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RESEARCH ARTICLE

Mapping of *Id* locus for dermal shank melanin in a Chinese indigenous chicken breed

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Abstract. The dermal shank pigmentation, one of the defining traits of chicken breeds, is caused by an abnormal deposition of melanin in the dermis of the shank. The abnormal deposition is controlled by the sex-linked inhibitor of dermal melanin (*Id*). In this study, we aim to locate the gene responsible for the dermal shank pigmentation in chickens by an association analysis and a differential expression analysis. Based on our results, 72 single-nucleotide polymorphisms (SNPs) located in Z chromosome (chrZ): 71–73 Mb (galGal3) were selected to further explore their relationships with the dermal shank pigmentation in pure lines of 96 Gushi hens and 96 Gushi hens with a yellow shank skin colour. The results of the association analysis showed that the SNPs located in chrZ: 72.58–72.99 Mb (galGal3) (chrZ: 79.02–79.44 Mb (galGal4)) are significantly associated with the dermal shank pigmentation. Based on the results of our previous studies and the present association analysis, the zinc-finger protein 608 (*ZNF608*), GRAM domain containing 3 (*GRAMD3*), aldehyde dehydrogenase 7 family member A1 (*ALDH7A1*), fem-1 homologue C (*FEM1C*), beta-1,4-galactosyltransferase 1 (*B4GALT1*) and versican (*VCAN*) genes were selected for the differential expression analysis. The gene expression profiles showed that the expression of *GRAMD3* gene in the dermis tissues of the shank was significantly ($P = 0.010738 < 0.05$) higher in 350-day-old Gushi chickens characterized by the dermal shank pigmentation than in one-day-old Gushi chickens. The dermal shank pigmentation was not present in the one-day-old Gushi chickens. Additionally, the results of the association analysis and the expression analysis showed that *GRAMD3* could be the most likely candidate gene for the *Id* locus. However, we did not detect a mutation, i.e. significantly associated with this trait within *GRAMD3*. Therefore, we concluded that the variations located in the flanking region of *GRAMD3* led to the abnormal expression of *GRAMD3*, which requires further study.

Keywords. Gushi chicken; sex-linked inhibitor of dermal melanin; *GRAMD3* gene; shank skin colour.

Introduction

The phenotypic diversity of pigmentation in wild animals and domesticated animals is one of the most observed characteristics by biologists and breeders. The phenotype of the shank skin colour is a defining trait of the breeds of chickens, and it is due to the interaction between the melanins and carotenoids that were deposited in the dermis and epidermis, respectively. There are two loci, *–w* and *id* that control the pigmentation of the epidermis and dermis tissues. The '*W*' gene which is responsible for the white skin trait is dominant to '*w*', which is the yellow skin gene. These alleles affect the 'epidermal-outer layer', and therefore influence the shank skin colour. It has been shown that the yellow-skin gene is associated with the gene encoding beta-carotene dioxygenase 2 (*BCDO2*) (Eriksson *et al.* 2008; Jin *et al.* 2016). The sex-linked dermal gene '*Id*' (no pigment) is dominant to '*id*' (dermal-black pigment), and affects the 'dermal-under layer' colour to allow the pigmentation of the shank in the dermal layer (Wh 1974; McGibbon 1979). Using the three-point test cross, Bitgood (1988) mapped '*Id*' on the distal end of the long arm of chrZ, and is 13.7 ±

Jiguo Xu and Shudai Lin contributed equally to this work.

Figure 1. Four types of shank skin colours in the Gushi breed. From left to right, white shank (*Id*), yellow shank (*Id*), green shank (*id*) and gray shank (*id*).

2.2 cM proximal to the centromere from the sex-linked barring gene (*B*). Finally, Hellstrom *et al.* (2010) confirmed that sex-linked barring is controlled by the *CDKN2A*/*B* locus, which is located in chrZ: 78.45–78.47 Mb (galGal4). It was found that a SNP, rs14686603 located at chrZ: 78.8 Mb (galGal4) showed the highest association with *Id* by a genomewide SNP–trait association analysis (Dorshorst *et al.* 2010). Unfortunately, now we do not know which gene is responsible for the trait of dermal shank melanin.

Gushi chicken, a famous indigenous chicken breed in China has four types of shank skin colour (white, yellow, green and slate) (figure 1). The green and slate shank skin colours are controlled by *id* locus which do not express in one day-old chicks (McGibbon 1974). In this study, we used Gushi chicken as the experimental material to locate the gene responsible for the dermal shank melanin. Therefore, we hypothesized that it is the variations located in flanking region of GRAM domain containing 3 (*GRAMD3*) leading to abnormal expression of *GRAMD3* and further resulting in the dermal shank melanin.

Materials and methods

Animals and DNA preparations

All experimental procedures in this study were approved by the Animal Care and Use Committee of the South China Agricultural University (Guangzhou, China) with approval number SCAU#0011. All efforts were made to minimize animal suffering. A total of 96 Gushi chickens exhibiting dermal shank melanin and 96 hens exhibiting yellow shank were obtained from Sangao Agriculture and Animal Husbandry Limited by Share, Henan, China and were selected to fine-map the *Id* locus for the dermal shank melanin.

At 52 weeks of age, 1 mL blood samples were withdrawn from all the chickens (192 birds) through the wing veins into the syringe containing 2% EDTA, which was used as an anticoagulant. DNA was extracted from the anticoagulated blood using the E.Z.N.A NRBC Blood DNA kit (Omega Bio-Tek, Norcross, USA). All steps were performed according to manufacturer's protocol. The DNA concentration was determined by measuring the optical density in a Nanodrop 2000 spectrophotometer at a 260/280 nm ratio, and the genomic DNA was then stored at –20°C.

RNA isolation and RNA reverse transcription

Six one-day-old female Gushi chickens that did not present the green shanks phenotype and six 350-day-old female Gushi chickens characterized by green shanks were obtained from Sangao Agriculture and Animal Husbandry by Share (Gushi, Henan, China). The dermis of the shanks was used for the expression analysis of candidate genes. The tissue samples were collected immediately after slaughter, frozen in liquid nitrogen and stored at –80°C until RNA extraction.

Total RNA was extracted using the RNAiso Plus kit (TaKaRa, Otsu, Japan) according to the manufacturer's instructions. The obtained RNA was treated with RQ1 Rnase-Free DNAse (Promega, Madison, USA) to remove any contaminating genomic DNA. Reverse transcription was carried out with the PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa) following the manufacturer's protocol. The synthesis of cDNA was completed in a mixture of 2 μg total RNA, 4.0 μL 5× Prime Script Buffer and 1 mL PrimeScript RT enzyme mix. The final reaction of the reverse transcription was performed at 37°C for 15 min and then at 85°C for 5 s.

SNP selection and genotyping

Based on previous results (Dorshorst *et al.* 2010), 72 SNPs (table 1) in chrZ: 71–73 Mb (galGal3) were selected for genotyping and exploring their relationships with the dermal shank melanin. Genotyping was carried out using the method of SNaPshot at Shanghai Generay Biotech (Shanghai, China).

Differential expression analysis

Tissues (dermis of the shank) were collected from six one-day-old birds and six 350-day-old birds from the populations with a green shank skin colour. Based on the results of previous studies (Dorshorst *et al.* 2010) and our analysis, six genes (*ZNF608*, *GRAMD3*, *ALDH7A1*, *FEM1C*, *B4GALT1* and *VCAN*) were selected for the differential expression analysis (table 2). Seven pairs of

Table 1. Information on the SNPs used for the association analysis.

	ID	Position (galGal3)	Position (galGal4)		ID	Position (galGal3)	Position (galGal4)
1	rs13725801	71022114	72731540	37	rs14686419	72157813	78613328
2	rs16127503	71039969	72749395	38	rs14686433	72178181	78632834
3	rs14782500	71060472	72769512	39	rs14686488	72196844	78649798
4	rs14782525	71079924	72786369	40	rs14686528	72238081	78688784
5	rs14782575	71100595	72807040	41	rs16684253	72257626	78708984
6	rs16778990	71120024	72825602	42	rs16684257	72278533	78729296
7	rs14782624	71159311	72864259	43	rs14686595	72297580	78747305
8	rs16779030	71179138	72885049	44	rs14686603	72310603	78758539
9	rs14782638	71200695	72906004	45	rs14686622	72317505	78765442
10	rs16779053	71221800	72925268	46	rs14686663	72334644	78782581
11	rs14782714	71240702	72943005	47	rs16684327	72355396	78801496
12	rs14782726	71262488	72964548	48	rs14686694	72374862	78820216
13	rs16127710	71299885	73034643	49	rs14686730	72417274	78861724
14	rs16779177	71320720	73054600	50	rs14066525	72436610	22554377
15	rs14782873	71341502	73074282	51	rs14686099	72495385	78925422
16	rs14782887	71358807	73092315	52	rs16744781	72515547	78944784
17	rs16127787	71380471	73112336	53	rs15992089	72535947	78980734
18	rs16127795	71396911	73128133	54	rs16683926	72556021	79000375
19	rs14782931	71420515	73150799	55	rs16683912	72576864	79023115
20	rs14782946	71443637	73172685	56	rs15992063	72598250	79044143
21	rs16127848	71461014	73190062	57	rs16683889	72617074	79062820
22	rs14782954	71482820	73210225	58	rs13800898	72637157	79081016
23	rs16127963	71520373	73256550	59	rs16683874	72680440	79124704
24	rs16127915	71543533	73355832	60	rs16683857	72704397	79167014
25	rs14722276	71624390	73438616	61	rs15992011	72724332	79185213
26	rs14686145	71903009	78355278	62	rs14685829	72761365	79222165
27	rs14686180	71937085	78389354	63	rs16683841	72783845	79243478
28	rs14686204	71957162	78408656	64	rs13800879	72801669	79260281
29	rs16684046	71997010	78453130	65	rs16683817	72848222	79304744
30	rs14686280	72017296	78474063	66	rs14685777	72886244	79341792
31	rs14686315	72037030	78493126	67	rs14685769	72928718	79382121
32	rs15992378	72057191	78512005	68	rs13800864	72946342	79399745
33	rs14686361	72076859	78535859	69	rs16683794	72965628	79419152
34	rs16684177	72097375	78554442	70	rs14685747	72986102	79439369
35	rs14686376	72117727	78573931	71	rs14685736	72999921	79455142
36	rs15992526	72137811	78593731	72	rs14779590	67143188	68668193

Table 2. Information on the genes used for the expression analysis.

Gene	Position	
	galGal3	galGal4
ZNF608	73, 032, 178–73, 076, 141	79, 449, 231–79, 529, 636
GRAMD3	72, 329, 744–72, 344, 700	78, 776, 484–78, 809, 738
ALDH7A1	72, 301, 386–72, 320, 592	78, 750, 396–78, 768, 531
FEM1C	72, 270, 610–72, 284, 822	78, 721, 490–78, 735, 188
B4GALT1	68, 711, 927–68, 722, 880	70, 457, 341–70, 474, 687
VCAN	61, 308, 618–61, 409, 263	62, 474, 981–62, 580, 840

primers were designed by Primer 5.0 to test the six candidate genes and the housekeeping gene *β-actin* using real time (RT)-PCR (table 3). All primer pairs were designed to span at least one intron and synthesized by Sangon Biotechnology (Shanghai, China).

The expression level of the candidate genes was examined by RT-PCR with the SYBR Green Real-Time PCR Master mix (Bio-Rad, Hercules, USA). The qRT-PCR reactions were performed in a final volume of 20 μL containing 10 μL SsoFast EvaGreen Supermix (Bio-Rad), 0.3 μL primers (10 μM), 2.5 μL cDNA and 6.9 μL RNase-free water according to the manufacturer's protocol (Bio-Rad). For each sample, PCR was conducted in triplicate using an ABI7500 real-time PCR system (Applied Biosystems, Foster City, USA).

The two-step method was used for qRT-PCR as follows: 95°C for 2 min, 39 cycles at 95°C for 15 s and 58°C for 30 s, the fluorescent signal was captured in each cycle

Table 3. Primers used for real-time PCR.

Primer name	Primer sequence	T_m (°C)
GRAMD3-F	5′-ggctgatcgtcccacaactctg-3′	58
GRAMD3-R	5′-tgcgtgcgtcggaatgga-3′	
ZNF608-F	5′-cccaaccatccagcctatcgag-3′	58
ZNF608-R	5′-ttgggctggtattgactttctcc-3′	
VCAN-F	5′-aaggcattgtatcgctggctgttg-3′	58
VCAN-R	5′-ccagccagcatcacattgttcaaa-3′	
B4GALT1-F	5′-ggcagcagctagattatggagtgt-3′	58
B4GALT1-R	5′-ttggttggctgtagcacttgtag-3′	
FEM1C-F	5′-cgtgcttgatgatttcctgttaca-3′	58
FEM1C-R	5′-tggtttgcacatgctgggtca-3′	
ALDH7A1-F	5′-gcgggtcagcgatgcacaac-3′	58
ALDH7A1-R	5′-gggcgatttataacctttccacc-3′	
β-actin-F	5′-gagaaattgtgcgtgacatca-3′	58
β-actin-R	5′-cctgaacctctcattgcca-3′	

followed by a melting curve analysis (65–95°C, 5 s). After the amplification, the dissociation curve was analysed using Bio-Rad Manager software, and the data were normalized to the geometric mean of *β-actin* which was used as a reference gene. The relative expression levels of the candidate genes in the dermis of the shank were calculated by the $2^{-\Delta\Delta Ct}$ equation ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$, $\Delta\Delta Ct = \Delta Ct - \Delta Ct_{max}$). The difference in the expression levels between the one-day-old and 350-day-old chickens was tested for significance using the unpaired *t*-test with $P < 0.05$.

Association analysis based on the SNPs across GRAMD3

The results of the association analysis and differential expression analysis showed that *GRAMD3* might be a candidate gene for *Id*. To further confirm the relationship between *GRAMD3* and the dermis of the shank, 31 SNPs near *GRAMD3* were selected, and their relationships with the locus of *Id* were explored (table 4). Among these SNPs, SNPs 1–8 are located in the interior and flanking regions of *GRAMD3*. SNPs 9–25 are located in regions that were significantly associated with the green shank trait in the initial association analysis. SNPs 26–31 are located on both sides of the SNP that was significantly associated with the green shank trait in a previous study (Siwek *et al.* 2013).

Statistical analysis

The association analysis was carried out using the case–control module of Haploview.

Results

Detection of the region associated with dermal shank melanin

The results of the association analysis show that 26 SNPs were found to be significantly associated with the green

Table 4. Information on the variations used in genotyping.

	ID	Variation	Position galGal3	Position galGal4		ID	Variation	Position galGal3	Position galGal4
1	*GRAMD3-1*	*A/G*	72326106	78774041	17	rs14685868	*A/G*	72727164	79188003
2	*GRAMD3-2*	*TTTG/–*	72328291–72328295	78776227–78776231	18	rs16683848	*A/G*	72732727	79193566
3	*GRAMD3-3*	*C/T*	72333810	78781746	19	rs16683847	*C/T*	72739961	79200800
4	*GRAMD3-4*	*A/C*	72340970	78788906	20	rs13800889	*A/G*	72747191	79207990
5	*GRAMD3-5*	*T/C*	72346142	78793680	21	rs14685844	*T/G*	72753184	79213983
6	*GRAMD3-6*	*C/T*	72352587	78799402	22	rs14685832	*A/C*	72759372	79220171
7	*GRAMD3-7*	*T/A*	72358104	78804204	23	rs14685829	*G/T*	72761366	79222165
8	*GRAMD3-8*	*T/G*	72361802	78807902	24	rs13800887	*C/T*	72775129	79234761
9	rs16683874	*C/G*	72680441	79124704	25	rs16683841	*A/G*	72783846	79243478
10	rs16683873	*A/G*	72680476	79124739	26	rs53829076l	*C/T*	72961028	79414551
11	rs14685905	*G/A*	72686506	79130769	27	rs16683782	*C/T*	72975740	79429265
12	rs15992024	*A/–*	72694476	79157078	28	rs15991876	*A/G*	72981724	79434990
13	rs16683861	*T/A*	72701276	79163892	29	rs14685750	*A/G*	72985598	79438864
14	rs16683856	*A/C*	72708342	79170521	30	rs16683774	*A/G*	72988984	79442250
15	rs14685875	*A/T*	72713334	79174800	31	rs14685734	*A/G*	73005051	79460271
16	rs15992019	*G/A*	72720687	79181567					

Table 5. The SNPs significantly associated with the green shank trait.

No.	ID	χ^2	*P*	Position (galGal3)	Position (galGal4)
36	rs15992526	36.196	1.78E-09	72137811	78593731
63	rs16683841	36	1.97E-09	72783845	79243478
59	rs16683874	35.769	2.22E-09	72680440	79124704
62	rs14685829	35.769	2.22E-09	72761365	79222165
70	rs14685747	24.391	7.86E-07	72986102	79439369
57	rs16683889	22.359	2.26E-06	72617074	79062820
55	rs16683912	21.084	4.4E-06	72576864	79023115
19	rs16127795	20.799	5.1E-06	71420515	73128133
22	rs14782954	19.51	0.00001	71482820	73210225
60	rs16683857	19.456	1.03E-05	72704397	79167014
16	rs14782873	19.33	0.000011	71358807	73074282
48	rs14686694	19.143	1.21E-05	72374862	78820216
28	rs14686204	9.799	0.0017	71957162	78408656
12	rs14782726	8.195	0.0042	71262488	72964548
42	rs16684257	7.822	0.0052	72278533	78729296
64	rs13800879	7.319	0.0068	72801669	79260281
53	rs15992089	7.274	0.007	72535947	78980734

shanks trait ($P < 0.05$). In addition, the SNPs rs16127795, rs14782954, rs14782873 and rs16779177 were located in the region of 71.26–72.98 Mb (galGal3). The SNPs that were significantly associated with the green shanks trait ($P < 0.01$) were mainly concentrated in a relatively continuous interval between rs14685747 and rs16683912 (galGal3, table 5). In the vicinity of this region, there are four functional genes, including *ZNF608*, *GRAMD3*, *ALDH7A* and *FEM1C*.

Expression of candidate genes

Based on the results of the association analysis and previous studies (Dorshorst *et al.* 2010), *VCAN*, *B4GALT1*, *ALDH7A*, *FEM1C*, *GRAMD3* and *ZNF608* were selected for the differential expression analysis. We examined the expression levels of these genes in the dermal tissue of shanks by RT-PCR. The gene expression profiles showed that the expression level of the *GRAMD3* gene in the dermis tissues was significantly higher in the 350-day-old Gushi chickens characterized by green shanks than that in the one-day-old Gushi chickens that did not present the green shanks phenotype (figure 2, a–f). These results indicate that *GRAMD3* is the best candidate gene for the *Id* locus.

Association analysis based on the SNPs across GRAMD3

The results of the association analysis and expression analysis showed that the *GRAMD3* gene is likely involved in the formation of dermal shank melanin. Therefore, we examined the relationships between variations of *GRAMD3* and the trait of green shanks. In addition, the results of the association analysis demonstrated that there is no significant association between the SNPs located on *GRAMD3* and the trait. However, the significant associations between the mutations and the trait were all located in the flanking region of *GRAMD3* (table 6). Moreover, the genotyping results explained that all SNPs that were significantly associated with the dermal shank melanin are completely linked to each other.

Discussion

Studies on the origin, differentiation, proliferation and migration of melanoblasts in Silkie chicken have found that the melanoblasts in Silkie chicken can migrate in the ventral pathway, which is normally reserved for cells of neuronal and glial lineages, and the dorsolateral pathway (Hallet and Ferrand 1984; Erickson 1993; Lecoin *et al.* 1995; Jacobs-Cohen *et al.* 2002). In addition to the abnormal migration, the continuous proliferation of melanocytes was also observed because a large number of melanocytes were observed throughout the body of the Silkie embryo (Reedy *et al.* 1998; Faraco *et al.* 2001). Ferrand and L'Hermite (1985) found that the embryo extracts can increase the proliferation of quail melanoblasts when grafted into Silkie embryos. Now, scientists have confirmed that a complex genomic rearrangement involving endothelin 3, causes dermal hyperpigmentation in the chicken (Dorshorst *et al.* 2011). These results suggest that '*D*' may be responsible for the abnormal migration of melanocytes.

Based on previous studies, our association analysis results and our expression analysis results, we suggest that the *GRAMD3* gene is a candidate gene for *Id.* To date, not much research has been performed on the *GRAMD3* gene, and there is no evidence regarding the involvement

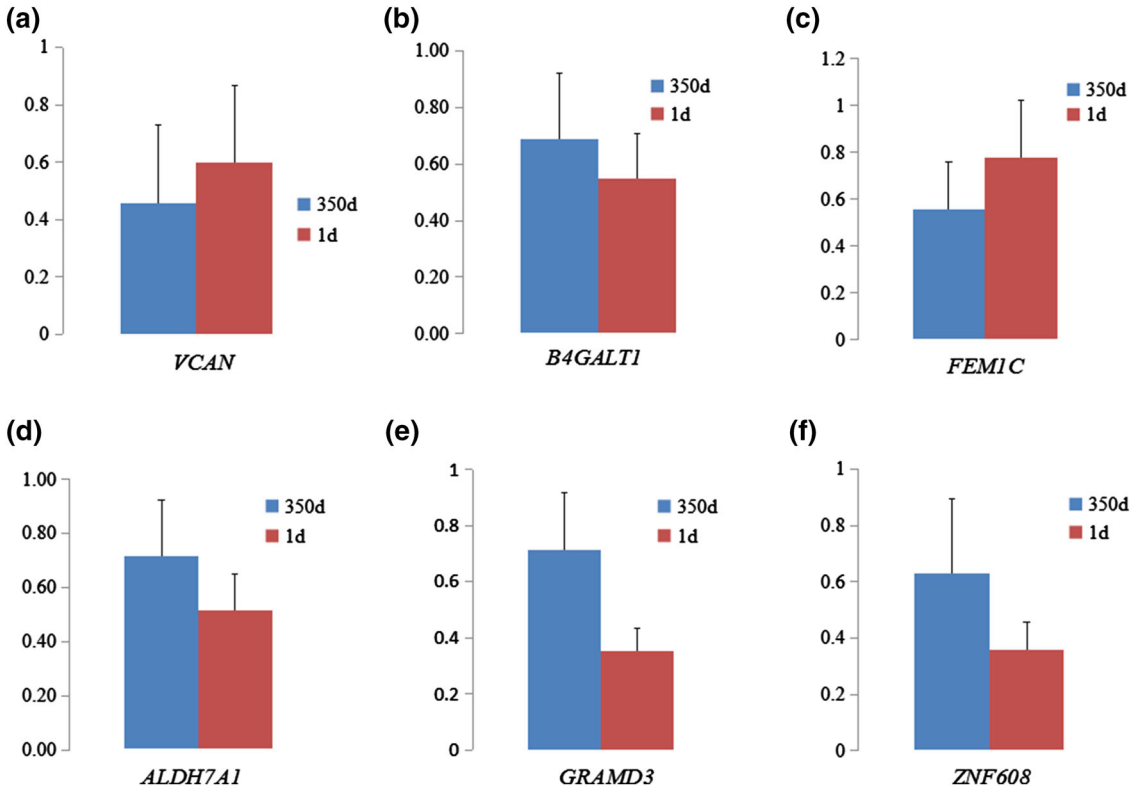

Figure 2. Expression levels of each candidate gene. (a) *VCAN*, (b) *B4GALT1*, (c) *FEM1C*, (d) *ALDH7A1*, (e) *GRAMD3* and (f) *ZNF608*. Each bar represents the mean ± SEM ($n = 3$). *$P < 0.05$.

Table 6. Results of the association analysis.

ID	Position (galGal3)	Position (galGal4)	χ^2	P
rs16683874	72680441	79124704	23.279	0.0016
rs16683873	72680476	79124739	23.279	0.0016
rs14685905	72686506	79130769	23.279	0.0016
rs16683847	72739961	79200800	23.279	0.0016
rs13800889	72747191	79207990	23.279	0.0016
rs14685844	72753184	79213983	23.279	0.0016
rs14685832	72759372	79220171	23.279	0.0016
rs14685829	72761366	79222165	23.279	0.0016
rs16683841	72783846	79243478	23.279	0.0016

of *GRAMD3* in cell migration. However, there are some other evidence that support our hypothesis in addition to our results.

First, *GRAMD3* has the same structural domain as *Rab27a*, which is involved in the capture of melanosomes. The mutated *Rab27a* gene can lead to Griscelli syndrome 2. Griscelli syndrome is an inherited condition characterized by unusually light (hypopigmented) skin and light silvery-gray hair starting at infancy (Vincent *et al.* 2010).

Second, the *GRAMD3* gene is highly expressed in human retinal pigment epithelial cells (RPEs) (Strunnikova *et al.* 2010). The *GRAMD3* gene was found to be highly expressed in human RPE by comparing the profiles of native and cultured human foetal and adult RPEs to the Novartis expression database. The high expression level of *GRAMD3* in RPEs indicates that it may be involved in the formation of the pigmented phenotype (Strunnikova *et al.* 2010).

Third, it is reported that a SNP located on chrZ 79.4 Mb (galGal4) was found to be significantly associated with the *Id* locus ($P = 2.11e-07$) (Siwek *et al.* 2013). *GRAMD3* is located upstream of this SNP. Although, no SNPs within *GRAMD3* were found to be significantly associated with the shank trait, *GRAMD3* is the closest gene to the SNPs that was significantly associated with this trait.

Fourth, using Tibetan chickens as material, Li *et al.* (2014) identified three SNPs located at chrZ: 78.5–79.2 Mb (galGal4) through the GWAS method that were

significantly associated with the dermal shank pigmentation in chickens. A portion of *GRAMD3* is located within this region (galGal4) (Li *et al.* 2014).

In addition to the above-mentioned studies, Tian *et al.* (2014) identified a region between rs16127903 (chrZ: 73219016, galGal4) and rs14685542 (chrZ: 79740470, galGal4) that was closely linked to *Id*, which suggested that methyl thio-adenosine phosphorylase (*MTAP*) could be the most likely candidate gene through an expression analysis. However, they did not determine the specific SNP(s) that was completely linked to the dermal shank pigmentation in Silkie chickens. In summary, there is no clear result on the *Id* corresponding gene, which requires further studies to validate our hypothesis.

In conclusion, based on our results from the association analysis and expression analysis, we deduced that *GRAMD3* could be the most likely candidate gene. Because we did not detect mutations within *GRAMD3* that were significantly associated with the dermal shank pigmentation, we concluded that it was the variations in the flanking region of *GRAMD3* that led to the abnormal expression of *GRAMD3*, and then influenced the dermal shank pigmentation.

Acknowledgements

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