polymorphism information content (PIC) analysis showed that g.17924G>A in the cows population belonged to the intermediate polymorphism level. The results preliminarily indicated that g.17924 G>A of FASN gene is a potential genetic marker for application of marker-assisted selection programmers to improve milk fat percentage in Chinese dairy cattle.

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Authors' Contribution

MC designed the study. XH, PW and QL conducted the experiments. ZZ and YL analyzed the data and drafted the manuscript. RD, YY, YA and MC helped in preparation of the manuscript.

Key words

Association analysis, Chinese Holstein cows, FASN gene, Milk production traits, SNPs

INTRODUCTION

Nowadays, milk is one of the crucial nutrients in people's daily diet, in which fatty acid content and composition determine the nutritional value of milk. Fatty acid synthase (FASN) is a multi-enzyme complex whose main function is to catalyze the synthesis of fatty acids in the intercellular substance (Ye *et al.*, 2021). The expression level of FASN was significantly increased in a variety of tumor cells and pre-tumor tissues, and its overexpression was closely related to tumor cell proliferation, metastasis, anti-apoptosis (Polonio-Alcalá *et al.*, 2020). As a central regulator of lipid metabolism, FASN could rewire tumor cells to obtain greater energy flexibility to achieve their high energy requirements, therefore, FASN was also

considered as a tumor marker (Fhu and Ali, 2020). As an important metabolic enzyme in fatty acid synthesis during the adult stage, FASN also played a central role during the embryo development in mammals (Chirala *et al.*, 2003). FASN was required for mammary gland development and milk production during lactation (Suburu *et al.*, 2014). The expressions of fatty acid synthase and its encoding gene (FASN) were studied at molecular biology level (Abe *et al.*, 2009; Alim *et al.*, 2014; Roy *et al.*, 2005; Schennink *et al.*, 2009; Li *et al.*, 2016).

FASN gene had been cloned and sequenced in mouse, chicken, human, goose and bovine (Amy *et al.*, 1990; Chirala *et al.*, 1997; Jayakumar *et al.*, 1995; Kameda *et al.*, 1991; Roy *et al.*, 2005). Bovine FASN is located on chromosome 19q22 (Roy *et al.*, 2001) and its expression in brain, testis and adipose tissue is higher than that in liver and heart (Roy *et al.*, 2005). Bovine FASN gene (AF285607) consists of 42 exons. TE domain in this gene is important in fatty acid synthesis as a substrate-binding site. TE domain determines the chain length of the fatty acids and effects on the fatty acid composition of bovine adipose tissue (Zhang *et al.*, 2008). KR domain which was close to the TE domain can affect the function of TE domain by the changing the structure of the substrate-

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binding site (Abe *et al.*, 2009).

The objectives of this study were to analyze the polymorphism of TE domain of *FASN* gene by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and to examine the relationship between *FASN* gene and milk production traits in Chinese Holstein cows. The results could provide basic molecular data for marker assisted selection research in Chinese Holstein cows.

MATERIALS AND METHODS

Blood sample collection and DNA preparation

Jugular blood samples (10 ml per cow) using acid citrate dextrose as an anticoagulant were collected from 407 Chinese Holstein cows calved in 2019 (first, second, or third parity) originated from four dairy farms (139 cows from the 1st farm, 131 cows from the 2nd farm, 69 cows from the 3rd farm and 68 cows from the 4th farm, respectively) in Hebei province, P.R. China.

The 407 Chinese Holstein cows were the progeny of five bulls (n=78, 79, 81, 83, 86) selected randomly.

Calving was partitioned into four 3-month seasons: March through to May (season 1, spring, n=105), June through to August (season 2, summer, n=98), September

through to November (season 3, autumn, n=110) and December through to February (season 4, winter, n=94). Genomic DNA was extracted from whole blood by the phenol-chloroform method, and then dissolved in TE buffer (10 mmol/l Tris-HCl (pH 8.0), 1 mmol/l EDTA (pH 8.0)) and kept at -20°C.

Primer sequences and PCR amplification

Eleven pairs of primers (P1-P11) were designed to screen the SNP at KR and TE domain according to the DNA sequence of bovine *FASN* gene (GenBank Accession No. AF285607). Primers were synthesized by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, P.R. China). Primer sequence, product size, amplified region and annealing temperature were listed in Table I.

The 25 µL polymerase chain reaction (PCR) volume contained approximately 2.5 µL 10 × PCR buffer, 0.5 µmol MgCl₂, 0.5 µmol dNTPs, 1.0 µL of 10 µmol/L each primer, 1.0 µL of 2.5 U/µL *Taq* DNA polymerase (Tiangen, Beijing, P.R. China), 150 ng genomic DNA. Amplified conditions were as follows: 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s, extension at 72°C for 30 s, a final extension at 72 °C for 10 min.

Table I. Primer sequence, product size, amplified region and annealing temperature for bovine *FASN* gene.

Primer name	Primer sequence (5'→3')	Annealing temperature (°C)	Product size	Amplified region
P1	F: GCATGGCCATCTTCCTGAAGAAC R: CTGAATGACCACTTTGCCGATGT	58	224 bp	Exon32
P2	F: GTACGTGAGGAAGAGCAGGAGGC R: CTGTGCGGATCCCAGAGC	67	202 bp	Intron 32
P3	F: CCAGACCTTCATTTGCCAATCCTC R: CCCCTAAAGCCGTCCTCACCA	55	215 bp	Intron 32
P4	F: GTCCTGAGAGATGCCATGCT R: CTGTCCAAGTTCAGGGTGCC	68	91 bp	Intron 32-Exon 33
P5	F: ACCCGGGAGGCATGCCCAG R: GCCGACGCTTCTCACATAT	65	132 bp	Intron 33-Exon 34
P6	F: GTGCAGTGGGGTGCGATTG R: CCAGGATGTGAGTCACAGCCTT	65	234 bp	Intron 34-Exon 35
P7	F: TGACTTGGCCACCGTCAACC R: CATCAGCTGTGCCAGCCTGC	60	181 bp	Intron 35-Exon 36
P8	F: TTGACACGGCTCAACTCGGTG R: CTTGCCATCCCCAACTCCCTT	63	185 bp	Exon 37
P9	F: GCAGCGGCACCCCTGGACA R: TGAGTGTAGGCCATCACGAAGG	62	223 bp	Intron 37-Exon 38
P10	F: GCCACCGTCGAGCTGATCGTGC R: GCTCGGCCAGGGAGCTGTGAAT	63	315 bp	Exon 40
P11	F: GGGACGACCGCGTTAAATAG R: CCGGGTTCCCGACTCGCAACT	62	188 bp	5' promoter region

SSCP detection and genotyping

The 3 μ L PCR product was mixed with 7 μ L gel loading solution (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 10 mmol/L EDTA (pH 8.0), 10% glycerol). The mixture was agitated and denatured at 98°C for 10 min, and quickly placed on ice for 7 min and loaded on 12% neutral polyacrylamide gels (acrylamide: bisacrylamide= 29:1). Electrophoresis was performed in 1 $\times$ Tris borate (pH 8.3)-EDTA buffer at 110 volts for 17 - 22 h at 4 °C. After electrophoresis, the DNA fragments in the gels were visualized by silver nitrate staining, photographed and analyzed by using an AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA). SSCP genotypes were identified by mobility shift due to conformational difference of the single-stranded DNAs of the amplified fragments by each primer, which was caused by nucleotide variation.

Cloning and sequencing

The PCR products with the variation detected by SSCP were cloned and sequenced according to the method of M.X. Chu (Chu *et al.*, 2012).

Statistical analysis

Hardy-Weinberg equilibrium (HWE) and population parameters including gene heterozygosity (H_e), effective allele numbers (N_e) and polymorphism information content (PIC), were calculated using PopGene 32.

The association between each SNP and difference of five milk production traits (milk yield at 305 d, protein percentage, fat percentage, lactose percentage and dry matter percentage) were evaluated by the following linear model using general linear model procedure (GLM) of SAS (Ver 8.12) (SAS Institute Inc., Cary, NC, USA).

$$y_{ijklmn} = \mu + B_i + H_j + P_k + CS_l + G_m + e_{ijklmn}$$

Where y_{ijklmn} is observation of milk production traits; μ is overall mean; B_i is the fixed effect of the i^{th} bull ($i=1, 2, 3, 4, 5$); H_j is the fixed effect of the j^{th} herd ($j=1, 2, 3, 4$); P_k is the fixed effect of the k^{th} parity ($k=1, 2, 3$); CS_l is the fixed effect of the l^{th} calving season ($l=1, 2, 3, 4$); G_m is the fixed effect of the m^{th} genotype ($m=1, 2, 3$); e_{ijklmn} is the random error effect of each observation.

RESULTS

Bovine FASN gene

The KR and TE domains in *FASN* gene of Chinese Holstein cows were successfully PCR amplified (Fig. 1). The sizes of amplification fragments were consistent with the target ones and had a good specificity, which could be directly analyzed by SSCP.

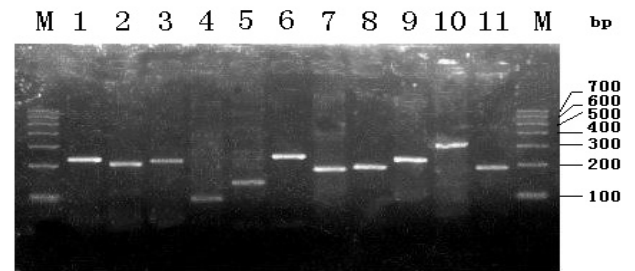


Fig. 1. PCR amplification products of eleven pairs of primers. M: 700 bp DNA marker; 1~11: PCR products of primers P1~P11.

The results of SSCP analysis showed that only the PCR products amplified by P3 and P8 displayed polymorphisms. Three genotypes were detected by primer P3 (Fig. 2A, GG, GA and AA) and primer P8 (Fig. 2B, GG, GA and AA) respectively. For primer P3, sequencing result revealed that g.16024 G>A was existed between genotype GG and genotype AA. For primer P8, g.17924 G>A was existed between genotype GG and genotype AA (Fig. 3). The non-synonymous g.16024G>A, g.17924G>A in KR and TE domains respectively resulted in a non-conservative substitution of alanine by threonine. The frequencies of G allele and GG genotype of g.16024 G>A and g.17924 G>A were higher in Chinese Holstein cows (Table II).

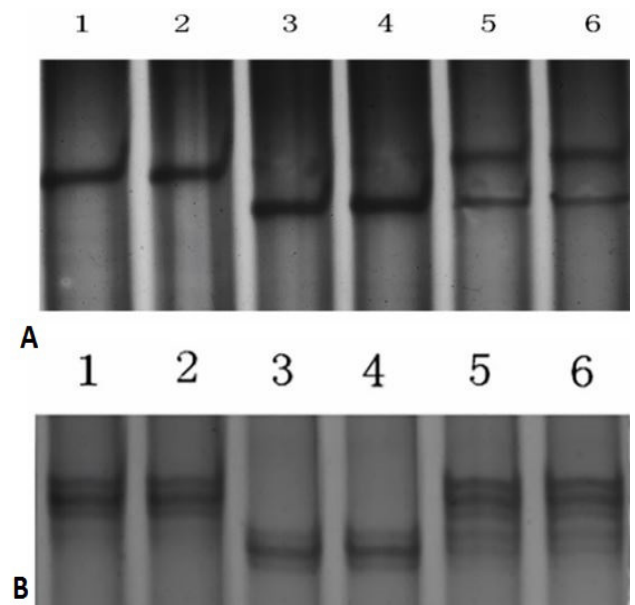


Fig. 2. SSCP analysis of PCR amplification products of primer P3 (A) and primer P8 (B) in bovine *FASN* gene. 1,2: GG genotype; 3,4: AA genotype; 5,6: GA genotype.

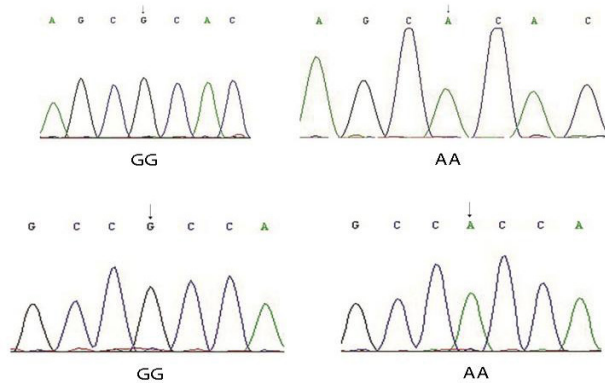


Fig. 3. Sequence comparison of GG and AA genotypes of primer P3 and GG and AA genotypes of primer P8 in bovine *FASN* gene.

Table II. Allele and genotype frequencies of *FASN* gene g.16024G>A and g.17924G>A in Chinese Holstein cows.

Locus	Genotype	Number of samples	Genotype frequency	Allele	Allele frequency
g.16024G>A	GG	308	0.757	G	0.873
	GA	95	0.233	A	0.127
	AA	4	0.010		
g.17924G>A	GG	199	0.489	G	0.701
	GA	173	0.425	A	0.299
	AA	35	0.086		

Genetic characteristics of *FASN* gene

Genetic characteristics of *FASN* gene in Chinese Holstein cows were analyzed. g.16024 G>A and g.17924 G>A of *FASN* gene were in Hardy-Weinberg equilibrium in Chinese Holstein cows (Table III), which indicated that the genotype frequencies of the two loci were not affected by selection, mutation or migration. Generally, polymorphism information content (PIC) was classified into the following three types: low polymorphism (PIC value < 0.25), intermediate polymorphism (0.25 < PIC value < 0.5) and high polymorphism (PIC value > 0.5) (Serrote *et al.*, 2020). According to this classification of PIC, the g.17924 G>A in Chinese Holstein belonged to the intermediate polymorphism level, which indicated that there was a greater variation and selective potentiality in this locus. It might obtain a greater genetic progress for selection.

Association of *FASN* gene polymorphisms with milk production traits

The least squares means and standard errors for the five milk production traits of different genotypes in Chinese Holstein cows were given in Table IV. The mutation g.16024 G>A had no significant effect on milk production traits (milk yield at 305 d, protein percentage, fat percentage, lactose percentage and dry matter percentage) ($P > 0.05$). Regarding to the mutation g.17924 G>A, the fat percentage of AA genotype was higher than the other genotypes ($P < 0.05$). There was no significant difference on fat percentage between genotypes GG and GA ($P > 0.05$) in Chinese Holstein cows (Table IV).

Table III. Genetic characteristics of *FASN* gene g.16024G>A and g.17924G>A in Chinese Holstein cows.

Locus	Hardy-Weinberg equilibrium $\chi^2(P)$	Polymorphism information content (PIC)	Heterozygosity (He)	Effective number of alleles (Ne)
g.16024G>A	1.27(0.529)	0.197	0.221	1.284
g.17924G>A	0.09(0.956)	0.331	0.419	1.721

Table IV. Least squares means and standard errors for milk production traits of different genotypes of *FASN* gene in Holstein cows.

Locus	Genotype	Number of samples	Milk yield at 305 d (kg)	Protein percentage (%)	Fat percentage (%)	Lactose percentage (%)	Dry matter percentage (%)
g.16024G>A	GG	308	7166.3 ^a ±176.4	3.11 ^a ±0.03	3.70 ^a ±0.06	4.72 ^a ±0.02	12.23 ^a ±0.10
	GA	95	6783.8 ^a ±216.6	3.08 ^a ±0.06	3.71 ^a ±0.10	4.75 ^a ±0.05	12.22 ^a ±0.18
	AA	4	6361.8 ^a ±250.1	3.09 ^a ±0.10	3.68 ^a ±0.12	4.74 ^a ±0.08	12.23 ^a ±0.23
g.17924G>A	GG	199	7150.3 ^a ±256.3	3.08 ^a ±0.04	3.66 ^b ±0.09	4.74 ^a ±0.03	12.29 ^a ±0.13
	GA	173	6953.1 ^a ±195.1	3.09 ^a ±0.05	3.67 ^b ±0.10	4.73 ^a ±0.02	12.11 ^a ±0.12
	AA	35	7181.0 ^a ±442.4	3.21 ^a ±0.11	4.13 ^a ±0.14	4.70 ^a ±0.07	12.51 ^a ±0.24

Note: Least squares means with the same superscript for the same locus means that there was no significant difference ($P > 0.05$). Least squares means with the different superscripts for the same locus differ ($P < 0.05$).

DISCUSSION

Polymorphisms of bovine FASN gene

In this study, we identified two SNPs by PCR-SSCP previously reported, g.16024 A>G (Abe *et al.*, 2009), and g.17924 G>A (Abe *et al.*, 2009; Morris *et al.*, 2007; Zhang *et al.*, 2008). It had been found that the g.17924 A>G SNP was located in the thioesterase domain of the FASN protein, and changed from polar, neutral, and hydrophilic to nonpolar, aliphatic, and hydrophobic, which had an effect on increasing fat deposition in Korean cattle (Oh *et al.*, 2018). Morris *et al.* (2007) identified five mutations in bovine *FASN* gene, g.17250-17251 AT indel, g.15603 G>A in introns, g.16907 T>C, g.15531 C>A and g.17924 A>G in exons, of which only g.17924 A>G caused an amino acid.

Substitution of threonine to alanine in FASN protein. In the meantime, researchers detected three single nucleotide mutations, g.17924 A>G reported by Morris (Morris *et al.*, 2007), and two silent mutations g.18663 T>C and g.18727 C>T in Angus cattle (Zhang *et al.*, 2008). Previous studies had detected four known mutations (g.16907 T>C, g.17924 A>G, g.18663 T>C and g.18727 C>T) in coding region of *FASN* gene and five novel mutations (g.8805 C>T [Ala>Val], g.13126 T>C [Tyr>His], g.15532 C>A [Leu>Ile], g.16024 A>G [Thr>Ala] and g.16039 T>C [Trp>Arg]) resulted in amino acid changed in F2 population from Japanese Black cross with Limousin cattle (Abe *et al.*, 2009). The bovine *FASN* gene showed abundant polymorphisms affecting the fat related traits in different breeds. The difference of allele frequency may be due to the effect of artificial selection in different cattle breeds.

Association between FASN gene and milk production traits

Bovine *FASN* is located on BTA19 where several quantitative trait loci (QTL) affecting milk-fat content and related traits (Boichard *et al.*, 2003; Bouwman *et al.*, 2014; Uemoto *et al.*, 2011; Bhuiyan *et al.*, 2018). It had been reported that the frequencies of g.763 G>C and g.16009 A>G were significantly different in Holsteins with high and low breeding values for milk-fat content (Roy *et al.*, 2006). The individuals with haplotype C-G were associated with a high production of fat, while those with haplotype G-A were associated with a low production of milk fat. Some researchers concluded that mutation g.763 G>C in bovine *FASN* exon 1 increased milk fat content in dairy cattle due to the decrease of bovine *FASN* promoter activity in vitro (Ordovas *et al.*, 2008). Morris found that the substitution g.15531 C>A or g.15603 G>A had effects on the C14:0 percentage of adipose fat and milk fat in the

opposite direction (Morris *et al.*, 2007). Researchers also found that cows with the *FASN* GG genotype, the protein percentage was higher, but the A allele was associated with higher milk, protein and fat yields than the G allele (Čítek *et al.*, 2021). It had been proved that g.17924 A>G of *FASN* gene was significantly associated with fatty acid composition of longissimus dorsi muscle of Angus bulls (Zhang *et al.*, 2008) and significantly associated with the decreasing of milk fat percentage ($P < 0.05$) in Dutch Holstein-Friesian cows (Schennink *et al.*, 2009). In addition, Ye's study in Mediterranean buffalo found that the two mutations g.7164 G>A and g.8927 T>C were also significantly related to peak milk production and protein percentage, respectively (Ye *et al.*, 2021). Besides, it had been reported that the haplotypes (TW and AR) of g.16024 A>G and g.16039 T>C segregated in F2 individuals from Japanese Black and Limousin cattle and had a significant effect on the fatty acid composition of back fat, intermuscular fat, and intramuscular fat. The two mutations similarly determined the contribution to the fatty acid composition of intramuscular fat in two commercial Japanese Black half-sibling populations (Abe *et al.*, 2009).

CONCLUSION

Our study indicated that allele A of g.17924 G>A of *FASN* gene was significantly associated with increased fat percentage in Chinese Holstein cows. The results of this study also showed that g.17924 G>A mutation might provide useful information in terms of predicting the milk fat percentage, or that allele A of g.17924 G>A of *FASN* gene may be applied as a potential genetic marker for improving milk fat percentage in dairy cattle.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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