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ASSOCIATION OF ALPHA-A GLOBIN GENE POLYMORPHISM WITH ITS EXPRESSION LEVEL IN RACING PIGEONS

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ABSTRACT

The objective of the study was to investigate possible effect of the polymorphism in pigeon α^A-*globin* (*AGLOB*) gene on its expression level. The g.5768C>T substitution located in $3'$ flanking region of the gene was analyzed. PCR-RFLP (*Ava*II) method was used to identify the SNP. The gene expression analyses were performed using qPCR. The investigation indicated a difference but not significant in the α^A-*globin* gene expression levels between pigeons carrying different g.5768C>T genotypes. The $AGLOB^{CC}$ genotype was associated with 2.46-fold higher expression level of pigeon α^A-*globin* gene compared to $AGLOB^{TT}$ genotype. The lower expression of α^A-*globin* gene in $AGLOB^{TT}$ pigeons may be caused by the presence of cis-acting regulatory elements in the analysed gene region.

Key words: α^A-*globin* gene, racing pigeons, qPCR, SNP

INTRODUCTION

Pigeons have developed a strong relation with humans. There is evidence that cave-dwelling humans exploited Rock Doves for food at least 67 thousand years ago [Blasco et al. 2014]. In the ancient Middle East, doves were kept in towers; their droppings with high nitrogen content were a major source of organic fertilizer [El Gemaiey 2016]. People used pigeons to send war reports home from the front. The discovery of the pigeon's homing ability opened unforeseen possibilities that extend beyond that of a messenger. Nevertheless, sports and gambling are as old as war [Jerolmack 2007], therefore intensive management and breeding for meat or sport, isolate pigeons from external selective pressures were performed [Marom et al. 2018]. Moreover, sport pigeons are under continuous selection to improve their speed, spatial orientation, and endurance during long flights, however, there are many factors affect these traits in racing pigeons [Ramadan et al. 2018], so searching a reliable and precisely gene marker is crucial for now.

There are many tools for selection, but the most common types of markers in genomes are SNPs [Goldstein and Cavalleri 2005, Jacob et al. 2018], nowadays, genetic markers have been used to predict racing performance in pigeons [Proskura et al. 2014, Proskura et al. 2015, Proskura et al. 2017, Ramadan et al. 2018, Dybus et al. 2018]. Recently, in whole-genome study, the *CASK* gene was marked as an important genetic factor for fast flight, long endurance, and accurate navigation was revealed [Gazda et al. 2018].

There are a large number of polymorphisms/mutations in globin gene in hemoglobinopathies context [Alaithan et al. 2018]. In birds, Galen et al. [2015] reported that a single point mutation in the *βA-globin* gene changed the hemoglobin function. Targa et al. [1993] identified regulatory elements at the $3'$-side of the chicken and duck α-globin gene domains. Furthermore, García-González and Recillas-Targa [2014] described regulatory element which modulates the activity of the chicken α-globin $3'$ enhancer. In pigeons, two SNPs were detected in α^A-*globin* gene, one of them in $3'$ region of the gene [Dybus et al. 2008].

The aim of this study was to investigate the association between polymorphism in $3'$ region of the α^A-*globin* gene and relative expression level of the gene in the three genotype group of racing pigeons.

MATERIAL AND METHODS

Ten homing pigeons (5 hens and 5 cocks) averaged six months old from the West Pomeranian University's loft were used in the study. The pigeons used in this study were reared in one loft and feed upon the same diet of dry mixed grains (35 grams per pigeon a day), free access to minerals (grit) and water. For the expression gene analysis ten pigeons were selected from the group of all individuals (~70) kept in the loft, by previous genotyping [Dybus et al. 2008]. Gender identification was determined as described by Griffiths et al. [1998]. The analysed polymorphic site is located in the $3'$ flanking sequence (at a distance of 65 nucleotides from the end of the $3'$UTR) of the α^A-globin gene (NW_004973488.1).

DNA was isolated from whole blood using salting-out procedure (Epicentre Biotechnologies, Madison, WI, USA). The PCR-RFLP method (with *Ava*II enzyme) was used to identify the pigeons' g.5768C>T SNP in the $3'$ region of the α^A-globin gene [GeneBank: NW_004973488.1]. PCRs were carried out in 15 μl (~60 ng of DNA, 15 pmol of each primer, 1.5 μl of 10×PCR buffer, 1.5 mM MgCl$_2$, 200 μM dNTP, 0.3 unit of *Taq* polymerase; EUR$_X$, Gdańsk, Poland). The following thermal profile was applied: 5 min / 94°C followed by 33 cycles (94°C / 20 s, 61°C / 30 s and 72°C / 30 s) and 72 °C / 5 minutes. Amplicons were digested with 2 units of *Ava*II (New England Biolabs, Beverly, MA, USA) at 37°C and separated (120 V, 50 min) in 4% agarose gel (Sigma-Aldrich, St Louis, MO, USA).

Total RNA was extracted from the 3 μl of blood immediately after its collection (Total RNA Kit, A&A Biotechnology, Poland). RNA samples were digested with 1 unit of DNase I (Qiagen, Hilden, Germany) to remove possible DNA contamination. Total RNA was finally eluted in 100 μl of RNase-free dH$_2$O (AppliChem, Darmstadt, Germany). RNA concentration was assessed (mean of three measurements) with the Qubit 2.0 fluorometer (Quant-iT^{TM} RNA BR Assay Kit; Invitrogen, Carlsbad, CA, USA). Two-step reverse transcription was performed using TranScriba Kit based on Oligo-dT$_{18}$ primers and MMLV reverse transcriptase (A&A Biotechnology, Poland) according to the vendor's protocol. Synthesized cDNA was stored in –20°C.

Intron-spanning primers bind in the exon 1 and 2 and led to amplify a 131 bp fragment of the *AGLOB* cDNA. The qPCR analyses were performed in Rotor Gene 6000 thermocycler (Corbett Research, UK). The qPCR mixture contained 7.5 μl Kapa Sybr Fast qPCR Mix (KAPA Biosystems, USA), ~10 ng of cDNA, 200 nM of each primer and nuclease-free water (AppliChem, Germany) up to 15 μl volume. The qPCR conditions were: 3 min / 95°C followed by 40 cycles (95°C / 3 s, 60°C / 3 s, 72°C / 20 s) and 72°C for 3 minutes. All samples were run in triplicates. No template control was included in every qPCR run.

The differences in relative level of *AGLOB* expression between the genotypes were tested using Kruskal Wallis non-parametric ANOVA, while the differences between sexes were analysed using Mann-Whitney U test [Livak and Schmittgen 2001, Pfaffl 2001]. All PCR primers (for PCR-RFLP and qPCR) were designed with Primer3 software [Untergrasser et al. 2012] and presented in Table 1.

RESULTS AND DISCUSSION

The PCR-RFLP was performed to identify the genotypes ($AGLOB^{TT}$ – 165 and 38 bp, $AGLOB^{CT}$ – 165, 152, 38, 13 bp, $AGLOB^{CC}$ – 152, 38, 13 bp) in all young pigeons kept at the University's loft. Sex of pigeons was determined by PCR using CHD1 primers (Fig. 1) [Griffiths et al. 1998]. However, only ten individuals with no relationship based on the accurate pedigree were selected for qPCR analysis. The relative expression level of *AGLOB* gene was determined in selected six-month individuals (5 from both genders). The average *AGLOB* gene expression level, which is calculated by the average relative quantity (RQ of *AGLOB* gene expression was normalized using *18S rRNA* gene expression values) in the females group was 1.58-fold higher than that of males, the aver-

Table 1. Primer sequences used for PCR-RFLP and real-time PCR analyses

Tabela 1. Sekwencje starterów użytych do analiz PCR-RFLP i PCR w czasie rzeczywistym

Gene Gen	Primer sequences Sekwencje starterów	Size (bp) Wielkość (pz)	RE	Accession No. Nr akcesyjny
AGLOB	F 5′-CATGGCTAGAGCTGGACACA-3′ R 5′-AGCCCATTTCACCTACATGC-3′	203	*Ava*II	NW_004973488.1
18S rRNA	F 5′-CATGGCCGTTCTTAGTTGGT-3′ R 5′-GAACGCCACTTGTCCCTCTA-3′	131	–	AF173630.1
*AGLOB**	F 5′-CTGTCTGCCAACGACAAGAG-3′ R 5′-TGGGGGAAGTAGGTCTTGGT-3′	131	–	NW_004973488.1

*primers used for qPCR were designed to span an exon-exon junction
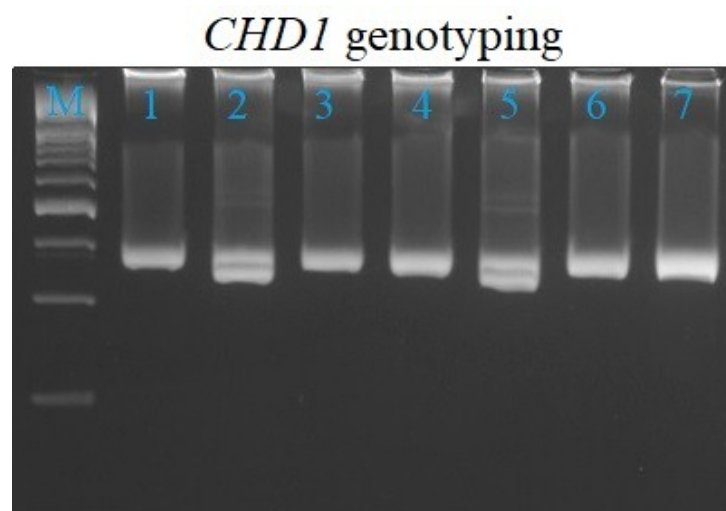*startery stosowane do qPCR zaprojektowano tak, aby obejmowały połączenie ekson-ekson

Fig. 1. Results of sex-specific *CHD1* genotyping; M – DNA marker (GeneRuler 100 bp Plus DNA Ladder); lines 1, 3, 4, 6 and 7 – males; lines 2, 5 – females

Rys. 1. Wyniki genotypowania *CHD1* zależnego od płci; M – marker DNA (GeneRuler 100 bp Plus DNA Ladder); linie 1, 3, 4, 6 i 7 – samce; linie 2, 5 – samice
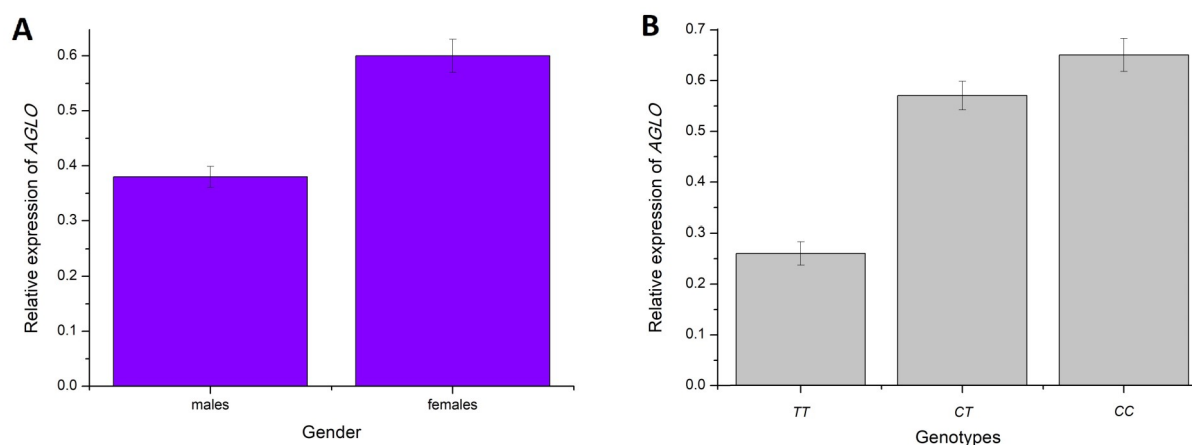

Fig. 2. The average relative expression level of *AGLOB* in samples divided into two categories – gender (A) and *AGLO/Ava*II genotypes (B)

Rys. 2. Średni względny poziom ekspresji *AGLOB* w próbkach podzielonych na dwie kategorie – płeć (A) i genotypy *AGLO/Ava*II (B)

age Ct values were 19.05 and 20.87 for females and males groups, respectively. Only numerical, not statistical significance differences between gender were confirmed.

RQ of *AGLOB* gene expression analysis focused on individuals divided into three genotype groups. The average Ct values in group of $AGLOB^{TT}$ and $AGLOB^{CT}$ individuals were slightly similar (20.76 and 20.45, respectively). The lowest values (18.99) were observed for $AGLOB^{CC}$ ones. The relative expression level of *AGLOB* gene (normalized using *18S rRNA* gene expression values) was the highest in the $AGLOB^{CC}$ individuals (RQ =

0.65). The RQ value for $AGLOB^{CT}$ pigeons was 0.57 and the lowest was recorded in the $AGLOB^{TT}$ (RQ = 0.26, Fig. 2).

Gene expression can be controlled at the transcriptional and translational level through the characteristic non-coding DNA sequences called cis-regulatory elements. These elements may be located in the vicinity or at a long distance from the coding sequence [Daniel et al. 2014]. Factors that enhance or silence gene transcription also play an important role in gene expression. Both can influence the gene expression regardless of the location
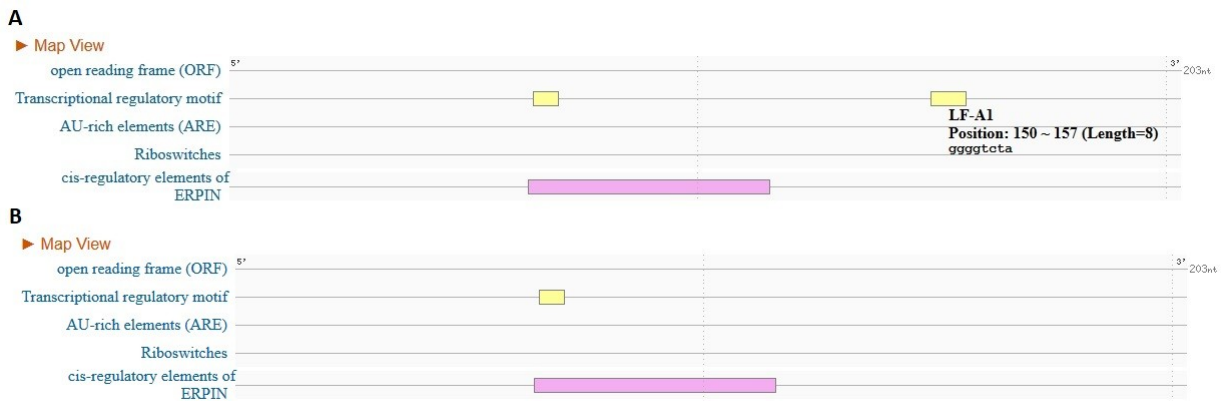
Fig. 3.   The map view of analysed region (RegRNA 2.0). The presence of LF-A1 motif in polymorphic region of homozygote *TT* (A); Lack of LF-A1 motif in polymorphic region of homozygote *CC* (B). The PCR product sequence of *Columba livia* was compared to *Gallus gallus* sequence according to RegRNA 2.0 database

Rys. 3.   Widok mapy analizowanego regionu (RegRNA 2.0). Obecność motywu LF-A1 w regionie polimorficznym homozygoty *TT* (A); brak motywu LF-A1 w obszarze polimorficznym homozygoty *CC* (B). Sekwencję produktu PCR *Columba livia* porównano z sekwencją *Gallus gallus* wg bazy danych RegRNA 2.0
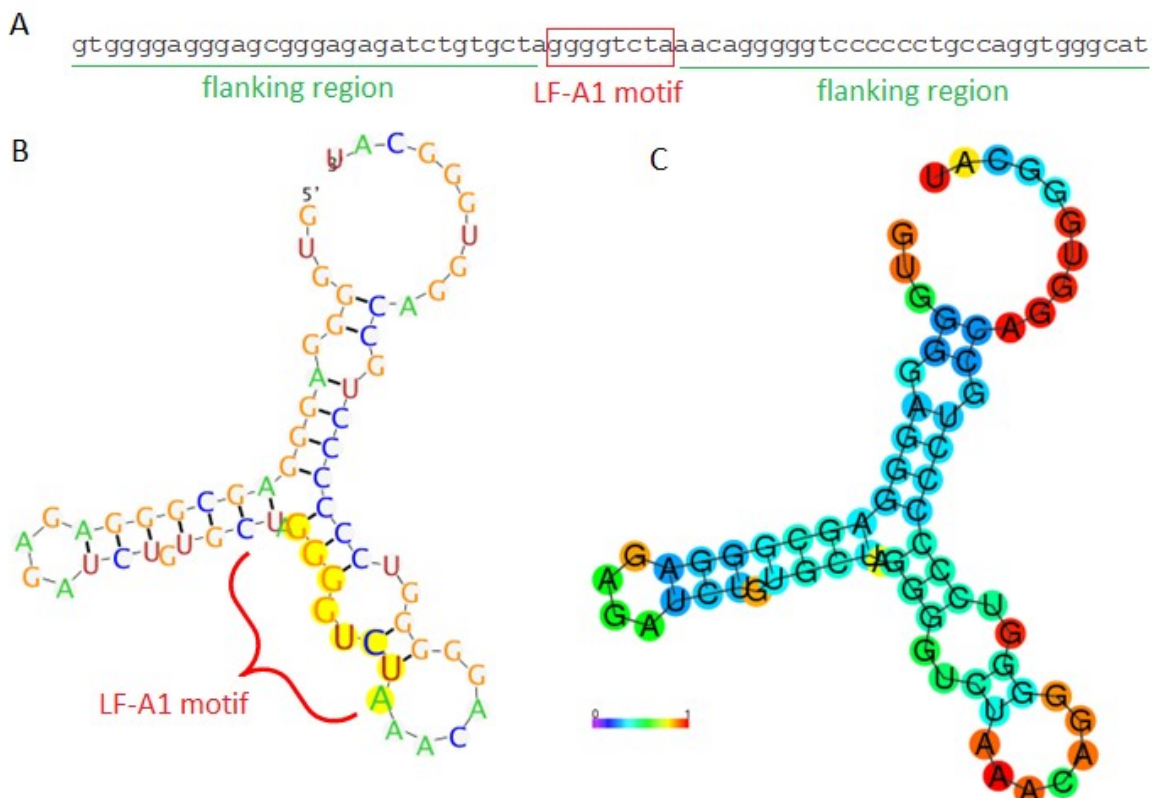

Fig. 4.   The LF-A1 motif with the flanking regions (A); The RNA secondary structure. Graph of predictive RNA (B); RNA fold reliability information of pair probabilities (C)

Rys. 4.   Motyw LF-A1 z regionami flankującymi (A); drugorzędowa struktura RNA; schemat przewidywanego RNA (B); przewidywany układ RNA na postawie prawdopodobieństwa par (C)

in the genome [Pennacchio et al. 2013]. The number of transcripts in a cell that code for a particular protein is not equal to the amount of protein produced. Changes in the efficiency of protein synthesis among others depended on the elements controlling the translation, mostly located in the UTR sequences [Holcik and Pestova 2007].

In all vertebrates, α- and β-globin genes play the same role in the body, i.e. oxygen transport in erythroid cells. However, the division of the globin gene into two families (α- and β-globin), and their location on different chromosomes caused large differences in the molecular mechanism of their expression regulation. In contrast to the family of β-globin genes, α-globins do not have characteristic promoter sequences such as the TATA box core promoter (5′-TATAAA-3′), or the inseparable CAT box regulatory sequence (5′-GCCAATCT-3′). Different cis-regulatory elements of both globin families are related to the possibility of transcription factor access. In contrast to the β-globin gene, α-globin genes are constantly exposed to transcription factors in the open chromatin structure. The absence of promoter sequences (TATA or CAT) and the location of α-globin genes near CpG islands make them similar to housekeeping genes [Forget and Hardison 2009].

The coding sequence (with introns and UTRs) of the pigeon α^A-globin gene is 828 base pairs. The gene consists of three exons. Non-translated regions have a length of 40 bp (5′UTR) and 100 bp (3′UTR) [GenBankNW_004973488.1]. The mRNA half-life is relatively long and ranges from 24 to 60 hours [Lodish and Small 1976, Ross and Sullivan 1985]. The 3′UTR region plays a significant role in gene activity and post-transcriptional gene expression [Hilleren and Parker 1999, Mitchell and Tollervey 2000], mainly by modulating mRNA stability by trans-regulatory or non-coding RNAs [Liebhaber and Russell 1998, Moss 2000]. The 3′UTR region of the α-globin mRNA contains a CRE (C-rich element) stabilizing element called α-complex [Wang et al. 1995, Wang and Kiledjian 2000]. This complex of riboproteins correlates with the stability of mRNA by binding to the 3′UTR of the gene [Weiss and Liebhaber 1995, Wang et al. 1999].

The sequences of the two homozygote genotypes (g.5768C>T) were subjected to bioinformatics analysis using the RegRNA 2.0 program [Chang et al. 2013], in order to locate cis-regulatory regions. The analysis showed the presence of a regulatory motif for the *LF-A1* transcription factor for *TT* genotype (Fig. 3). The occurrence of this motif was not confirmed in *CC* genotype. The analysis of individuals carrying different *AGLOB* genotypes (g.5768C>T) showed differences in the relative expression level, with the highest value was recorded for $AGLOB^{CC}$ ones.

The *LF-A1* transcription factor, a protein with a molecular weight of about 40 kDa, binds to an 8-nucleotide regulatory motif with a TGGACT/CT/C or TGGCCC consensus sequence (Fig. 4). This fragment is identified in the promoter regions of many liver-specific genes, e.g. in the promoter sequence of haptoglobin or apolipoprotein [Ramji et al. 1991]. According to TiGER (Tissue-Specific Gene Expression and Regulation), *LF-A1* factor has 30 co-regulating transcription factors, including tissue-specific ones characteristic of the brain, muscles or bone tissue [Liu et al. 2008]. The *LF-A1* factor occurring in *TT* individuals may effect on the expression of the pigeon α^A-globin gene, however, there is no evidence at the current stage of research. Recently, a very low frequency of allele *T* (0.077) and only *CT* genotype detected (no *TT* individuals) in the group of homing pigeons was reported [Dybus et al. 2018].

CONCLUSIONS

In conclusion, the effect of the SNP in the 3′ flanking region of the α^A-globin gene on the level of its expression has been demonstrated. The highest expression was observed in individuals carrying the common *CC* genotype, while the lowest was in a very rare *TT* one. The fragment of the α^A-globin gene under the study was analysed for the presence of cis-regulatory elements. The analysis showed the presence of a regulatory motif for the *LF-A1* transcription factor in *TT* homozygous individuals. The occurrence of this motif has not been confirmed in individuals with the *CC* genotype. Lower expression level of the α^A-globin gene in *TT* pigeons may reduce their racing performance.

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ZWIĄZEK MIĘDZY POLIMORFIZMEM W GENIE ALFA-A GLOBINY A POZIOMEM JEGO EKSPRESJI U GOŁĘBI POCZTOWYCH

STRESZCZENIE

Celem niniejszej pracy było wykazanie potencjalnej zależności pomiędzy polimorfizmem (g.5768C>T) w genie α^A-globiny (AGLOB), a jego wpływem na poziom ekspresji badanego genu. Analizowana substytucja g.5768C>T zlokalizowana jest w regionie 3′-flankującym. Genotypownie przeprowadzono w oparciu o analizę długości fragmentów restrykcyjnych (PCR-RFLP) powstałych w wyniku trawienia produktów PCR endonukleazą AvaII. Analizę ekspresji genu przeprowadzono metodą qPCR. W wyniku przeprowadzonego doświadczenia wykazano różnice w poziomie ekspresji genu α^A-globiny u osobników o różnych genotypach (g.5768C>T). Osobniki $AGLOB^{CC}$ charakteryzowały się 2,46-krotnie wyższym poziomem ekspresji w porównaniu z osobnikami o genotypach $AGLOB^{TT}$. Przyczyną niższego poziomu ekspresji genu α^A-globiny u gołębi o genotypie $AGLOB^{TT}$ może być prawdopodobnie obecność substytucji g.5768C>T w obrębie elementów cis-regulatorowych w analizowanym regionie genu.

Słowa kluczowe: region 3′-flankujący, α^A-globina, gołębie, qPCR

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