Two single nucleotide polymorphisms sites in \(\alpha_1\)-AT gene and their association with somatic cell score in Chinese Holstein cows

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**Abstract**

**Background:** Alpha 1-antitrypsin (\(\alpha_1\)-AT) may affect the susceptibility of mastitis in dairy cattle for its possible role in the protection of lactoferrin from proteolytic degradation in the mammary. Milk somatic cell score (SCS) is a logarithmic transformation of the milk somatic cell count widely used as an index to evaluate mastitis. To study the relationships of \(\alpha_1\)-AT gene and SCS in Chinese Holstein cows, methods of PCR-SSCP, DNA sequencing, PCR-RFLP, and CRS-PCR technologies were used to detect single nucleotide polymorphisms sites in \(\alpha_1\)-AT gene.

**Results:** Two polymorphic sites at G5503A and G5746C of \(\alpha_1\)-AT gene were found. AA (0.3633), AB (0.4644) and BB (0.1723) genotypes were detected at G5503A site, CC (0.3483), CD (0.4906) and DD (0.1611) genotypes were found at G5746C in Chinese Holstein cows. Least squares mean of SCS for individuals with BB genotype was significantly lower than that with AA and AB genotype \((p < 0.01)\), and that with AB genotype was significantly lower than that with AA \((p < 0.05)\). There was no significant difference among individuals with CC, CD and DD genotypes \((p > 0.05)\). Least squares mean of SCS for individuals with BBDD genotype combination were significantly lower than those with AACC and AACD \((p < 0.05)\).

**Conclusions:** Statistical analysis indicated that B allele and BBDD genotype combination of \(\alpha_1\)-AT can improve mastitis resistance in dairy cattle.

**Keywords:** SNP, \(\alpha_1\)-AT gene, Polymorphism, Somatic cell score, Chinese Holstein cows

**Background**

\(\alpha_1\)-antitrypsin (\(\alpha_1\)-AT) or alpha 1-protease inhibitor (\(\alpha_1\)-PI), is mainly synthesized in the animals’ liver while it exists in plants and microorganisms, as well. As a member of serine protease inhibitor protein subfamily, \(\alpha_1\)-AT inhibits the target proteases by a specific mechanism, which depends on a change in conformation [1]. The \(\alpha_1\)-AT could be isolated from cow’s milk, its molecular size ranges from 56 to 64 kDa and it possesses the characteristic of cell membrane permeability. The \(\alpha_1\)-AT can suppress the reaction of trypsin and elastase, while it is not active against plasmin [2]. Chowanadisai and Lönnerdal [3] had reported that \(\alpha_1\)-AT was expressed in mammary gland epithelial cells, and it can protect lactoferrin from digestion by pancreatic in vitro, which suggested that it had the potential of protecting milk proteins by inhibiting proteases in the gastrointestinal tract of infants. The \(\alpha_1\)-AT might be the predominant protease inhibitor in milk, for its ability to inhibit both trypsin and chymotrypsin, which are the primary pancreatic proteases [3]. The \(\alpha_1\)-AT protein in bovine milk may inhibit the hydrolysis of trypsin on lactoferrin [4, 5]. Heihavand-Kheiripour et al. [6] suggested that \(\alpha_1\)-AT protein may affect the susceptibility of mastitis in dairy cattle for its possible role in the protection of lactoferrin from proteolytic degradation in the mammary.
The bovine \( \alpha_1 \)-AT gene is located on chromosome 21 (approximately 9 kb of genomic DNA), consists of five exons, and encodes a protein with 416 amino acids [7]. Sinha et al. [8] had isolated a whole cDNA clone coding for bovine \( \alpha_1 \)-AT from a \( \lambda \)gt11 bovine liver cDNA library using a human \( \alpha_1 \)-AT cDNA as a probe. Five single nucleotide polymorphisms (SNPs) in coding regions of the bovine \( \alpha_1 \)-AT gene were found by direct sequencing of reverse transcription-polymerase chain reaction (RT-PCR) products from a wide range of cattle tissues. The relationship of these SNPs with the economic traits in North American Holstein population was studied [9]. Meanwhile, researchers had reported that \( \alpha_1 \)-AT gene was associated with milk production traits in dairy cattle [10–12].

Since it is difficult to measure the mastitis phenotype using a direct index, milk SCS has been most widely used as an index to evaluate mastitis [13]. Milk SCS is a logarithmic transformation of the milk somatic cell count (SCC) to achieve normality of distribution in statistics which has positive correlation with clinical mastitis [14–17]. In the present study, two single nucleotide polymorphisms sites of \( \alpha_1 \)-AT gene were found in Chinese Holstein cows and their relationships with SCS were also studied; the results may provide a theoretical basis for marker-assisted selection of mastitis resistance in Chinese Holstein cows.

Results

PCR amplification and SSCP detection

The \( \alpha_1 \)-AT gene in 267 Chinese Holstein cows was amplified using the designed primers (Table 1). The results showed that the amplification fragment sizes were consistent with the target ones. The PCR products of three pairs of primers were all analyzed by SSCP. Polymorphisms were only found in the amplified fragments in the results of P1 and P3 primers. P1 and P3 primers were adopted for following analysis.

Sequencing analysis

The products of primer P1, P3 were cloned and sequenced. The sequences were compared by DNA-MAN software (Lynnon Biosoft Inc., San Ramon, USA). In the second exon of \( \alpha_1 \)-AT gene, one mutation of \( G \rightarrow A \) was found at the 5503 bp site, which generated the \( Sphl \) restriction enzyme site and caused no amino acid change. Another mutation of \( G \rightarrow C \) was found at the 5746 bp site, which also caused no amino acid change (Fig. 1).

RFLP detection

\( Sphl \) analysis of the G5503A site

The 978 bp PCR products were completely digested with \( Sphl \) restriction endonuclease and genetic polymorphisms of \( \alpha_1 \)-AT were investigated by PCR-RFLP.

![Fig. 1 Two SNPs of \( \alpha_1 \)-AT gene in Chinese Holstein cows](image-url)
As shown in Fig. 2, three genotypes, AA (978 bp), AB (978/688/290 bp) and BB (688/290 bp), were found in 267 Chinese Holstein cows.

**Hinf I analysis of the G5746C site**

Because of no restriction enzyme site existing in G5746C, the restriction enzyme site of *Hinf I* was introduced by created restriction site-PCR (CRS-PCR) based on primer P3. The 149 bp PCR products were digested by *Hinf I* restriction endonuclease; then, the genetic polymorphisms could be detected by PCR-RFLP. As shown in Fig. 3, three genotypes, CC (131 bp), CD (149/131 bp) and DD (149 bp), were found in 267 Chinese Holstein cows.

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**Table 1** Primer sequence, product size, location and annealing temperature of α1-AT gene in Chinese Holstein cows

| Primer | Primer sequence (5′→3′) | Product size (bp) | Location* | Annealing temperature (°C) |
|--------|--------------------------|------------------|-----------|--------------------------|
| P1     | F: GCCATTGTTCTGAGTCTTTTC | 978              | 5216-5235 | 54                       |
|        | R: TCCCCATACCCATTTGGATT  |                  | 6174-6193 |                          |
| P2     | F: GGCACCAACTGAAAAAAACAC | 175              | 8060-8081 | 59                       |
|        | R: AGCCCTATCGCTGAAGACCT  |                  | 8215-8234 |                          |
| P3     | F: CCTTGGCGATGCTTCTCCCTG | 149              | 5616-5635 | 55                       |
|        | R: CTGGTGTTTTGGGTGATT    |                  | 5747-5764 |                          |

* Base positions corresponding to NC_007319 of GenBank
Frequencies of allele and its genotype

The frequencies of alleles and genotypes of \( \alpha_1 \)-AT gene in experimental Holstein cows were presented in Table 2. At locus of G5503A and G5746C, the frequencies of AA, AB, BB and CC, CD, DD genotypes were 0.3633, 0.4644, 0.1723 and 0.3483, 0.4906, 0.1611, respectively, while the frequencies of A, B, C and D allele were 0.5955, 0.4045, 0.5936 and 0.4064, respectively. Chi square fitness test of the mutation site showed that the locus of G5503A and G5746C were in Hardy–Weinberg equilibrium in these Chinese Holstein cows \((p > 0.05)\). Polymorphism Information Content (PIC) of the polymorphic sites G5503C and G5746C were >0.3, indicative of moderate polymorphism; this finding implies that the selection potential of the sites was relatively large (Table 3).

Genotype combination analysis

The two polymorphic sites have generated nine genotype combinations (Table 4). The frequencies of AACC, AACD, AADD, ABCC, ABCD, ABDD, BBCC, BBCD and BBDD were 0.2697, 0.0899, 0.0075, 0.0749, 0.3408, 0.0412, 0.0037, 0.0599 and 0.1124, respectively. Further analysis showed that these nine genotype combinations were composed of the four haplotypes of A+C, A+D, B+C and B+D, the frequency of haplotype A+D was the lowest, while that of the A+C haplotype was the highest.

The relationship between the polymorphism of \( \alpha_1 \)-AT gene and SCS in Chinese Holstein cows

For the polymorphic sites of G5503A and G5746C, the results of variance analysis indicated that the bull, herd, parity and calving season had a significant effect on SCS \((p < 0.05)\). Least squares means and standard errors for SCS of different genotypes and genotype combinations were listed in Tables 5 and 6. The results showed that the least squares mean of SCS of BB genotype was significantly lower than that of AA and AB genotypes \((p < 0.05)\), and the least squares mean of SCS of AB genotype was significantly lower than that of AA genotype \((p < 0.05)\). The least squares means of SCS of AB genotype had no significant difference between CC, CD and DD genotypes \((p > 0.05)\). The result of variance analysis of genotype combinations showed that the least squares mean of SCS of BBDD genotype combination was significantly lower than that of AACC and AACD genotype combinations \((p < 0.05)\). In regard to mastitis resistance, BB was the most favorable genotype, AA was the most unfavorable genotype, BBDD was the most favorable genotype combination, and AACC was the most unfavorable genotype combination. The results preliminarily indicated that allele B of G5503A polymorphic site of \( \alpha_1 \)-AT gene is a potential DNA marker for improving mastitis resistance in Chinese Holstein cow.

Discussion

Like the other traits in cows, yield and quality of milk were affected by factors of heredity, nutrition and environment [18–21]. At genetic level, several molecular marker genes which associated with milk production

| Table 2 | Allele and genotype frequencies of \( \alpha_1 \)-AT gene in Chinese Holstein cows |
|----------|---------------------------------|-----------------|-----------------|
| Polymorphic site | Genotype | Number of samples | Genotype frequency | Allele | Allele frequency |
| G5503A | AA | 97 | 0.3633 | A | 0.5955 |
| AB | 124 | 0.4644 | B | 0.4045 |
| BB | 46 | 0.1723 | |
| G5746C | CC | 93 | 0.3483 | C | 0.5936 |
| CD | 131 | 0.4906 | D | 0.4064 |
| DD | 43 | 0.1611 | | | |

| Table 3 | Genetic characteristics of two polymorphic sites in Chinese Holstein cows |
|----------|---------------------------------|-----------------|
| Polymorphic site | \( \chi^2 (\rho) \) | Polymorphism information content | Heterozygosity | Effective number of alleles |
| G5503A | 0.35 (0.841) | 0.366 | 0.482 | 1.93 |
| G5746C | 0.00 (0.999) | 0.367 | 0.485 | 1.94 |

| Table 4 | Frequencies of genotype combinations of the two polymorphic sites |
|----------|---------------------------------|-----------------|
| Genotype combination | Genotype combination frequencies |
| AACC | 72 (0.2697) |
| AACD | 24 (0.0899) |
| AADD | 2 (0.0075) |
| ABCC | 20 (0.0749) |
| ABCD | 91 (0.3408) |
| ABDD | 11 (0.0412) |
| BBCC | 1 (0.0037) |
| BBCD | 16 (0.0599) |
| BBDD | 30 (0.1124) |
traits were detected in various dairy cows over the years [22–26]. As one of the marker genes, five SNPs of the α1-AT gene (at positions 164, 269, 284, 407 and 989 bp on mRNA) were identified in North American Holsteins by Khatib et al. [9]. Researchers found that haplotype D was associated with a significant increase in milk, fat percentage and protein percentage, while haplotypes B and C were associated with an increase in milk protein percentage [9, 27]. The 668 and 999 bp fragments of 5′ flanking region in the α1-AT gene were amplified in Chinese Holsteins, and the C-T and T-C SNPs were identified at nucleotide +3142 and +4408 bp by sequencing, in which, the allele B had a significant impact on milk yield and fat/ protein ratio, and the allele F had a significant influence on milk fat percentage in Chinese Holstein cows [28].

In the present study, two mutations of G5503A and G5746C in α1-AT gene were detected in Chinese Holstein cows, which were consistent with the mutations at 164 and 407 bp in its mRNA, while the mutations at 269, 284 and 989 bp were not detected. The two mutations in this study caused no amino acid change, similar to studies made by other authors [6, 10]. It should be noted that, although SNPs in α1-AT gene were not functional mutations, they were possibly in linkage disequilibrium with a certain functional polymorphic site in the α1-AT gene or other closely linked gene(s) influencing milk-related traits [6, 7].

Over the years, most dairy breeding goals aim at increasing the milk production by high-strength artificial selection while ignored health traits such as mastitis resistance. A higher yielding cow results in a higher risk of mastitis, which would lead to an involuntary and premature culling of milking cows for defective udder characteristics and decreased milk yield [13, 14]. In the breeding of cattle, dairy breeds were less resistant to mastitis than dual-purpose breeds [29, 30]. The α1-AT gene was treated as a candidate gene of milk production trait, since α1-AT can protect breast tissue and milk protein from excessive hydrolysis damage. In addition, as a kind of multifunctional serine proteinase inhibitor, α1-AT protein also displays a wide range of anti-inflammatory properties [31]. Cow mastitis could cause the increased permeability between the blood and breast milk. Large amounts of neutrophil elastases could be secreted to decompose the pathogenic bacteria. As a marker protein of a cow with mastitis, the concentration of α1-AT protein will be higher than the health individuals. For the protective mechanism for the body of α1-AT protein, it was supposed that the somatic cell count in milk might be associated with the content of α1-AT protein in serum.

### Conclusions

The relationship between the two single nucleotide polymorphisms sites of α1-AT gene and SCC/SCS in Chinese Holstein cow milk was explored in the present study. The relationships between the mutations of G5503A and G5746C with SCS in Chinese Holstein cow were analyzed by least square method, and the results showed that the genotype combination of BBDD maybe a favorable combination for mastitis resistance, which means that B allele and BD haplotype in this study can be used as the genetic markers for mastitis resistance in Chinese Holstein cows. Further studies focused on associating the various polymorphisms in α1-AT gene with SCS in different regions and populations of Holstein cows are necessary to be carried out. Based on these results, further progress could be made to determine whether the α1-AT gene can be recognized as the candidate gene of mastitis resistance.

### Methods

**Blood sample collection and DNA preparation**

In 2015, jugular blood samples (10 ml per cow) were collected from a total of 267 Chinese Holstein cows calved
SCC in milk samples were estimated by MilkoScan (FOSS 6000, Denmark) with unit of cells μl⁻¹. SCS was measured according to the calculation formula: $SCS = \left( \log_2 \frac{SCC}{100000} \right) + 3$.

**Primer design**

Three pairs of primers were designed according to bovine α1-AT gene sequence (GenBank accession number NC_007319) using Primer 5.0 and Oligo 6.0 software, and synthesized by Shanghai Invitrogen Biotechnology Co., Ltd.

**Cloning and sequencing**

Fifteen different individuals were selected randomly for PCR amplification. The PCR products were rapidly recovered by DNA purification kit, and the DNA fragments were inserted into pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacture’s instructions. The recombinant plasmid was transformed into competent Escherichia coli DH5α; then, the positive clones were identified and sent to Beijing Jin Wei Zhi Company for sequencing.

**PCR-SSCP detection**

Polymerase chain reaction (PCR) was carried out in 25 μl volume containing 2.5 μl × 10 PCR buffer, 1.0 μl of each primer (forward and reverse, 10 μM), 2.5 μl MgCl₂ (20 mM), 2.0 μl dNTPs (2 mM), 1.0 μl Taq DNA polymerase (2.5 U μl⁻¹, SABC, Beijing, China), 3.0 μl DNA template (50 ng μl⁻¹) and 12 μl of dH₂O. Amplification conditions were as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 59 °C for 60 s, extension at 72 °C for 15 s with a final extension step at 72 °C for 10 min on Mastercycler™ 5333 (Eppendorf AG, Hamburg, Germany).

PCR products of primer P2 were used for SSCP analysis. A volume of 3 μl PCR product was transferred into the Eppendorf tube, mixed with 7 μl gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mmol l⁻¹ EDTA (pH 8.0) and 10% glycerol. The mixture was centrifuged and denaturated at 98 °C for 10 min, then chilled on ice for 7 min and loaded on 10-12% neutral polyacrylamide gels (acr ylamide:bisacrylamide = 29–39:1). Electrophoresis was performed in × 1 Tris borate (pH 8.3)-EDTA buffer at 9–15 V cm⁻¹ for 14–16 h at 4 °C. After electrophoresis, the DNA fragments in the gels were visualized by silver staining, photographed and analyzed using an AlphaInnotech™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

**Restriction fragment length polymorphism (RFLP) analysis**

Restriction enzyme reaction system mixture (10 μl volume) were incubated at 37 °C overnight (contained 3 μl of PCR products, 0.5 μl restriction enzyme, 1 μl corresponding ×10 reaction buffer, 5.5 μl ultrapure H₂O). The enzyme-digested products were visualized by 2% agarose gel electrophoresis, and identified by silver nitrate staining, then photographed and analyzed.

Based on the results of sequence of P1, one mutation was revealed in exon 3 and restriction enzyme SpHl was used to detect it. The PCR products of P3 were digested by SpHl (NEB, Beijing, China) with 10 μl volume containing 5 μl of PCR product, 0.5 μl of 10 U μl⁻¹ restriction enzyme SpHl, 1 μl of ×10 reaction buffer, 3.5 μl H₂O, incubated at 37 °C for 5 h. The mixtures were detected by 3.0% agarose gels and were genotyped.

**Creating restriction site (CRS)**

When there was no available restriction enzyme site for PCR-RFLP analysis, CRS combined with PCR amplification is a simple and efficient method that could be used to detect single nucleotide polymorphisms (SNPs) genotypes [8]. One-mismatch bases are used in a primer P3 in this study to create a restriction site for PCR. Then the PCR products can be genotyped in the same way as RFLP.

**Statistical analysis**

A mutation was assumed that appearing on a base site, and the computational formula of genotype frequency in resultant three genotypes (AA, AB, BB) were as follows:

\[
f(AA) = \frac{n_{AA}}{n_{AA} + n_{AB} + n_{BB}}; \quad f(AB) = \frac{n_{AB}}{n_{AA} + n_{AB} + n_{BB}}; \\
f(BB) = \frac{n_{BB}}{n_{AA} + n_{AB} + n_{BB}}
\]
note: $n_{AA}$ is the number of AA, $n_{AB}$ is the number of AB, $n_{BB}$ is the number of BB.

Computational formula of allele frequency:

$$p = \frac{2n_{AA} + n_{AB}}{2(n_{AA} + n_{AB} + n_{BB})}; \quad q = \frac{2n_{BB} + n_{AB}}{2(n_{AA} + n_{AB} + n_{BB})}$$

note: $p$ is the allele frequency of A, $q$ is the allele frequency of B.

Computational formula of heterozygosity ($He$):

$$He = 1 - \sum_{i=1}^{n} p_i^2$$

note: $n$ is the number of allele, $p_i$ is the allele frequency of the $i$th allele.

Computational formula of effective number of alleles ($Ne$):

$$Ne = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$$

note: $n$ is the number of allele, $p_i$ is the allele frequency of the $i$th allele, and $p_{ij}$ is the allele frequency of the $j$th allele.

The following statistical model was fitted to compare difference of SCS among different genotypes in Chinese Holstein cows.

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

where $y_{ijklmn}$ is phenotypic value of SCS; $\mu$ is population 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. Analysis was performed using the general linear model procedure of SAS (Ver 8.12) (SAS Institute Inc., North Carolina, USA). Mean separation procedures were performed using a least significant difference test.

**Authors’ contributions**

This study was designed by XFG, WPH and MXC. XFG and WPH performed all the experimental analyses and prepared all the figures and tables. XFG analyzed the data and drafted the manuscript. XZL, QLL, XYW, RD, QYL, XLL, YFA and WPH contributed to revisions of the manuscript. MXC assisted in explaining the results and revised the final version of the manuscript. All authors read and approved the final manuscript.

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**Acknowledgements**

This work was supported by Tian Yi Hui Yuan Biological co., LTD and Hebei Dairy co., LTD.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

All authors have consented the manuscript been published.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAHCAAS2015335). We comply with all the points claimed by the committee.

**Funding**

This work was funded by Agricultural Science and Technology Innovation Program (ASTIP-IA513), National Key Technology Research and Development Program (2006BAD04A10) and Hebei Province Youth Talent Project (812014048) of China.

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Received:** 3 December 2016  **Accepted:** 4 April 2017

**Published online:** 13 April 2017

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