ASSESSMENT OF *ANXA9* SNP EFFECTS ON MILK PERFORMANCE TRAITS IN POLISH HOLSTEIN-FRIESIAN COWS

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ABSTRACT

The aim of this study was to estimate associations between the *ANXA9 951A/G* polymorphism and milk performance traits (daily milk yield, protein yield, fat yield, protein content and fat content), as well as somatic cell count in milk in 990 Polish Holstein-Friesian cows. The statistical analysis confirmed its significance in shaping the analyzed traits. In most cases, the presence of the allele *A* was associated with a decrease in the values of the above traits, while in the first lactation a significant additive effect of this allele toward increasing the values of the daily milk yield and SCC was found.

Key words: *ANXA9* SNP, additive and dominance effects, cattle, milk performance

INTRODUCTION

Annexin A9 (ANXA9) is the protein associated with the cytoplasmic membrane. It belongs to a family of proteins that bind phospholipids in Ca^{+2} dependent way [Moss and Morgan 2004]. Annexins have different functions in the cell. They are involved in the signal processing, endocytosis, membrane trafficking, calcium channel formation, and regulation of the inflammatory response in the cell cycle. ANXA9, whose activity is not regulated by Ca^{+2}, is considered to be a specific protein in this family. The expression of the *ANXA9* gene was demonstrated in various tissues, particularly in those which are characterized by rapid metabolism of fatty acids, such as mammary tissue during lactation [Calvo et al. 2006].

The gene encoding the ANXA9 was mapped on the bovine chromosome 3. As a result of different studies, QTLs for some milk performance traits were mapped on the bovine chromosome 3. Moreover, a QTL region with effect on fat yield, in which *ANXA9* gene is located has been found [Ashwell et al. 2004, Calvo et al. 2006, Ogorevc et al. 2009]. Polymorphic sites within the coding and noncoding sequences were also identified [Calvo et al. 2006]. However, the results of the analyses on the relationship between polymorphism in this gene and milk traits are scarce.

The biological role of the protein encoded by the discussed gene, the presence of transcripts in the mammary gland, as well as chromosomal location indicate its validity as candidate gene for traits related to milk performance in dairy cattle. In order to verify the potential of existing knowledge base on the subject and to further complement it, the studies involving the following were undertaken: determining the frequencies of alleles and genotypes of SNP located in the gene encoding the ANXA9 in herd of cows belonging to Polish Holstein-Friesian breed; to establish if there are any statistically significant associations between the *ANXA9* polymorphism and milk performance traits (milk yield, protein yield, fat yield, protein content and fat content), as well as somatic cell count in milk in the population of cows under study.

MATERIAL AND METHODS

Material

The study was conducted in a herd of 990 Polish Holstein-Friesian cows kept on a farm located in the

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north-western region of Poland. All animals lived under similar environmental conditions. They were kept in one free-stall barn and milked twice a day in a herringbone-type milking parlour. The cows had *ad libitum* access to water from individual automatic drinking vessels and were fed an identical standard TMR (total mixed ration) diet. Additionally, during milking each cow was given specially selected feed concentrate suited to its current physiological condition and milk yield. The cows were of different ages and different lactations (from first to sixth).

The cows were covered under a milk performance monitoring system based on the A4 milk recording method and the data on their pedigree background and milk performance was collected in the SYMLEK animal recording system. The data concerning milk production traits was derived from the results of monthly test milkings. A total of 39,470 test milkings were performed. The cows were daughters of 307 different bulls, the average number of daughters per one bull being 3.23. The cows were characterized by different participation of Holstein-Friesian genes, varying from 37.5% to 100%.

Genotyping

Blood samples were taken from the external jugular vein and then placed in tubes containing K_3EDTA. Genomic DNA was obtained using MasterPureTM DNA Purification Kit for Blood (Symbios) in accordance with the attached instructions. The DNA samples suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA) were stored at 4 degrees Celsius.

The study covered the *951A/G* polymorphic site located within the *ANXA9* gene coding sequences. It results in the H84R substitution at the polypeptide level [Martínez-Royo et al. 2010].

Genotypes were determined based on the method available in the literature, although some modifications were made to optimize the procedures. To amplify the desired fragment of the *ANXA9* (241 base pair), the following primers were used: 5'-*TCC CAG ACC TTG TCA TTT CC*-3' and 5'-*CTC CTG GGA ATC AGT GTG GT*-3'. Primers were designed based on the sequence available in GeneBank at the AY785287 accession number and by using the Primer^{-3} (v. 0.4.0) software tool [Calvo et al. 2006].

The PCR proceeded in a total volume of 20 μL mixture with the following composition: approximately 60–70 ng DNA, 15 pmol of each primer, 0.5 mM dNTP, 4 mM MgCl$_2$, *Taq* buffer and 0.4 U of *Taq* polymerase (A&A Biotechnology). The PCR thermal conditions were as follows: the initial denaturation for 3 minutes at 95°C; then denaturation step for 45 s at 94°C, annealing step for 45 s at 55°C, elongation step for 45 s at 72°C (30 cycles); then a final elongation step for 5 minutes at 72°C.

The PCR products were digested using the *Nla*III endonuclease, then the restriction fragments were separated on 2% agarose gels with ethidium bromide. The results were read using the photodocumentation of electrophoretic separation, and image storage system (Vilber Lourmat®).

Statistical methods

The results were recorded and analyzed statistically to examine the genetic structure of the population under study, i.e. to determine allele and genotype frequencies for the *ANXA9* gene polymorphism. Subsequently, a statistical analysis was conducted to search for associations between the polymorphism and daily milk yield and also protein, fat, SCC content in milk. SCC was transformed logarithmically to normalize the distribution of this trait. The calculations were made separately for lactations I to VI (lactation VI also including subsequent lactations, if any).

To estimate any such associations, a mixed model (containing both random and fixed effects) was applied. Apart from genotype, the following factors were included as sources of variation: standard factors such as test day, birth year/season and day in milk (DIM), and the additive polygenic effect (genetic background effect). Additionally, because studied traits are repeatable over time, the model also included fixed environmental effect. The adopted statistical model also included the dominant effect, as estimated with the natural and orthogonal interactions NOIA model [Alvarez-Castro and Carlborg 2007]. To estimate the genetic effects of the examined *locus*, single-trait mixed multiple regression models were used. The overall formula for the selected models was as follows:

$$Y_{jklmn} = \mu + G_j + TD_k + YS_l + DIM_m + \\ + A_n + PE + e_{jklmn}$$

where:

Y_{jklmn} — daily milk yield or fat yield or fat content or protein yield or protein content or somatic cell count (lnSCC) of cow with *j*-th genotype in *k*-th class of milking test year/month, *l*-th year/season of birth class, *m*-th day in milk and *n*-th father group;

μ — mean value of the analyzed trait in the population;

G_j — additive (*add*) and dominance (*dom*) effects of *ANXA9* gene;

DT_k — effect of *k*-th class of milking test year/month;

YS_l — effect of *l*-th birth year/season class;

DIM_m — effect of *m*-th DIM (day in milk);

A_n — random additive polygenic effect of *n*-th father group;

PE — random permanent environmental effect;

e_{jklmn} — random residual effect of cow with j-th genotype in k-th year/month of milking test, l-th year/season of birth, m-th day in milk and n-th father group.

RESULTS

The frequencies of the analyzed *ANXA9 951A/G* alleles and genotypes are presented in the Table 1. Within the cows' herd the *AA* genotype was the most frequent. Population was in Hardy-Weinberg equilibrium.

Table 2 presents the estimated additive and dominant effects of the allele *A* in relation to milk performance traits of the analyzed cow herd. This gene has been involved in shaping daily milk yield, fat yield, fat content, protein yield, protein content and SCC in milk. In most cases, the presence of the allele *A* was associated with a decrease in the values of the above traits.

When analyzing daily milk yield, a significant additive effect of the allele *A* which resulted in a decrease in the value of this trait was found in lactations III and IV, while a positive effect associated with its increase – in lactation I. Allele *A* was significantly associated with a fat yield decreasing in II, III and IV lactations, as well as with milk fat content decreasing in all the analyzed lactations.

The presence of this allele was also resulted in a protein yield decrease, which was observed in lactations III and IV, as well as in a milk protein content decrease, which was found in II, VI and subsequent lactations. In the case of somatic cell count, the analysis showed a significant additive effect of the *ANXA9* SNP in I, II, V and VI lactations except the second one. The effect of the allele *A* toward increasing SCC was found in the first lactation, while in the remaining ones mentioned above a decrease in the somatic cells count in milk was observed.

Based on the statistical analysis, the dominance of the allele *A* in shaping the analyzed traits was found. With regard to milk yield, this effect was statistically significant in lactations I and IV, and resulted in an increased and decreased value of this trait, respectively. A significant dominant effect of the allele *A* was also found with regard to the fat yield and milk fat content in lactations II and IV. In the case of protein yield, a statistically significant dominant effect of the above allele was observed in IV lactation. When analyzing the somatic cell count, a significant dominant effect of the allele *A* was found in lactation II, III, V and VI. Its effect resulted in a decreased SCC.

DISCUSSION

Cow's milk is an important component of the human diet. It is a valuable source of essential nutrients [Čítek et al. 2019]. The production of market milk in Poland is based on the Holstein-Friesian breed [Litwińczuk and Barłowska 2014]. Polish Holstein-Friesian Black and White variety cattle are the most frequently represented in Poland. The breed was created by crossing a local lowland Black and White breed with Holstein-Friesian cattle imported from the United States and Canada since the 1970s [Litwińczuk and Barłowska 2014]. In 2018, an average of 691,836.3 cows of this breed were subject to performance recording, which constituted 85% of the entire population assessed. Polish Holstein-Friesian cows were characterized by the highest milk yield, 8,519 kg [PZHiPBM 2019].

The goal of the breeding program for Polish Holstein-Friesian cattle is to achieve the greatest genetic progress in improving production traits, with particular emphasis on the milk yield and quality (the yield of fat and protein, the content of fat, protein, lactose and dry matter in milk). While implementing the breeding program, it is also assumed to improve functional traits such as fertility, udder health, longevity, milking rate and milking behavior, as well as type and body conformation traits. The systematic development of performance control with extensive use of the possibilities offered by genomic assessment may contribute to the optimization of genetic progress [PZHiPBM 2019].

Table 1. Observed (N obs) and expected (N exp) numbers of *ANXA9* genotypes and frequency of genotypes and alleles

Tabela 1. Obserwowana (N obs) i przewidywana (N exp) liczebność genotypów *ANXA9* oraz frekwencja genotypów i alleli

Genotype Genotyp	N obs	N exp	obs-exp	(obs-exp)^2	Frequency Frekwencja	Allele Allel	Frequency Frekwencja
AG	384	410.150	−26.150	1.667	38.788	A	70.707
AA	508	494.901	13.099	0.347	51.313	G	29.293
GG	98	84.949	13.051	2.005	9.899		
Sum – Suma	990	990.000	0.000	4.019	100.000		100.000

chi-square (χ^2)= 4.018797, df = 2, P = 0.134071 – population in Hardy-Weinberg equilibrium.
chi-square (χ^2)= 4,018797, df = 2, P = 0.134071 – populacja w równowadze Hardy'ego-Weinberga.

Table 2. Additive and dominance effects of *ANXA9* gene (allele *A*) in relation to milk production traits in the herd under study

Tabela 2. Efekty addytywne i dominacyjne genu *ANXA9* (allel A) w odniesieniu do cech użytkowości mlecznej w badanym stadzie

Lacta-tion Kolejna laktacja	Factor Czynnik	Daily milk yield, kg Dobowa wydajność mleka, kg		Fat yield, kg Wydajność tłuszczu, kg		Fat content, % Zawartość tłuszczu, %		Protein yield, kg Wydajność białka, kg		Protein content, % Zawartość białka, %		Somatic cell count, ln(SCC/ml) Liczba komórek somatycznych ln(SCC/ml)	
		Effect Efekt	SE	Effect Efekt	SE	Effect Efekt	SE	Effect Efekt	SE	Effect Efekt	SE	Effect Efekt	SE
I	Intercept wyraz wolny	33.492	0.763	1.190	0.033	3.521	0.085	1.162	0.215	3.123	0.042	4.830	0.151
	ANXA9 add	0.723**	0.244	0.009	0.010	–0.064*	0.027	0.016	0.069	–0.025	0.013	0.105*	0.048
	ANXA9 dom	0.920**	0.327	0.018	0.014	–0.068	0.036	0.025	0.092	–0.025	0.018	0.055	0.064
II	Intercept wyraz wolny	46.922	0.534	1.767	0.025	3.689	0.056	1.513	0.018	3.137	0.025	4.555	0.092
	ANXA9 add	0.133	0.294	–0.047**	0.014	–0.168**	0.031	–0.011	0.010	–0.053**	0.014	–0.109*	0.050
	ANXA9 dom	0.445	0.393	–0.038*	0.018	–0.177**	0.041	0.008	0.013	–0.032	0.019	–0.199**	0.067
III	Intercept wyraz wolny	46.638	0.302	1.841	0.014	3.937	0.031	1.494	0.010	3.137	0.013	5.053	0.049
	ANXA9 add	–0.778*	0.359	–0.064**	0.017	–0.112**	0.037	–0.037**	0.012	–0.032	0.016	0.035	0.058
	ANXA9 dom	–0.386	0.482	–0.019	0.023	–0.026	0.050	–0.016	0.016	–0.007	0.021	–0.153*	0.078
IV	Intercept wyraz wolny	47.270	0.318	1.861	0.015	3.947	0.033	1.504	0.011	3.115	0.013	5.293	0.050
	ANXA9 add	–2.077**	0.480	–0.129**	0.023	–0.170**	0.050	–0.081**	0.016	–0.031	0.020	0.146	0.076
	ANXA9 dom	–2.852**	0.639	–0.158**	0.030	–0.164**	0.067	–0.100**	0.021	0.002	0.027	0.034	0.101
V	Intercept wyraz wolny	45.562	0.373	1.795	0.018	3.936	0.041	1.449	0.012	3.098	0.016	5.442	0.063
	ANXA9 add	0.402	0.645	–0.046	0.032	–0.198**	0.071	–0.001	0.022	–0.040	0.027	–0.404**	0.109
	ANXA9 dom	0.245	0.850	–0.027	0.042	–0.108	0.093	–0.001	0.028	–0.010	0.036	–0.427**	0.144
VI	Intercept wyraz wolny	42.372	0.765	1.738	0.036	4.211	0.080	1.330	0.025	3.122	0.032	5.662	0.122
	ANXA9 add	0.810	0.851	0.009	0.040	–0.207*	0.089	0.002	0.028	–0.126**	0.035	–0.525**	0.136
	ANXA9 dom	0.167	1.124	0.019	0.053	–0.101	0.118	–0.003	0.037	–0.069	0.047	–0.665**	0.179
Total Ogółem	Intercept wyraz wolny	39.169	0.371	1.464	0.017	3.719	0.037	1.245	0.039	3.102	0.016	5.208	0.061
	ANXA9 add	–0.167	0.166	–0.044	0.008	–0.131	0.016	–0.021	0.017	–0.049	0.007	–0.036	0.027
	ANXA9 dom	–0.080	0.223	–0.030	0.010	–0.101	0.022	–0.013	0.023	–0.033	0.010	–0.148	0.036

** statistically significant at P ≤ 0.01; * statistically significant at P ≤ 0.05; SE – standard error.

** istotne statystycznie przy P ≤ 0,01; * istotne statystycznie przy P ≤ 0,05; SE – błąd standardowy.

The economic significance of milk production and health-promoting effects of milk have resulted in the implementation of numerous studies on polymorphisms in candidate genes related to milk performance traits. Research focuses on analyzing the relationship between the values of these traits and polymorphic variants of different genes. The genes encoding different proteins, such as milk proteins, enzymes involved in the metabolism of fatty acids, proteins binding and transporting fatty acids, some hormones, transcription factors and many others are analyzed in this aspect [Szewczuk et al. 2011, Cieślińska et al. 2019, Thuy et al. 2018, Kiyici et al. 2019].

The approach to identify mutations in candidate genes is not losing popularity. The *ANXA9* gene may be useful in this type of research. The analyzed *951A/G* SNP is located in the coding sequence of the gene and results in an amino acid substitution at the protein level. Moreover, the *ANXA9* is located in a region in which the

QTL for milk fat content and other milk production related traits were previously mapped [Ashwell et al. 2004, Ogorevc et al. 2009]. So, the analyzed SNP may be also linked to another polymorphism, which significantly affects the milk performance traits.

In this study the *ANXA9* allele *A* turned out to have higher frequency in Polish Holstein-Friesian cows, which is consistent with the results obtained by Martínez-Royo et al. [2010] on the Spanish Holstein-Friesian cattle and Kulig et al. [2010] on Jersey cows.

Martínez-Royo et al. [2010] reported significant associations between this SNP and milk and fat yield of Spanish Holstein-Friesian cows with the highest value of this trait in *GG* cows. Kulig et al. [2013] found that the polymorphism in the *ANXA9* was associated with the breeding value for fat content in Polish Holstein-Friesian cows; the *GG* individuals were characterized by the lowest value of this trait. When analyzing milk performance traits in the present study, a significant additive and dominance effects of the allele *A* was found in different lactations, generally resulting in a decrease in the value of the traits. Such a discrepancy might have several reasons, among other different statistical models used. The use of EBV contains information from relatives, which is reflected in the results obtained [Chinyere et al. 2014]. In the study conducted by Kulig et al. [2010] no association were found between the SNP and milk production traits in Jersey cows. On the other hand, significant association were found between the genotypes and the SCC in milk, wherein significantly lower value of ln SCC was found in *AA* and *AG* cows than in *GG* animals. The assessment of the analyzed *ANXA9 951A/G* SNP effect in the present study, taking into account additive and dominance effects, confirmed its statistical significance in shaping somatic cell count. The presence of the allele *A* was also resulted in a SCC decrease.

CONCLUSION

The obtained results give some insight into the potential involvement of the studied *ANXA9 951A/G* polymorphism in shaping the milk performance traits in Polish Holstein-Friesian cattle. The statistical analysis, including additive and dominance effects, confirmed that in most cases, the presence of the allele *A* was generally associated with a decrease in the values of the above traits. Further research are necessary to assess the *ANXA9* polymorphism helpfulness in selection strategies aimed at improving milk performance traits in Holstein-Friesian cows.

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OCENA WPŁYWU SNP W GENIE *ANXA9* NA CECHY UŻYTKOWOŚCI MLECZNEJ KRÓW POLSKICH HOLSZYŃSKO-FRYZYJSKICH

STRESZCZENIE

Celem badania była ocena zależności między polimorfizmem *ANXA9 951A/G* a cechami użytkowości mlecznej (dobowej wydajności mleka, wydajności białka, wydajności tłuszczu, zawartości białka i zawartości tłuszczu), a także liczbą komórek somatycznych w mleku w stadzie 990 krów polskich holsztyńsko-fryzyjskich. Analiza statystyczna uwzględniająca ocenę efektów addytywnych i dominacyjnych, potwierdziła jego znaczenie w kształtowaniu analizowanych cech. W większości przypadków obecność allelu *A* wiązała się ze zmniejszeniem wartości powyższych cech, podczas gdy w pierwszej laktacji stwierdzono znaczący efekt addytywny tego allelu w kierunku zwiększenia wartości dobowej wydajności mleka i SCC.

Słowa kluczowe: *ANXA9* SNP, efekty addytywne i dominacyjne, bydło, użytkowość mleczna

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