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Article *in* Journal of Dairy Science · September 2021

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https://doi.org/10.3168/jds.2020-19943

Genetic parameters and weighted single-step genome-wide association study for supernumerary teats in Holstein cattle

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ABSTRACT

Supernumerary teats (SNT) are a common epidermal abnormality of udders in mammals. The SNT negatively affect machine milking ability, udder health, and animal welfare and sometimes act as reservoirs for undesirable bacteria, resulting in economic losses on calves and lactating cows due to the cost of SNT removal surgery, early culling, and low milk yield. This study aimed to analyze the incidence and genetic parameter of SNT and detect SNT-related genes in Chinese Holstein cattle. In this study, the incidence of SNT was recorded in 4,670 Chinese Holstein cattle (born between 2008 and 2017) from 2 farms, including 734 genotyped cows with 114,485 SNPs. The SNT had a total frequency of 9.8% and estimated heritability of 0.22 (SE = 0.07), which were obtained using a threshold model in the studied Chinese Holstein population. Furthermore, we calculated approximate genetic correlations between SNT and the following indicator traits: 12 milk production, 28 body conformation, 5 fertility and reproduction, 5 health, and 9 longevity. Generally, the estimated correlations, such as 305-d milk yield for third parity (−0.55; SE = 0.02) and age at first calving in heifer (0.19; SE = 0.03), were low to moderate. A single-step GWAS was implemented, and 10 genes associated with SNT located in BTA4 were identified. The region (112.70–112.90 Mb) on BTA4 showed the highest genetic variance for SNT. The quantitative trait loci on BTA4 was mapped into the *RARRES2* gene, which was previously shown to affect adipogenesis and hormone secretion. The *WIF1* gene, which was located in BTA5, was also considered as a candidate gene for SNT. Overall, these findings provide useful information for breeders who are interested in reducing SNT.

Key words: supernumerary teat, Chinese Holstein, genetic parameter, single-step genome-wide association study

INTRODUCTION

Supernumerary teats (**SNT**) are the epidermal appendages of udders in mammals. The SNT are an undesired and heritable trait in dairy cattle because they can reduce machine milking ability and affect the healthy condition of udders (Brka et al., 2002; Pausch et al., 2012; Joerg et al., 2014; Butty et al., 2017). In most Chinese modern dairy farms, SNT are usually removed roughly 10 d after the SNT animals are born. However, this procedure may cause numerous problems, including increased cost against infection and reduced animal welfare (Brka et al., 2002; Pausch et al., 2012; Joerg et al., 2014). Now, many elite dairy animals are excluded from breeding programs for their SNT (Martin et al., 2016).

In dairy cattle, the occurrence of SNT varies depending on breed and selection (Butty et al., 2017). The frequencies of the affected German Simmental, Brown Swiss, and Holstein populations are 44.3%, 31.2%, and 15.0%, respectively (Brka et al., 2000; Brka et al., 2002). Furthermore, in Swiss Brown Swiss, only 20% are found to be affected by SNT (Butty et al., 2017). The strict selection of clear udders on Swiss Brown Swiss for many years has caused a discrepancy between the 2 Brown Swiss populations (Butty et al., 2017). The estimated heritability for SNT goes from 0.09 to 0.63 in cattle, with most estimates being around 0.40 (Brka et al., 2002; Martin et al., 2016). There have also been SNT observed in goats, and heritability estimates fluctuating from 0.34 to 0.44 have been reported (Brka et al., 2007; Martin et al., 2016).

Received November 23, 2020.
Accepted June 29, 2021.
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The high heritability of SNT reveals that lowering the frequency and risk of SNT by genetic selection is feasible. However, the genetic basis and potential environmental factors of SNT remain unclear. Each farm has its own management style, which may result in variation in frequency of SNT. For instance, for the treatment toward SNT, Brka et al. (2002) reported that the frequency of SNT decreases by approximately 2% in the dairy cow population due to the removal of SNT by surgery. A previous study indicated that the frequency of bovine SNT will rise gradually with the increased parity of the dam, and a large difference exists between primiparous and multiparous cows (Brka et al., 2002). Some of the reasons provided by several studies are as follows: (1) multiparous cows are lactating during pregnancy and (2) the hormonal environment of the fetus differs in primiparous and multiparous cows (Brka et al., 2002; Pausch et al., 2012). The nipple development is easily influenced by hormones, such as testosterone (Hotchkiss et al., 2007) and estrogenic agents (i.e., methoxychlor and diethylstilbestrol; Golub et al., 2003), resulting in retarded growth of the nipple. Additionally, the environment of feeding, particularly in extreme weather, during gestation can affect the performance of offspring (Laporta et al., 2020), disrupt embryo development, and increase embryonic mortality (Biggers et al., 1987; de Barros and Paula-Lopes, 2018). The SNT of different bovine breeds are influenced in various degrees depending on their genetic background.

The single-step genome-wide association study (**ssGWAS**), which is based on the single-step genomic BLUP approach, was proposed by Wang et al. (2012). The weighted single-step genome-wide association study (**WssGWAS**) is derived from ssGWAS and uses all observed phenotypes, pedigree information, and genotypes simultaneously, leading to more powerful and precise results. The ssGWAS and WssGWAS can replace the traditional GWAS (Dikmen et al., 2013) and can be useful in understanding the underlying biological mechanism of SNT by identifying candidate genes or QTL. Several significant SNPs and genes about SNT, such as *TBX3*, *LEF1*, and *DKK2*, have been identified in Fleckvieh and Holstein population (Pausch et al., 2012; Joerg et al., 2014). The genes have been shown to affect the development of the mammary gland (Eblaghie et al., 2004; Logan and Nusse, 2004; Boras-Granic et al., 2006).

The present study aimed to explore the genetic basis of SNT in Chinese Holstein cattle. First, we have summarized the frequency of SNT for our studied population. Then, we have estimated the genetic parameters and mapped genomic regions highly associated with SNT via the WssGWAS. Our data and the candidate genes of SNT can provide new insights into SNT in dairy cattle.

MATERIALS AND METHODS

Animals and Data Sets

This study included 4,670 Chinese Holstein cows (459 SNT cows) that were born from 2008 to 2017 from 2 farms of the Beijing Sanyuan Dairy Farm Center in Beijing (40° N, 116° E), China. All 4,670 cows were used in calculation of SNT frequency. For 3,269 individuals (355 SNT cows) out of the 4,670 Chinese Holstein population, we had access to phenotypic data (farm-year-season of birth, parity of dam) and traditional pedigree information, and the population used for variance components estimation and WssGWAS for SNT. A total of 734 female individuals (78 SNT cows) within 3,269 individuals were genotyped using the Illumina 150K bovine BeadChip (Illumina Inc.) including 138,892 SNPs. The diagnosis of SNT was based on observation regarding the occurrence of any abnormal teat in udders except 4 usual and functional teats (Figure 1). Genotype imputation for missing SNPs was conducted using the BEAGLE software (version 5.1; Browning et al., 2018). The SNP markers were kept with a minor allele frequency >0.05, Hardy-Weinberg equilibrium exact test P-value above 10^{-6}, and known position and chromosome, using PLINK software (v1.07; Purcell et al., 2007). After the quality control of the SNP chip panel, 114,485 SNPs were used for heritability estimation and association study. The position of each SNP was determined using the reference bovine genome sequence ARS-UCD 1.2 assembly (http://www.ncbi.nlm.nih.gov/genome/guide/cow/).

Model and Analysis

All cattle in the pedigree were traced spanning up to 4 generations, and the pedigree information was provided by the Dairy Cattle Association of China (Beijing, China). The pedigree included 13,962 females and 1,572 males born from 1934 to 2017. A threshold model (Sorensen et al., 1995) used for variance components estimation and WssGWAS for SNT through the BLUPF90 software (version 1.3.25; Misztal et al., 2002) can be described as follows:

$$\lambda = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{a} + \mathbf{e},$$

where $\boldsymbol{\lambda}$ is the vector of unobserved liabilities, whereas the observed phenotype is binary (0: non-SNT cow, 1: SNT cow); $\boldsymbol{\beta}$ is a vector of fixed effects, including farm-

year-season of birth (farm means the farm where individuals lived, year means birth year of individuals, season of birth means the birth season of individual; 53 levels) and parity of dam (2 levels: primiparous and multiparous cows); **a** is the random genetic animal effect and assumed to follow $\mathbf{a} \sim N\left(0, \mathbf{H}\sigma_a^2\right)$, where σ_a^2 represents the additive genetic variance; **H** is the relationship matrix combining pedigree and genotype information as defined by Legarra et al. (2009); **X** and **Z** are the incidence matrices; and **e** is the residual effect and assumed to follow $e \sim N\left(0, \mathbf{I}\sigma_e^2\right)$, where **I** represents the identity matrix and σ_e^2 the residual variance. An inverse of the **H** matrix ($\mathbf{H}^{-1}$) allows for remarkably simpler computations (Aguilar et al., 2010) as follows:

$$\mathbf{H}^{-1} = \mathbf{A}^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & (\alpha\mathbf{G} + \beta\mathbf{A}_{22})^{-1} - \mathbf{A}_{22}^{-1} \end{bmatrix},$$

where $\mathbf{A}^{-1}$ is the inverse of the numerator relationship matrix; $\mathbf{A}_{22}^{-1}$ is the inverse of a pedigree-based relationship matrix for genotyped animals only; α and β are weighting factors with values of 0.95 and 0.05 in our analyses; $\mathbf{G}^{-1}$ is the inverse of the genomic relationship matrix. The **G** matrix can be constructed in accordance with the method of VanRaden (2008). The Bayesian inference implemented in the THRGIBBS1F90, POSTGIBBSF90, and BLUPF90 programs was used to estimate variance components and genomic breeding value (**GEBV**; Misztal et al., 2002; Tsuruta and Misztal, 2006). A total of 100,000 iterations were run, with the first 20,000 discarded as burn-in and thinned every 5 samples. The chain convergence was verified with the graphical evaluation of sampled values by using the "coda" package in R (Martyn et al., 2006). The heritability on the liability scale for the binomial character was transformed to the observed (0/1) scale by using the formula of Robertson and Lerner (1949).

The equation for predicting SNP effects as described in Wang et al. (2012) was as follows:

$$\hat{\boldsymbol{\theta}} = \mathbf{DW}'\left[\mathbf{WDW}'\right]^{-1}\hat{\mathbf{u}}_{\boldsymbol{g}},$$

where $\hat{\boldsymbol{\theta}}$ is the vector with estimated SNP effects; **D** is a diagonal matrix of weights for variance of SNP (initially $\mathbf{D} = \mathbf{I}$); **W** is a matrix relating genotypes of each locus to each individual; and $\hat{\mathbf{u}}_{\boldsymbol{g}}$ is the vector of GEBV for genotyped animals.

The estimates of SNP effects and weights for Wss-GWAS were calculated through the following steps (Wang et al., 2012):

1. Let $\mathbf{D} = \mathbf{I}$ in the first step.
2. Calculate $\mathbf{G} = \dfrac{\mathbf{WDW}'}{k}$, where $k = \sum_{j=1}^{N} 2p_j\left(1 - p_j\right)$, where p is the minor allele frequency of SNP, thus p_j is the minor allele frequency of the jth SNP. N denotes the number of SNP.
3. Calculate GEBV for the entire data set by using single-step genomic BLUP.
4. Calculate the SNP effects $\left(\hat{\mathbf{u}}\right)$ by GEBV: $\hat{\boldsymbol{\theta}} = \mathbf{DW}'\left[\mathbf{WDW}'\right]^{-1}\hat{\mathbf{u}}_{\boldsymbol{g}}$.

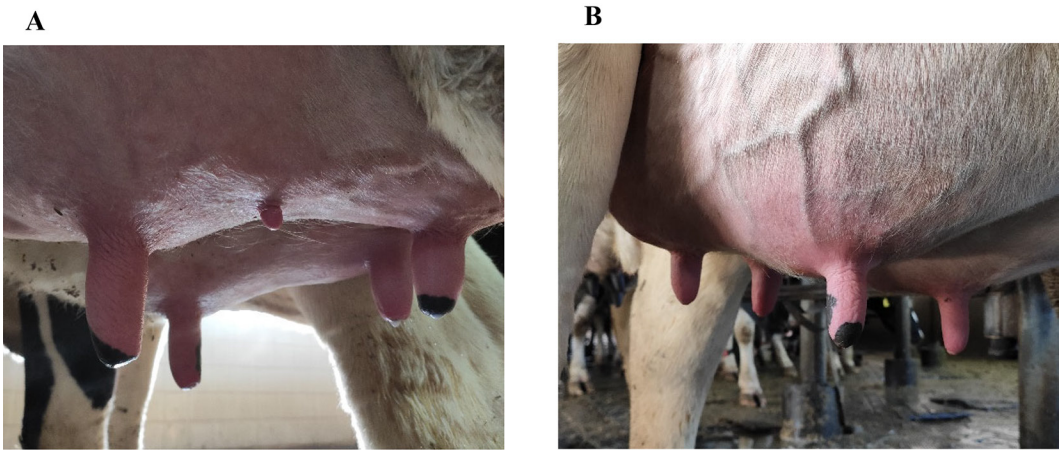

Figure 1. Chinese Holstein with the phenotypes of (A) 5 teats including 4 normal teats and 1 supernumerary teat and (B) 4 normal teats.

5. Calculate weight for each SNP: $d_j = \hat{\theta} 2 p_j \left(1 - p_j\right)$, where d is the weight of each SNP; thus, d_j is the weight of the jth SNP.
6. Normalize SNP weights to keep the total genetic variance constant.
7. Loop to step 2.

Iterations increase the weights of SNPs with large effects and decrease those with small effects (Wang et al., 2014). The procedure was run for 3 iterations on the basis of the accuracies of GEBV for our study.

The percentage of genetic variance explained by the ith 10-SNP sliding window was calculated as follows (Wang et al., 2014):

$$\frac{\mathrm{var}\left(u_i\right)}{\sigma_u^2} \times 100 = \frac{\mathrm{var}\left(\sum_{j=1}^{10} \mathbf{W}_j \hat{\theta}_j\right)}{\sigma_u^2} \times 100,$$

where u_i is the genetic value of the ith 10-SNP sliding window that consists of contiguous 10 SNPs, and no overlap exists among windows; $\mathrm{var}(u_i)$ is the genetic variance explained by the ith 10-SNP sliding window; σ_u^2 is the total additive genetic variance, $\mathbf{W}_j$ is the vector of gene content of the jth SNP for all individuals, $\hat{\theta}_j$ is the effect of the jth SNP within the ith 10-SNP window. Manhattan plots showing these windows were completed using ggplot2 (version 0.9.2; Wickham, 2016) and R (https://www.r-project.org/).

Calculation of Approximate Genetic Correlations

The 59 traits included in this section area as follows: 12 milk-related traits (milk yield, fat yield, protein yield, and SCS; only the first 3 parities were calculated in this part, and each parity was considered as a different trait), 28 body conformation traits [capacity (or frame), rump, feet and legs, mammary system, dairy character, conformation (final score), body size, stature, fore body (termed forepart), chest width, body depth, loin strength, rump width, rump angle, bone quality, foot angle, rear legs side view, rear legs rear view, udder depth, udder texture, median suspensory, fore attachment, fore teat placement, average teat length, rear attachment height, rear attachment width, rear teat placement, angularity], 5 fertility and reproduction traits (age at first calving – in heifer, age at first service – in heifer, interval from calving to first service – in cow, interval from first to last insemination – in cow, and interval from first to last insemination – in heifer), 5 health traits (fertility disorders, udder diseases, feet and legs diseases, digestive disorders, and metabolic disorders), and 9 longevity-indicator traits for both heifers and cows (heifer longevity: survival up to 60 d of age, survival up to 365 d of age, and survival until calving; cow longevity: survival at second, third, fourth, fifth, and sixth parities, and productive life). Given the limited access to raw data sets and multiple measurements for longitudinal traits, approximate genetic correlations between SNT with the other traits were estimated in accordance with the formula proposed by Calo et al. (1973).

Reliabilities and accuracies for EBV were calculated using the following equation:

$$\text{Reliability} = 1 - \frac{\mathrm{SEP}^2}{\sigma_a^2},$$

where **SEP** is the standard error of prediction and σ_a^2 is the direct additive genetic variance. Reliability is the square of accuracy.

Approximate genetic correlations were calculated by individual's EBV with reliability greater than 0.35 for both traits as follows (the average reliabilities for each trait pair are presented in Supplemental Tables S1–S4; https://doi.org/10.6084/m9.figshare.15098385.v1; Wen, 2021):

$$\hat{r}_{g1,2} = \frac{\sqrt{\left(\sum RL_1\right) \times \left(\sum RL_2\right)}}{\sum \left(RL_1 \times RL_2\right)} \times r_{1,2},$$

where $\hat{r}_{g1,2}$ is the approximate genetic correlation between traits 1 and 2; RL_1 and RL_2 are the reliabilities of traits 1 and 2, respectively; and $r_{1,2}$ is the Pearson correlation between EBV for traits 1 and 2. The standard error of the approximate genetic correlations were calculated as follows (Sokal and Rohlf, 1995):

$$\mathrm{SE} = \sqrt{\frac{1 - \hat{r}_{g1,2}^2}{n - 2}},$$

where n is the number of individuals with records. The Beijing Dairy Cattle Center (Beijing, China) provided EBV and the reliabilities of 59 traits from 677,983 Holstein cattle in early 2020.

Searching for Genes and Functional Annotation

In this present study, a significance threshold of 0.5% of total genetic variance explained by one window was

used. For the significant 10-SNP windows, genes were annotated on the basis of the starting and ending coordinates of each window by using R package 'Biomart' of Ensembl (Flicek et al., 2013). Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathways and Gene Ontology (**GO**) term were enriched via the "cluster Profiler" package (Yu et al., 2012) of R with cutoff P-value < 0.05.

RESULTS

Frequency and Heritability of SNT in Chinese Holstein Cattle

The frequency of SNT in this studied Chinese Holstein cows was 9.8%. In addition, farm B (14.9%) had significantly higher frequency of SNT than that of farm A (8.7%) in Chinese Holstein cows ($P < 0.05$, Table 1). Then, we calculated the frequency of SNT depending on the cow's birth season. Differences in SNT frequency among 4 seasons were noticed, showing relatively low value for cattle born in uncomfortable weather (summer or winter) in comparison with cows born in other seasons. The frequency of SNT at different birth seasons is shown in Supplemental Figure S1 (https://doi.org/10.6084/m9.figshare.15098385.v1; Wen, 2021). Additionally, the estimated heritability for SNT presence in this study was 0.22 (SE = 0.07) with genetic variance of 0.286. The heritability on the observed scale for SNT (0.06; SE = 0.02) was significantly lower than the liability estimate.

Approximate Genetic Correlation Between SNT and Other Traits

The approximate genetic correlations between SNT and milk performance traits are presented in Table 2. Most of the approximate genetic correlations between SNT and milk performance were negative and ranged from −0.55 (SE = 0.02; SNT with 305-d milk yield in the third parity) to −0.17 (SE = 0.02; SNT with 305-d fat yield in the first parity). The genetic correlations between SNT and 305-d SCS were close to 0. The correlations of milk performance and SNT varied among parities, and cattle with high parity was accompanied with low correlations between milk-related traits and SNT. The 305-d milk yield (−0.55; SE = 0.02) and 305-d fat yield (−0.51; SE = 0.03) in the third parity had the lowest negative relationship with SNT. For the 305-d protein yield, correlations with SNT in the second parity had the lowest value (−0.54; SE =0.01).

Moderate approximate genetic correlations were observed in Table 3 between SNT and traits about body conformation, which ranged from −0.17 (SE = 0.07; with side view of rear legs) to 0.39 (SE = 0.05; with rear udder height). The correlations of SNT with most udder characters for linear type traits [except front teat placement (−0.14; SE = 0.07)] were positive.

Unfavorable moderate approximate genetic correlations between SNT and longevity traits in the heifer and cow are shown in Table 4. The SNT showed a lowest negative correlation with survival up to 365 d (−0.30; SE = 0.01) in heifers. Correlations between SNT and longevity traits decreased from moderate (survival up to the second parity, −0.13; SE = 0.02) to 0 (survival up to the sixth parity, 0; SE = 0.05) as a result of increased parity in mature cows.

The approximate genetic correlations between SNT and fertility and disease traits are shown in Table 5. The SNT were unfavorably (positively) correlated with most fertility traits (e.g., age at first calving in heifer, interval from first to last inseminations in heifer, interval from first to last inseminations in cow, and age at first calving in heifer), with correlations ranging from 0.01 (SE = 0.01) to 0.19 (SE = 0.03). Negative genetic correlations were also found between the interval from calving to first service in cow with SNT (−0.15; SE = 0.03).

The metabolic diseases showed moderate and positive correlation with SNT (0.23; SE = 0.04), whereas

Table 1. Frequency of supernumerary teats (SNT) among different farms in Chinese Holstein cattle

Farm	Number of SNT cows	Total number of dairy cows	Frequency of SNT (%)
A	336	3,845	8.74[a]
B	123	825	14.91[b]
Total	459	4,670	9.83[a]

[a,b]Values with different superscripts within a column indicate a significant difference ($P < 0.05$).

Table 2. Approximate genetic correlations between supernumerary teats with milk-related traits in Chinese Holstein cattle[1]

Trait	N	$r_G \pm$ SE
305-d milk yield for third parity	1,656	−0.55 ± 0.02
305-d protein yield for second parity	3,617	−0.54 ± 0.01
305-d milk yield for second parity	3,525	−0.53 ± 0.01
305-d fat yield for third parity	1,126	−0.51 ± 0.03
305-d protein yield for third parity	2,398	−0.46 ± 0.02
305-d protein yield for first parity	3,029	−0.41 ± 0.02
305-d fat yield for second parity	1,164	−0.37 ± 0.03
305-d milk yield for first parity	3,721	−0.30 ± 0.02
305-d fat yield for first parity	1,575	−0.17 ± 0.02
305-d SCS for third parity	191	−0.07 ± 0.07
305-d SCS for first parity	346	0 ± 0.05
305-d SCS for second parity	259	0.02 ± 0.06

[1]N = number of animals with reliability greater than 0.35 that were used to calculate the approximate genetic correlations; r_G = approximate genetic correlation.

Table 3. Approximate genetic correlations between supernumerary teats with body conformation traits in Chinese Holstein cattle[1]

Trait	N	$r_G \pm SE$
Side view of rear legs	207	−0.17 ± 0.07
Front teat placement	207	−0.14 ± 0.07
Stature	4,564	−0.08 ± 0.01
Rump width	1,269	−0.05 ± 0.03
Foot angle	207	−0.02 ± 0.07
Rear teat placement	219	0.02 ± 0.07
Body size	3,669	0.03 ± 0.02
Body depth	357	0.04 ± 0.05
Feet and legs	119	0.05 ± 0.09
Rear view of rear legs	317	0.06 ± 0.06
Teat length	178	0.07 ± 0.08
Fore udder attachment	1,950	0.11 ± 0.02
Suspensory ligament	352	0.13 ± 0.05
Foot heel depth	331	0.13 ± 0.05
Capacity	300	0.14 ± 0.06
Rump	253	0.14 ± 0.06
Bone quality	242	0.15 ± 0.06
Chest width	307	0.15 ± 0.06
Rump angle	149	0.19 ± 0.08
Angularity	278	0.23 ± 0.06
Overall conformation	238	0.23 ± 0.06
Forepart	315	0.25 ± 0.05
Udder depth	201	0.26 ± 0.07
Rear udder width	674	0.26 ± 0.04
Milking system	272	0.30 ± 0.06
Udder texture	371	0.31 ± 0.05
Loin strength	408	0.34 ± 0.05
Rear udder height	409	0.39 ± 0.05

[1]N = number of animals with reliability greater than 0.35 that were used to calculate the approximate genetic correlations; r_G = approximate genetic correlation.

Table 4. Approximate genetic correlations between supernumerary teats with longevity traits in Chinese Holstein cattle[1]

Trait	N	$r_G \pm SE$
Survival up to 365 d	6,896	−0.3 ± 0.01
Survival up to 60 d	2,282	−0.25 ± 0.02
Productive life	2,038	−0.22 ± 0.02
Survival up to the second parity	3,078	−0.13 ± 0.02
Survival up to the third parity	946	−0.12 ± 0.03
Survival up to the forth parity	491	−0.06 ± 0.05
Survival until calving	6,043	−0.06 ± 0.01
Survival up to the fifth parity	403	−0.02 ± 0.05
Survival up to the sixth parity	445	0 ± 0.05

[1]N = number of animals with reliability greater than 0.35 that were used to calculate the approximate genetic correlations; r_G = approximate genetic correlation.

fertility and digestive disorders showed negative correlations [−0.22 (SE = 0.03) and −0.17 (SE = 0.07), respectively]. However, positive genetic correlations between SNT and the udder health, and feet and leg diseases were low and close to 0.

GWAS

The Manhattan plot for genetic variance explained by 10-SNP sliding windows for SNT is shown in Figure 2. Many windows with small effect regulated SNT occurrence jointly, indicating that SNT are a highly polygenic trait. This finding was consistent with the SNT study in goats (Martin et al., 2016). A clear peak within the informative window (112.70–112.90 Mb, Supplemental Table S5; https://doi.org/10.6084/m9.figshare.15098385.v1; Wen, 2021) for SNT on BTA4 was observed and explained 0.57% of the genetic variance. The region detected on this chromosome for SNT in the current study was reported for the first time in cattle. Our data suggested this important region that was linked to SNT. Eight possible known (*ZNF862*, *ATP6V0E2*, *LRRC61*, *RARRES2*, *REPIN1*, *ZNF775*, *GIMAP8*, *GIMAP7*) and 2 uncharacterized SNT associated genes were identified in our current analysis (Supplemental Table S6; https://doi.org/10.6084/m9.figshare.15098385.v1; Wen, 2021).

GWAS Signal Enrichment

Six significant KEGG pathways (i.e., collecting duct acid secretion, synaptic vesicle cycle, rheumatoid arthritis, oxidative phosphorylation, phagosome, human papillomavirus infection; $P < 0.05$) were identified on the

Table 5. Approximate genetic correlations between supernumerary teats with fertility and health traits in Chinese Holstein cattle[1]

Category	Trait	N	$r_G \pm SE$
Fertility	Interval from calving to first service – cow	1,312	−0.15 ± 0.03
	Age at first service – heifer	8,477	0.01 ± 0.01
	Interval from first to last inseminations – heifer	167	0.03 ± 0.08
	Interval from first to last inseminations – cow	705	0.08 ± 0.04
	Age at first calving – heifer	1,331	0.19 ± 0.03
Health	Fertility disorders	1,069	−0.22 ± 0.03
	Digestive disorders	284	−0.15 ± 0.06
	Udder health	1,506	0.02 ± 0.03
	Feet and leg diseases	246	0.06 ± 0.06
	Metabolic diseases	700	0.23 ± 0.04

[1]N = number of animals with reliability greater than 0.35 that were used to calculate the approximate genetic correlations; r_G = approximate genetic correlation.

basis of 8 mentioned coding genes by using the KEGG pathway analyses (Supplemental Table S7; https://doi.org/10.6084/m9.figshare.15098385.v1; Wen, 2021). Consistently, 50 significant GO terms were observed for 8 candidate genes (Supplemental Table S8; https://doi.org/10.6084/m9.figshare.15098385.v1; Wen, 2021).

DISCUSSION

The SNT have been little studied in dairy genetics. A deep understanding of the genetics underlying the SNT of dairy cattle to reduce its frequency and negative effect on milk production is vital for breeders. Our main objectives were to estimate the genetic parameters of SNT and their genetic correlations with functional traits for Holstein cattle and detect SNT-linked genes through the WssGWAS. Results suggested that SNT could be highly inherited and could compromise the productivity, health, and wellbeing of dairy cattle. The findings obtained through WssGWAS also provided information about key genes and regions for SNT. Although the negative effect of SNT was not massive, we could minimize the chances of SNT through intensive genetic selection programs for cattle.

The average frequency of SNT in our studied population was relatively low (9.8%) compared with those in previous studies on Holstein cattle (15.0%; Brka et al., 2000) and other breeds, such as German Simmental (44.3%) and Brown Swiss (31.2%; Brka et al., 2002). This result might be caused by different cattle populations. In addition, breed, environment, and selection could affect the occurrence of SNT (Brka et al., 2002).

The trait for SNT is highly heritable. The heritability for SNT in the current population was 0.22 (SE =0.07) but lower than that of other dual-purpose breeds such as German Simmental (n = 179,793; h^2 = 0.45; SE = 0.01) and Swiss Brown (n = 37,460; h^2 = 0.43; SE = 0.03; Brka et al., 2002). In addition, the heritability on the observed scale was considerably lower, and this finding was consistent with previous studies (Brka et al., 2002). The heritability on the liability scale can seize more variability and is uneasily affected by the disease incidence among herds (Misztal, 2017). Thus, several studies about genetic evaluation of SNT, such as Brka et al. (2002) and Martin et al. (2016), used the liability heritability. Limited pedigree depth led us to not consider inbreeding depression in the model for SNT. In future studies, we will expand the study population with more information from pedigree.

Previous studies about SNT heritability estimation in cattle were different from our study for the definition of SNT phenotype. Other researchers defined specific phenotypes on the basis of the areas of animals located, shapes of SNT, and the presence of SNT mammary gland in their models, which could explain the reason for their relatively high heritability (Brka et al., 2002; Joerg et al., 2014; Martin et al., 2016; Butty et al., 2017). Limited to the information we collected, the occurrence of SNT only was considered in the current study. However, the heritability we estimated was within the normal range found for SNT in cattle (0.15–0.44; Brka et al., 2002). This finding meant SNT were influenced by genetics, and selection programs could consider selecting against SNT.

Cattle with SNT have relatively low milk production and quality. Our previous work compared the milk yield between individuals with and without SNT in the Chinese Holstein population. We observed that the daily milk yield, peak milk yield, and lactation persistency of non-SNT cows were always better than those of cows with SNT, but the results did not reach a significant level (Wen et al., 2019). In the current study, SNT were

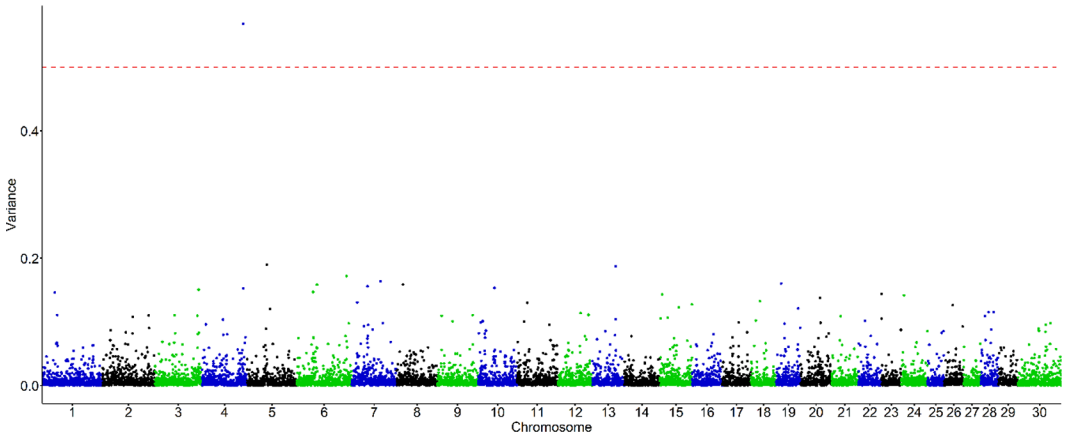

Figure 2. Manhattan plots for the genetic variance (%) explained by each 10-SNP sliding window for supernumerary teats in Holstein dairy cattle.

negatively correlated with 305-d milk yield, 305-d fat yield, and 305-d protein yield in genetics, which could explain the difference of phenotype for milk production between SNT and non-SNT cows. The SNT are easily invaded by bacteria through the teat canal (Hardwick et al., 2020), thus potentially predisposing cows to mastitis and leading to low milk production. The incidence of subclinical mastitis has been reduced in Dutch dairy calves after removing SNT (Santman-Berends et al., 2012). Nonetheless, correlations between SNT and 305-d SCS were close to 0 because SCS was easily influenced by the environment, management, and other factors in farms, not only by SNT.

The SNT were positively correlated with many body development traits, such as udder texture (0.31; SE = 0.05), forepart (0.25; SE = 0.05), udder depth (0.26; SE = 0.07), and chest width (0.15; SE = 0.06), indicating that SNT and body development traits in cattle might be regulated by a similar underlying mechanism. For example, the *VRTN* mutation was identified because it can increase vertebral and teat number, and enlarge body size in Chinese pigs (Yang et al., 2016). The SNT also showed positive genetic correlations with udder-related traits in body conformation except the front teat placement (−0.14; SE = 0.07).

The SNT showed unfavorable relationship with longevity traits, and the lowest value of the correlations was −0.30 (SE = 0.01) in survival up to 365 d for heifer. Calves with SNT may suffer from abnormality and many diseases, resulting in being culled early. Supportive evidence shows that humans with SNT are susceptible to congenital heart anomalies (Urbani, 2004), kidney malformation (Brown and Schwartz, 2004), and urologic diseases (Méhes et al., 1987). In addition, diseases around kidney and urology in SNT individuals supported the findings on high positive correlation (0.23; SE = 0.04) between SNT and metabolic diseases. In addition, few studies provided biological reasons for the favorable (negative) correlations of SNT with fertility disorders and digestive disorders [−0.22 (SE = 0.03) and −0.15 (SE = 0.06), respectively].

Unfavorably (positively) low to moderate approximate genetic correlations between SNT and several fertility traits were observed, indicating that SNT were genetically correlated with a long time of heifer's first calving and long interval from first to last inseminations in heifers and cows. In addition, the highest unfavorable correlation appeared between SNT and the age at first calving in heifer, which was considered as an important predictor of subsequent milk yield (Jairath et al., 1995; Haworth et al., 2008). Older age at first calving in heifers results in the need for replacement heifers and increased costs for rearing (Tozer and Heinrichs, 2001). In addition, van Pelt et al. (2016) reported that calving at a young age results in high survival rates. However, only the correlation value between SNT and interval from calving to first service in cows was negative (−0.15; SE = 0.03). Gernand and König (2017) found the genetic correlation between fertility disorders and interval from calving to first service in cows reached 0.80 ± 0.06, which supported our genetic correlation results of SNT with fertility disorders and interval from calving to first service in cows.

The abnormality of udders, SNT, is unfavorable for milk production, longevity, health, and fertility traits and causes economic losses. In addition, SNT are associated positively with body development. Our findings showed that individuals with SNT may have low milk production and may be susceptible for diseases and culled early. Several GWAS about SNT have been already conducted in German Fleckvieh (Pausch et al., 2012) and Holstein breeds (Joerg et al., 2014), and several have identified some genes with a major effect on SNT (e.g., *LGR5* and *LEF1*; Pausch et al., 2012; Butty et al., 2017). The phenotypes of these GWAS included the areas of animals located, shapes of SNT, and the presence of SNT mammary gland (Pausch et al., 2012; Joerg et al., 2014; Butty et al., 2017). In the current study, we focused on the occurrence of SNT and detected a series of novel genes for this trait.

We discovered a region (112.70–112.90 Mb) on BTA4 that showed the strongest effect on SNT occurrence in GWAS. The region with the largest proportion of variance contains the *RARRES2*. Previous studies documented that *RARRES2* regulates lipid metabolism and adipogenesis and mediates hormone secretion through gonadal functions in mice and humans (Goralski et al., 2007; Reverchon et al., 2015). Previous literature supported that the SNT occurrence may result from the abnormal secretion of hormone (Brka et al., 2002; Hotchkiss et al., 2007).

The variance explained by our window on BTA5 (48.65–48.79 Mb) is 0.17%, which is much lower than 1%. However, BTA5 is still regarded as another important chromosome because some critical SNT-related QTL in cattle were identified on it (Pausch et al., 2012; Butty et al., 2017). One QTL on BTA5 was detected by GWAS in the dual-purpose Fleckvieh breed, and this region contains cluster sequences (i.e., *LOC783893* and *LOC783966*; Pausch et al., 2012). Butty et al. (2017) found *LGR5*, a gene on BTA5 that can serve as a candidate gene for SNT in Swiss Brown, by performing GWAS with a mixed model. Subsequently, we found that the expression level of *LGR5* in the blood of SNT cows was significantly higher than that of non-SNT cows (Wen et al., 2019). Gene *LGR5* and many others

on BTA5 are involved in the Wnt signaling pathway, and this signaling pathway regulates cell proliferation and differentiation and mammary gland development (Chu et al., 2004; Clevers, 2006; Nusse and Clevers, 2017). In the current study, the *WIF1* located on BTA5 was associated with SNT of dairy cattle. The *WIF1* gene encoded a conserved Wnt-binding protein, which could inhibit the activation of Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway takes part in diverse processes, including cell proliferation and differentiation during embryogenesis (Zachary and Stephane, 2018). The Wnt/β-catenin signaling pathway also plays a critical role in controlling the tissue expansion and remodeling during mammary gland development (Tocci et al., 2020; Chu et al., 2004). When the Wnt/β-catenin signaling pathway is activated abnormally, it vastly promotes cell proliferation, differentiation in diverse stages, and adult tissue homeostasis (Clevers, 2006). Therefore, *RARRES2* and *WIF1* genes may play important roles in the occurrence of SNT.

Furthermore, only few studies investigated the association of the identified 6 significant signaling pathways with mammary tissue in dairy cattle. Among 50 GO terms, the most significant 2 terms were associated with insulin (i.e., regulation of cellular response to insulin stimulus and insulin receptor signaling pathway). Neville and Picciano (1997) revealed that the interaction of insulin with its receptor is critical to normal mammary differentiation and milk secretion. Functional enrichment and annotation suggested that *RARRES2* and *WIF1* can be considered as important candidate genes for SNT in Chinese Holstein cattle.

Building a unified standard for SNT, including position, shape, and mammary gland, is one of our purposes in future research. Our study has a limitation of small data size, which may lead to biased results. Thus, expanding the population and exploring the phenotype can efficiently improve the power for detecting genomic regions related to SNT in dairy cattle. Genomic regions can be used to improve the accuracy of genomic prediction for SNT.

CONCLUSIONS

The estimated heritability of SNT was moderate (0.22; SE = 0.07) in the studied Chinese Holstein population. The approximate genetic correlations between SNT and traits of milk production, longevity, health, and fertility were low to moderate and unfavorable. The SNT were associated positively with body development. The region (112.70–112.90 Mb) on BTA4 might play crucial roles in the development of SNT. Thus, selection programs could be considered against SNT in dairy cattle.

ACKNOWLEDGMENTS

This work was funded by the NSFC-PSF joint project (grant no. 31961143009, Beijing, China), Beijing Dairy Industry Innovation Team (grant no. BAIC06, Beijing, China), Beijing Natural Science Foundation (grant no. 6182021, Beijing, China), and Earmarked Fund for Modern Agro-industry Technology Research System (grant no. CARS-36). The authors thank the editor and 2 anonymous reviewers for their comments and suggestions that greatly contributed to improve our manuscript. Furthermore, the authors are grateful to the Beijing Dairy Cattle Center (BDCC, Beijing, China) and Beijing Sunlon Livestock Development Company Limited (Beijing, China) for facilitating the data collection and access to production records, and the members of the COWINFO Laboratory at China Agriculture University (Beijing, China) for collecting the data, especially Wenqin Qiu [Allflex (China) Intelligent Technology Company Limited, Beijing, China], Shaokan Chen (Beijing Sunlon Livestock Development Company Limited, Beijing, China), and Muhammad Zahoor Khan (Gomal University, Dera Ismail Khan, Pakistan). The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be constructed as a potential conflict of interest.

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