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Original Research

Using Linear Discriminant Analysis to Characterize Novel Single Nucleotide Polymorphisms and Expression Profile Changes in Genes of Three Breeds of Rabbit (*Oryctolagus cuniculus*)

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The objectives of this study were to investigate polymorphisms and changes in expression patterns of the genes *FGF5*, *PGAM2*, *TLR2* and *IL10* in V-line, Baladi Black and Baladi Red rabbits. Blood samples were collected from 180 healthy rabbits (n = 60 for each breed) for DNA extraction and DNA sequencing. At 3 mo of age, 20 randomly selected females from each breed were euthanized for gene expression quantification in muscle and spleen samples. PCR-DNA sequencing revealed single nucleotide polymorphisms (SNPs) among the 3 breeds that provided a monomorphic pattern for 3 of the 4 genes analyzed. Linear discriminant analysis (LDA) was used to classify the SNPs of these genes in the 3 breeds. The overall percentage of correctly classified cases for the model was 75%, with percentages of 100% for *FGF5*, 63% for *IL10*, and 100% for *TLR2*. Breed was a significant predictor for gene classification with estimation (1.00). Expression profiles of the genes were higher in V-line as compared with Baladi Black or Baladi Red. The LDA discriminated the 3 breeds using results of the gene expression profile as predictors for classification. Overall, 73% of the cases were correctly classified by gene expression. The identified SNPs, along with changes in mRNA levels of *FGF5*, *PGAM2*, *TLR2*, and *IL10*, could provide a biomarker for efficient characterization of rabbit breeds and could thus help develop marker assisted selection for growth and immune traits in rabbits.

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Wild rabbits are commonly considered to have been first domesticated in 600 A.D. when French medieval monks began to keep rabbits in cages for food;[23] newly born rabbits were not considered to be meat and therefore rabbits were valued as a meat substitution during Lent.[30,45] In the family *Leporidae* of the order Lagomorpha, the domestic rabbit (*Oryctolagus cuniculus domesticus*) is a subspecies of the European rabbit, *Oryctolagus cuniculus*. *Oryctolagus cuniculus* includes the European rabbit species and its descendants, the world's 305 breeds of domestic rabbit. European rabbits today exist in the wild on every continent except Asia and Antarctica.[19,65,96]

Rabbits are considered a valuable food species due to their rapid breeding and high-quality meat. Rabbit meat is leaner than that of other animals, is tender and easily digested, and has high value protein and low sodium, cholesterol and fat content. Consequently, a diet high in rabbit meat is particularly recommended for the elderly, children and pregnant women.[20,28,86]

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Phenotypic and genetic characterization studies of native rabbits can be used to identify and improve breeding and conservation programs.[32] The genetic structure of a population is the result of many factors, such as geographical and ecological influences, which may cause variation and division of the populations. Moreover, genetic drift, gene flow, and the balance between them can also contribute to shaping the genetic structure of a population.[1,63] Genetic structure can be characterized using a variety of proxy molecular markers, depending on the aim of breeder.[37] In recent years, analysis of single nucleotide polymorphisms (SNPs) has begun to dominate the field of genetic structure analysis. New technologies using SNPs or whole genome scanning may build upon previous achievements in biodiversity assessment and genetic characterization of breeds. Such approaches could provide a more thorough understanding of molecular basis of functional diversity within a species.[37]

Fibroblast growth factors (FGFs) are small polypeptides that share certain common structural characteristics. Many FGF signal peptides are secreted into the extracellular environment where they contribute to normal development, wound healing, and tumor development and progression.[69] To date, 23 FGF family members have been identified in humans, where they are widely present in various tissues.[72] One member of the FGF

family is *FGF5*, which stimulates growth and proliferation in multiple cell types and is considered a promising candidate gene for economically important quantitative traits in rabbits.[40,90] In rabbits, the *FGF5* gene is present on chromosome 15, contains 3 exons, and has a molecular weight of 34 kDa.[18,38,102]

Phosphoglycerate mutase (PGAM) is an enzyme of the glycolytic pathway that converts 3-phosphoglycerate into 2-phosphoglycerate.[29] In mammalian tissues, PGAM is a dimer of 2 distinct 30 kDa subunits, including the ubiquitously expressed brain form (B form, known also as PGAM1) and the muscle form (M form, known also as PGAM2) expressed only in adult skeletal and cardiac muscle.[97] The 2 forms generate 3 types of PGAM dimers (MM, BB, and MB).[48,101] The isozyme pattern of human PGAM2 is regulated developmentally during myogenesis. Some mutations cause PGAM2 deficiency in humans, resulting in a serious muscle dysfunction with exercise intolerance, cramps, myoglobinuria, scattered atrophic and hypertrophic fibers.[86,87] In pigs, PGAM2 is expressed at a high level in skeletal muscle during all stages of development, and is related to growth, feed conversion, and slaughter traits.[29,71] PGAM2 has also key roles in the glycolysis process controlling postnatal development and related meat quality parameters.[25]

Toll-like receptors (TLRs) are single-pass membrane-spanning proteins that are usually expressed on sentinel cells such as macrophages and dendritic cells.[21] They recognize conserved molecules known as pathogen-associated molecular patterns (PAMPs), which are derived from the extracellular domain constituent of leucine-rich repeat (LRR) domains of pathogens.[3] TLRs also initiate the early immune response in innate and acquired immunity.[89] Differences in recognition of PAMPs by TLRs may be attributed to gene polymorphisms.[46,88] TLR2 recognizes PAMPs, mycobacterial lipoglycan and other bacterial cell wall macro-amphiphiles.[5,73] *TLR2* is expressed on monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes, B cells, and T cells.[13]

Interleukin 10 (IL10) is an antiinflammatory cytokine encoded by *IL10* gene, which consists of 5 exons.[66] IL10 is a homodimer with each subunit consisting of 178 amino-acids.[100] It is synthesized mainly by monocytes and to a lesser extent by T cell subsets, monocytes/macrophages, mast cells, keratinocytes, eosinophils, epithelial cells and natural killer cells.[70] IL10 was initially reported to suppress cytokine secretion, antigen presentation and CD4+ T cell activation.[4,49] The IL10 gene also has a pleiotropic effect in immune-regulation and inflammation through down-regulation of the expression of Th1 cytokines, MHC class II antigens, and costimulatory molecules on macrophages. It also enhances B-cell survival, proliferation, and antibody production.[36,85] Expression of *IL10* is tightly regulated at transcriptional and posttranscriptional levels, which may involve control of mRNA stability via AU-rich elements.[68]

Few studies have explored the molecular characterization of *FGF5*, *PGAM2*, *TLR2* and *IL10* genes in rabbits[64,91,97,103] particularly in the V-line, Baladi Black and Baladi Red breeds, and variation in expression profiles of these genes in different breeds of rabbits has not been studied. Consequently, the objective of the current study was to characterize the genetic structure of V-line, Baladi Black and Baladi Red breeds of rabbit using DNA sequencing to identify polymorphisms at loci of functional genes encoding *FGF5, PGAM2, TLR2* and *IL10*. A further aim of the study was to link the genetic structures with the expression profiles of these genes by using real time PCR.

Materials and Methods

Ethics Statement. The collection of samples and care of rabbits used in this study followed guidelines of Mansoura University and the protocol of the study was approved by the Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University (code R/32).

Rabbit breeds. This study evaluated the genetic structures of 3 rabbit breeds (V-line, Baladi black, and Baladi Red) and the probability of connecting variation in these genetic structures to the expression profiles of growth and immune genes. The Animal Science Department (UPV, Valencia, Spain), selected V line as a synthetic material for litter size at weaning. The V-line was used in this study due to its unique characteristics. One such characteristic is Valencia's long history of selection[34] in an area whose climatic conditions are similar to the Nile delta in Egypt. Another feature is that V-line rabbits have been tested in hot climates such as Adana in Turkey or Zagazig in Egypt, and this line demonstrated better performance than other exotic breeds, as stated at the First International Hot Climate Rabbit Development Conference in Cairo[98] and the 6th World Rabbit Congress in Toulouse.[84] Recent findings obtained in Saudi Arabia for V-line rabbits have reinforced the excellent heat adaptability of this breed.[51]

Baladi is an Egyptian breed resulting from a crossbreed of native rabbits with Flemish Giants for many generations at The Stations of Poultry Breeding Section of the Ministry of Agriculture.[8,32] The Baladi breed was created as a heat-resistant breed that can cope with the Egyptian climate and is primarily used for meat production. A breeding strategy used for the development of 3 native strains: Baladi Red, Baladi White and Baladi Black aims favoring of highest growth and immune breed.[52]

This study used 180 healthy rabbits (n = 60 of each breed). The 60 rabbits of each breed consisted of 15 males, representing 25% of the total group, and 45 females, representing the remaining 75%. Rabbits belonged to the El-Serw Animal Production Research Station, Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. Rabbits were 3 mo old and their body weights ranged from 2.0 to 2.7 kg.

Housing. Rabbits were housed in open-sided hutches with wire bottoms. Breeding rabbits were housed separately in galvanized wire hutches (40 × 60 × 50 cm) fitted with a watering nipple system and a manual feeder. A metal nest box (40 × 40 × 40 cm) was attached to the doe's cage for kindling and nursing kits. Diazinon 20% and bashbori fire were routinely used to wash cages and nest boxes before and after each kindling to eliminate mange. Rabbits infested with mage were injected with Ivomac (Ivermectin injection 2%) biweekly for nonpregnant does and kits, while Benzylbenzoate cream (Pharanoia, India) was used for pregnant does. Urine and feces were cleaned daily every morning. Environmental temperature was maintained at about 27 °C (80.6 °F). Ventilation and fresh air were provided by fans to minimize the accumulation of ammonia in the building. In the rabbitry, photoperiod was 16L: 8D. Breeding rabbits were housed individually in galvanized wire hutches (40 × 60 × 50 cm) fitted with a watering nipple system and a manual feeder.

Feeding and watering. Commercial pelleted rations (Kent rabbit ration, Egypt) containing crude protein (not less than 18.5%), crude fiber (11.5%) and lipids (2.5%) were fed to rabbits of all ages conforming with National Research Council (NRC).[60] Fresh tap water was supplied ad libitum. Just before weaning, prophylactic antibiotics and anticoccidia medications were supplied in drinking water for 3 to 5 d. Kits were injected every 2

wk with multivitamins (Oasis, Egypt) until weaned to avoid any shortages and counter declining immunity associated with weaning. In addition, injectable vit-E and selenium were administered for fertility enhancement. Bucks and does (male and female) were clinically healthy and were vaccinated against Pasteurellosis and infectious rabbit hemorrhagic disease at 6 and 10 wk, respectively.

Doe management and mating. Mating was done randomly; however, parent-offspring, full sibling, and half sibling mating was avoided. With a ratio of 1 buck to 3 does from each breed, each doe was mated with the corresponding buck of the same breed. Each doe was palpated to determine pregnancy at 10 d of mating and those who were diagnosed as nonpregnant were returned to the same buck. On day 27 of pregnancy, nest boxes were supplied with rice straw to assist the doe in building a nest for her offspring.

Litter management. Immediately after birth, litter checks were carried out and dead kits were withdrawn. In addition, the numbers of live and dead kits was recorded, and all kits were weighed. Cool kits were warmed with warm towels quickly as possible in winter. All litters were examined regularly from birth; dead kits were removed as soon as they were identified. At the age of 4 wk, litters were weaned, ear tagged and separated in cages. Kits were raised under similar conditions as the parents.

Experimental samples. Blood samples were collected aseptically from the ear vein in all rabbits of each breed into tubes containing disodium EDTA as an anticoagulant for DNA extraction. At 3 mo, all females were numbered, and 20 randomly selected females were euthanized for collection of muscle and spleen samples. Females were euthanized by IP injection of large dose (20 to 60 mg/kg) of sodium thiopental (Archimedes, France).[42] Only females were used for gene analysis. Collected tissue samples were washed in phosphate buffer saline (PBS), snap frozen in liquid nitrogen and stored at -80 °C for quantification of gene expression.

DNA extraction. Extraction of the genomic DNA from whole blood was done using Gene JET whole blood genomic DNA extraction kit according to the manufacturer's recommendations (Thermo scientific, Lithuania). Quality, purity and concentration of DNA were screened by Nanodrop, (Uv-Vis spectrophotometer Q5000) for further analysis.

Polymerase Chain Reaction (PCR). PCR was carried out for amplification of fragments for exon 3 of *FGF5* (255-bp), exon 1 of *PGAM2* (488-bp), part of coding sequence (CDS) of *TLR2* (683-bp), and exon 4 of *IL10* (255-bp) genes. The primers for *FGF5, TLR2,* and *IL10* genes were based on previous studies.[64,91,103] Little information is available on the rabbit *PGAM2* gene; therefore, primer sequences for *PGAM2* were designed according to the PubMed published sequence of *Oryctolagus cuniculus* (XM_002713845.3). The primers used in the amplification are shown in Table 1. The PCR mixture was performed with a final volume of 100 µL in a thermal cycler. Each reaction volume contained 6 µL DNA, 41 µL H_2O (dd water), 50 µL PCR master mix (Jena Bioscience, Germany), and 1.5 µL of each primer. The reaction mixture was subjected to an initial denaturation temperature of 94 °C for 4 min. The cycling consisted of 30 cycles at 94 °C for 1 min for denaturation, 1 min at annealing temperatures (as shown in Table 1), I min at 72 °C for extension, and a final extension at 72 °C for 7 min. Samples were then held at 4 °C. Representative results were detected by agarose gel electrophoresis and fragment patterns were then visualized under UV using a gel documentation system (Gel Doc and Alpha-chem Imager, USA).

DNA sequencing and polymorphism detection. Before DNA sequencing, primer dimers, nonspecific bands, and other impurities were removed. As described previously,[10] purification of PCR products with target bands was carried out using a PCR purification kit according to manufacturer recommendations (Bioscience # pp-201×s/ Jena, Germany). Quantification of PCR product was carried out using Nanodrop (Uv-Vis spectrophotometer Q5000/USA) to yield high product and to ensure adequate concentrations and purity of the PCR products.[9] To detect SNPs in the investigated genes of the 3 breeds, PCR products containing the target band were DNA sequenced in forward and reverse directions using ABI 3730XL DNA sequencer (Applied Biosystem, Foster City, CA), using the enzymatic chain terminator technique developed previously.[77]

Analysis of DNA sequencing data was carried out by chromas 1.45 and blast 2.0 software.[6] Differences were classified as SNPs between PCR products of investigated genes and as reference sequences available in GenBank. Based on data alignment of DNA sequencing, variation of amino acid sequence of the investigated genes between the 3 breeds was performed using the MEGA4 software package.[82]

Total RNA extraction, reverse transcription and quantitative real time PCR. Total RNA was extracted from muscle and spleen tissues in the randomly selected 20 females of the 3 breeds using Trizol reagent based on manufacturer instructions (RNeasy Mini Ki, Catalogue no.74104). The amount of extracted RNA was quantified and quality determined using NanoDrop ND-1000 Spectrophotometer. The cDNA of each sample was synthesized according to the manufacturer's protocol (Thermo Fisher, Catalog no, EP0441). Absolute quantification of mRNA level of *FGF5*, *PGAM2*, *TLR2*, and *IL10* was performed by real-time PCR using SYBR Green PCR Master Mix (Quantitect SYBR green PCR kit, Catalog no, 204141). Primer sequences were designed according to the PubMed published sequence of *Oryctolagus cuniculus* as shown in Table 2. The housekeeping gene *ACTB* was used as a constitutive control for normalization. The reaction mixture was carried out in a total volume of 25 µL consisted of 3 µl total RNA, 4 µL 5× Trans Amp buffer, 0.25 µL reverse transcriptase, 0.5 µL of each primer, 12.5 µL 2× Quantitect SYBR green PCR master mix and 8.25 µL RNase free-water. The final reaction mixture was placed in a thermal cycler, (BioRadD, Japan) with the following program: reverse transcription at 50 °C for 30 min, primary denaturation at 94 °C for 10 min, 40 cycles of denaturation at 94 °C for 15 s, annealing temperatures as shown in Table 2, and extension at 72 °C for 30 s. At the end of the amplification phase, a melting curve analysis was performed to confirm the specificity of the PCR product. ΔCT of each sample was calculated for *FGF5, PGAM2*, *TLR2,* and *IL10* genes using threshold cycle (CT) values that were normalized to those of the *ACTB* gene. Lower ΔCT indicated increased expression.[58,67]

Statistical analysis. In this study, the statistical hypothesis was:

Ho: SNPs and expression profile changes in *FGF5, PGAM2*, *TLR2,* and *IL10* genes do not characterize V-line, Baladi Black and Baladi Red breeds

HA: SNPs and expression profile changes in *FGF5, PGAM2*, *TLR2,* and *IL10* genes characterize V-line, Black Baladi and Baladi Red breeds

Data were organized, summarized and analyzed using SPSS version 23, USA. χ^2 test ($\chi 2$) to compare frequencies of SNPs in different breeds of rabbit to determine the most dominant SNP. A discriminant analysis model was then carried out to check the significance of different determinants to discriminate and classify each type of gene as dependent variables using identified SNPs, and gene status as an independent variable. A second linear discriminant analysis model was used to discriminate the 3 breeds as dependent variable using gene type and gene

Table 1. Forward and reverse primer sequence, length of PCR product and annealing temperature for ***FGF5***, *PGAM2, TLR2* and *IL10* genes

Primer	Forward	Reverse	Annealing temperature (°C)	Length of PCR product (bp)	Reference
FGF5	5′ CCTATGCCTCAGCAATACATAGAACT -3′	5′- ATCCAAAGCGAAACTTGAGTCTG -3′	58 °C	288–bp	45
PGAM2	5′-GGCTGGACCGTTATAAGTGG -3′	5-′ CCTTGCTGATGGAGGTGTAG -3′	64 °C	488–bp	Current study
TLR2	5′ GGAAGCCTTTATGCCTTTGC -3′	TTCTCGCAGGCTGAATTTTT -3′	55 °C	683–bp	75
IL10	5′ TGACAGCCAAGGTCATTAACA -3′	5′ CGGGGAGCAGTCATTTAGAA -3′	62 °C	255–bp	65

Table 2. Oligonucleotide primers sequence, accession number, annealing temperature and PCR product size of *FGF5*, *PGAM2, TLR2* and *IL10* genes

Gene	Primer	Product length (bp)	Annealing temperature (°C)	Accession number	Source
FGF5	F5′ACAAACGAGGGAAAGCAAAG 3′ R5′CAGTAACAGTAAAGGAAAGT3′	120	56	KP682502.1	Current study
PGAM2	F5′ GCCACCATGGCCACCCACCG-3′ R5′ TCGGCCCCCTTCTCGCTCAG-3′	113	60	XM_002713845.3	Current study
TLR2	F5′TCCCAGACCTTTTAAGTTCT3′ R5′TCGGTGATTTTCACACTTCT3′	139	58	NM_001082781.1	Current study
IL10	F5′AAACAAGAGCAAGGCAGTGG3′ R5′CCCGGGGCATCACCTGCCCA3′	73	60	DQ437508.2	Current study

expression profile as predictors for classification process. The discriminant statistical model used for this analysis was:

$$DF = V1X1 + V2X2 + V3X3 + ... + VIXI$$

Where DF = discriminate function (score) of grouping variables, V = the standardized discriminant coefficient or loadings for the clinical signs (predictors), X = respondent's score for the clinical signs, I = the number of predictor variables.

The discriminant function coefficients V or standardized form β indicate the partial contribution of each clinical signs to the discrimination process. One way analysis of variance (ANOVA) and univariate general linear model were used to compare mean of gene expression between different breeds. Data are presented as means ± SEM (standard error means) and results were considered significant at $P < 0.05$.

Results

Molecular characterization of FGF5, PGAM2, TLR2 and IL10 genes. DNA sequencing of *FGF5* gene (255-bp) revealed 4 SNPs (submitted to GenBank with accession numbers gb|MT648833|, gb|MT648834|, gb|MT648835|, and gb|MT648836|) (Table 3). Out of the 4 SNPs, 3 characterized a number of Baladi Black rabbits, and 1 was specific for a number of Baladi Red rabbits. Nucleotide sequence variation of *FGF5* (288-bp) among the 3 breeds and reference sequences in GenBank (KP682502.1) confirmed all 4 identified SNPs. (Figure S1)

Regarding *PGAM2*, the DNA sequence of 488-bp elicited a monomorphic pattern (submitted to GenBank with accession number gb|MT675101|). (Figure S2) Nucleotide sequence alignment showed no variation among sequences of the 3 breeds. However, DNA sequencing of the *TLR2* gene (683-bp) revealed 25 SNPs (submitted to GenBank with accession numbers gb|MT664824|, gb|MT664825|, gb|MT664826|, gb|MT664827|, gb|MT664828|, gb|MT664829|, and gb|MT664830|) (Table 4). Out of 25 SNPs, 3 were specific for a number of V-line rabbits, 2 for a number of Baladi Red rabbits and 20 for a number of Baladi Black rabbits. Nucleotide sequence variation of *TLR2* gene (683-bp) among the 3 breeds and reference sequences available in GenBank (gb|NM_001082781.1|) confirmed all identified 25 SNPs. (Figure S3)

For *IL10*, DNA sequencing of 288-bp identified 62 SNPs among the 3 breeds (submitted to GenBank with accession number gb|MT684773|) (Table 5). Among the identified 60 SNPs, 36 were found in a number of Baldaldi Red rabbits, one ina number of V-line rabbits, 10 in a number of Baladi Black rabbits, and 15 in both Baladi Red and Baladi Black breeds. Nucleotide sequence variation of *IL10* gene (255-bp) among the 3 breeds and reference sequences available in GenBank (gb|DQ437508.2L|) confirmed all identified 62 SNPs. (Figure S4)

A significant difference ($P < 0.05$) was detected in frequencies of *FGF5*, *TLR2* and *IL10* genes among the 3 breeds by using χ^2 analysis to compare distributions of identified SNPs between V-line, Baladi Black and Baladi Red breeds. The identified SNPs and SNP status (0 = absent, 1= present) were used as variable loadings in linear discriminant analysis (LDA) for classification of the specific genes for each breed in the study. Overall, 75% of the model was correctly classified. Of the identified SNPs, 100% (12 of 12) were classified as the *FGF5* gene, 63% (121 of 192) were classified as the *IL10* gene and 100% (72 of 72) were classified as the *TLR2* gene. In the first LDA function, SNP status was the best predictor for breed classification with an estimate of -0.105. The second LDA function showed that breed was also the best predictor for gene classification with as estimate of 1.00 (Table 6).

Gene expression pattern. Levels of *FGF5, PGAM2, TLR 2* and *IL10* gene expression were significantly higher in V-line than in either Baladi Black or Baladi Red, as indicated by the lower values of ΔCT (Figure 1). A significant ($P < 0.05$) upregulation of *TLR2* and *IL10* was detected in Baladi Black as compared with Baladi Red, as indicated by significantly lower ΔCT values in Baladi Black. A significant ($P < 0.05$) interaction was found between gene type and breed when analyzing mRNA levels. Among all evaluated genes, the highest mRNA level was found for *FGF5* in V-line (-0.55600), and the lowest mRNA level was found for *TLR2* in Baladi Red (2.8860) as determined

Table 3. Distribution of SNPs of *FGF5* gene in V-line, Baladi Black and Baladi Red rabbits

SNPS	V-line	Baladi black	Baladi red	Total	Type of mutation	Amino acid number and type	Chi value	*P*–value
A32G	—-	36	—-	36/180	Nonsynonymous	11 E to G	90.00	<0.0001
C34A	—-	—-	37	37180	Nonsynonymous	12 Q to K	93.15	<0.0001
G38T	——	26		26/180	Nonsynonymous	13 G to V	60.77	<0.0001
G38A	——	33	——	33/180	Nonsynonymous	13 G to D	80.82	<0.0001

D= Aspartic acid; E= Glutamic acid; K= lysine and V= Valine.

Table 4. Distribution of SNPs of *TLR2* gene in V-line, Baladi Black and Baladi Red rabbits

SNPs	V-line	Baladi black	Baladi red	Total	Type of mutation	Amino acid number and type	Chi value	*P*–value
G28C	22	——	——	22/180	Nonsynonymous	10 G to R	50.12	<0.0001
G28T	38	——	——	38/180	Nonsynonymous	10 G to W	96.33	<0.0001
T35A——16	——	——	16	16/180	Nonsynonymous	12 M to K	35.12	<0.0001
A48C–15–	——	15	——	15/180	Synonymous	16 G	32.72	<0.0001
G61C–18–	——	18	——	18/180	Nonsynonymous	21 E to Q	40.00	<0.0001
C111G–18–	——	18	——	18/180	Synonymous	37 G	40.00	<0.0001
T411G——16—-	——	16	——	16/180	Synonymous	137 G	35.12	<0.0001
G427A–18–	——	18	——	18/180	Nonsynonymous	143 M to W	40.00	<0.0001
A485C–16–	——	16	——	16/180	Nonsynonymous	162 V to C	35.12	<0.0001
A487C–18–	——	18	——	18/180	Nonsynonymous	163 I to L	40.00	<0.0001
A493C–18–	——	18	——	18/180	Nonsynonymous	165 K to G	40.00	<0.0001
A504T–15–	——	15	——	15/180	Nonsynonymous	168 L to F	32.72	<0.0001
G511A–16–	——	16	——	16/180	Nonsynonymous	171 D to N	35.12	<0.0001
A531C–19–	——	19	——	19/180	Synonymous	177 S	42.48	<0.0001
T535C–15–	——	15	——	15/180	Nonsynonymous	179 S to P	32.72	<0.0001
G538A–19–	——	19	——	19/180	Nonsynonymous	180 E to K	42.48	<0.0001
T545G–20–	——	20	——	20/180	Nonsynonymous	182 V to G	45.00	<0.0001
A556C–18–	——	18	——	18/180	Nonsynonymous	186 T to P	40.00	<0.0001
T560G–15–	——	15	——	15/180	Nonsynonymous	187 V to G	32.72	<0.0001
T570G–19–	——	19	——	19/180	Nonsynonymous	190 S to R	42.48	<0.0001
A594T–17–	——	17	——	17/180	Synonymous	198 S	37.55	<0.0001
C597T–17–	——	20	——	20/180	Synonymous	199 S	45.00	<0.0001
A600C	——	22	——	22/180	Synonymous	200 S	50.12	<0.0001
T636C–22	——	——	18	18/180	Synonymous	212 S	40.00	<0.0001
G673C	26	——	——	26/180	Nonsynonymous	225A to P	60.77	<0.0001

A= Alanine; C= Cysteine; D= Aspartic acid; E= Glutamic acid; F= Phenylalanine; G= Glycine; I= Isoleucine; K= lysine; L= Leucine; M= Methionine; *n* = Asparagine; *P* = Proline; R= Argnine; S= Serine; T= Threonine; W= Tryptophan, and V= Valine.

respectively by significantly ($P < 0.05$) lower and higher values of ΔCT (Table 7).

Gene expression pattern and type of gene were used as variable loadings in LDA for classification of breed. Overall, 73% of the model correctly classified. Among the examined samples, 100% (20/20) were classified as V-line, 55% (11/20) were classified as Baladi Black, and 65% (13/20) were classified as Baladi Red (Table 8).

Discussion

Rabbits are an important meat species and are a necessary source of food in some countries. Molecular techniques for the detection of markers linked to production traits have become an important tool in the identification of rabbit species.[26] In breeding programs, data from associate genes has potential to improve the accuracy of selection for most economic quantitative traits.[41] Consequently, the use of genotype-based information provides a rapid and accurate way to improve the genetic makeup of rabbits, especially in the case of high-cost phenotypic assessment of economic traits in rabbits. Moreover, one of the major challenges when selecting for disease resistant animals is the exposure to pathogens. Challenging breeding animals is impractical due to the inability guarantee to ensure disease containment. Therefore, identification of DNA markers for disease resistance is widely studied and many useful SNPs have been reported.[61]

The rapid development of next-generation sequencing (NGS) technology has resulted in the availability of massive rabbit genome and transcriptome datasets. Whole-genome sequencing of a wide range of wild populations and domestic breeds was also performed to understand the genetic basis of rabbit domestication.[15] The whole genome and transcriptome of the most popular experimental rabbits, especially those related to hyperlipidemia and atherosclerosis, were provided in previous studies.[93] RabGTD was used to collect, process and display all published data of the rabbit genome and transcriptome generated by NGS.[104] The authors of that study collected the genome data from 79 samples, including 33 samples of wild

Table 5. Distribution of SNPs of *IL10* gene in V-line, Baladi Black and Baladi Red rabbits

SNPs	V-line	Baladi black	Baladi red	Total	Type of mutation	Amino acid number and type	Chi value	*P*–value
C45T	—	—	16	16/180	Synonymous	15 T	35.12	<0.0001
C55T	—	—	31	31/180	Nonsynonymous	19 P to S	74.89	<0.0001
G60T	—	—	32	32/180	Synonymous	20 V	77.84	<0.0001
A61G	—	—	32	32/180	Nonsynonymous	21 L to E	77.84	<0.0001
T135C	—	—	17	17/180	Synonymous	45 G	37.54	<0.0001
G136T	—	—	46	46/180	Nonsynonymous	46 A to S	123.6	<0.0001
C137G	—	—	30	30/180	Nonsynonymous	46 A to C	72.00	<0.0001
G139T	—	—	30	30/180	Nonsynonymous	47 A to S	72.00	<0.0001
A141G	—	—	44	44/180	Synonymous	47A	116.5	<0.0001
A145T	—	—	32	32/180	Synonymous	49S	77.84	<0.0001
G146C	—	—	32	32/180	Synonymous		77.84	<0.0001
G149T	—	—	14	14/180	Nonsynonymous	50 R to L	30.36	<0.0001
G149C	—	—	46	46/180	Nonsynonymous	50 R to P	123.6	<0.0001
G153C	—	—	44	44/180	Synonymous	51R	116.5	<0.0001
A158C	—	35	14	49/180	Nonsynonymous	53 Q to P	52.21	<0.0001
A158G	—	—	33	33/180	Nonsynonymous	53 Q to R	80.81	<0.0001
C161G	—	—	18	18/180	Nonsynonymous	54 A to G	40.00	<0.0001
C161A	—	—	39	39/180	Nonsynonymous	54 A to E	99.57	<0.0001
A162G	—	36	15	51/180	Synonymous	54A	53.68	<0.0001
G163C	32	—	—	32/180	Nonsynonymous	55 V to L	77.84	<0.0001
T164A	—	38	18	56/180	Nonsynonymous	55V to E	56.20	<0.0001
G169T	—	—	33	33/180	Nonsynonymous	57 E to L	80.81	<0.0001
A170T	—	—	31	31/180	Nonsynonymous		74.89	<0.0001
C174T	—	34	16	50/180	Synonymous	58 P	48.07	<0.0001
G176C	—	34	18	52/180	Nonsynonymous	58 G to A	46.94	<0.0001
C177G	—	34	18	52/180	Nonsynonymous	59 G to A	46.94	<0.0001
C177T	—	—	30	30/180	Synonymous	59 G	72.00	<0.0001
A178G	—	—	32	32/180	Nonsynonymous	60 S to G	49.15	<0.0001
T180A	—	37	17	54/180	Nonsynonymous	60 S to R	54.44	<0.0001
C183G	—	37	17	54/180	Synonymous	61 V	54.44	<0.0001
A184T	—	—	33	33/180	Nonsynonymous	62 T to S	80.81	<0.0001
C188T	—	38	58	96/180	Nonsynonymous	63 A to V	116.2	<0.0001
T189G	—	—	18	18/180	Synonymous	63 V	40.00	<0.0001
C191T	—	34	15	49/180	Nonsynonymous	64 S to F	48.85	<0.0001
A192T	—	—	18	18/180	Synonymous	64 S	40.00	<0.0001
A192C	—	35	14	49/180	Nonsynonymous	64 S to F	52.21	<0.0001
G193C	—	14	—	14/180	Nonsynonymous	65 G to R	30.36	<0.0001
G193A	—	—	32	32/180	Nonsynonymous		77.84	<0.0001
C195A	—	—	32	32/180	Nonsynonymous		77.84	<0.0001
A196G	—	36	16	52/180	Nonsynonymous	66 S to A	52.78	<0.0001
G197C	—	36	16	52/180	Nonsynonymous		52.78	<0.0001
G197A	—	36	34	70/180	Nonsynonymous	66 S to N	57.41	<0.0001
G200C	—	33	15	48/180	Nonsynonymous	67 R to T	46.53	<0.0001
A203G	—	14	—	14/180	Nonsynonymous	68 E to G	30.36	<0.0001
C205A	—	—	17	17/180	Nonsynonymous	69 H to N	37.54	<0.0001
G210T	—	—	56	56/180	Nonsynonymous	70 L to F	162.59	<0.0001
T212G	—	38	—	38/180	Nonsynonymous	71 F to C	96.33	<0.0001
T218A	—	—	33	33/180	Nonsynonymous	73 V to E	80.81	<0.0001
A221G	—	34	—	34/180	Nonsynonymous	74 K to R	83.83	<0.0001
G222A	—	34	—	34/180	Nonsynonymous	74 K to R	83.83	<0.0001
T223A	—	32	—	32/180	Nonsynonymous	75 C to S	77.84	<0.0001
C226T	—	—	47	47/180	Nonsynonymous	76 L to F	127.22	<0.0001
T228G	—	34	—	34/180	Synonymous	76 L	83.83	<0.0001
T229G	—	15	—	15/180	Nonsynonymous	77 W to G	32.72	<0.0001

Table 5. Continued

SNPs	V-line	Baladi black	Baladi red	Total	Type of mutation	Amino acid number and type	Chi value	*P*–value
G233T	——	——	33	33/180	Nonsynonymous	78 G to V	80.81	<0.0001
G234T	——	——	33	33/180	Nonsynonymous		80.81	<0.0001
T235G	——	34	——	34/180	Nonsynonymous	79 F to V	83.83	<0.0001
T236C	——	——	14	14/180	Nonsynonymous	79 F to S	30.36	<0.0001
T243A	——	——	48	48/180	Nonsynonymous	81 N to K	130.91	<0.0001
A245C	——	15	——	15/180	Nonsynonymous	82 A to D	32.72	<0.0001
C249T	——	——	45	45/180	Synonymous	83C	120.00	<0.0001
T250G	——	——	45	45/180	Nonsynonymous	84 S to A	120.00	<0.0001

A= Alanine; C= Cysteine; D= Aspartic acid; E= Glutamic acid; F= Phenylalanine; G= Glycine; I= Isoleucine; K= lysine; L= Leucine; M= Methionine; *n* = Asparagine; *P* = Proline; Q= Glutamine; R= Argnine; S= Serine; T= Threonine; W= Tryptophan, and V= Valine.

Table 6. Discriminant analysis for classification of type of genes and rabbit breeds

Classification results						
			Predicted group membership			
	Gene		*FGF5*	*IL10*	*TLR2*	Total
Original	Count	*FGF*	12	0	0	12
		TLR2	0	0	72	72
		IL10	42	121	29	192
	%	*FGF*	100	0	0	100
		TLR2	0	0	100	100
		IL10	22	63.	15	100

rabbits and 46 samples of domestic rabbits. The restriction-site-associated DNA sequencing (RAD-seq) approach was also employed to comprehensively discover genome-wide SNPs of 104 rabbits from 4 Chinese indigenous breeds.[74]

Genome-wide association studies (GWAS) represent a powerful approach to correlating SNPs and functional genes with quantitative traits.[59] SNPs associated with a specific trait can be considered as molecular markers for use in genomic selection[39] and as genetic markers.[79] The most important step in a GWAS is to acquire high-quality SNPs at the genome-wide level. Therefore, as a first step, using GWAS to identify SNPs associated with economically important traits would provide a basis for further improving the breeding efficiency of rabbits. Despite the great success of GWAS in animal science,[81] a lack of large-scale research studies linking important economic traits to candidate genes in rabbit has been limiting. Application of a GWAS identified using several candidate genes affecting the total number of teats in female European White rabbits (*Oryctolagus cuniculus*).[12] The genomic regions that are associated with intramuscular fatty acid composition in rabbits were also identified previously.[54,80] Another study carried out a GWAS for feed efficiency in cage-raised rabbits under full and restricted feeding.[76] Previous GWAS studies have also identified genetic variants associated with plausible economic traits in rabbits.[17,99]

The current study used PCR-DNA sequencing for molecular characterization of fragments of exon 3 of *FGF5* (288-bp), exon 1 of *PGAM2* (488-bp), part of CDS of *TLR2* (683-bp), and exon 4 of *IL10* (255-bp) in V-line, Baladi Black and Baladi Red breeds of domestic rabbits. A monomorphic pattern of the DNA sequence for *PGAM2* was found in all 3 breeds. SNPs were detected in *FGF5*, *TLR2*, and *IL10* genes. The identification of some SNPs specific to each breed is probably related to the founder effect associated with origin, history, evolution, and genetic constituents of each breed.[14]

This study is a novel investigation of the genetic structure of V-line, Baladi Black and Baladi Red breeds of rabbit using SNPs at the loci of functional genes encoding *FGF5, PGAM2, TLR2* and *IL10*. However, little information is available on the molecular characterization of such genes in other rabbit breeds.[64,91,97,103] In addition, results of experimental work on the molecular characterization of these genes are controversial. Previous work has reported gene polymorphisms, but has focused on other genetic markers such as restriction fragment length polymorphism(RFLP) and single strand conformation polymorphism (SSCP).[64,75] The current study explores polymorphisms via SNP genetic markers; this information could advance previous achievements in conservation decisions, biodiversity assessment, and genetic characterization of breeds.[37] The application of SNP genetic markers should provide a more comprehensive understanding of the molecular basis of functional diversity in rabbits. SNPs are also important in the search for linkages between a marker with a specific location in the genome and an unknown gene locus. Such associations allow a phenotypic effect to be assessed by identifying its genetic basis.[78] Our study also demonstrated that identified SNPs in each gene and SNP status (absent or present) can be used as variable loadings in LDA for classification of specific genes in the 3 breeds. LDA showed that breed was a best predictor for gene classification with estimate (1.00).

A previous study performed PCR using specific primers that were similar to those used in our study in order to amplify a 288-bp for *FGF5* exon 3 in 70 rabbits.[64] However, that study tested genetic polymorphisms using RFLP as a genetic marker. In that study, PCR-*TaqI* of 288-bp of *FGF5* showed a monomorphic pattern (TT genotype) in all tested animals that was confirmed by DNA sequencing.[64] In the current study, DNA sequencing of 288-bp of *FGF5* gene revealed 4 novel SNPs (submitted to GenBank with accession numbers gb|MT648833|, gb|MT648834|, gb|MT648835|, and gb|MT648836|) as compared with a matched reference sequence (KP682502.1). A previous study by others investigated polymorphisms of *PGAM2* in 3 breeds of rabbits (Tianfu black, Ira, and Champagne).[97] Three SNPs were identified by direct sequencing of 20 random individuals from the 3 breeds, including c.-10C

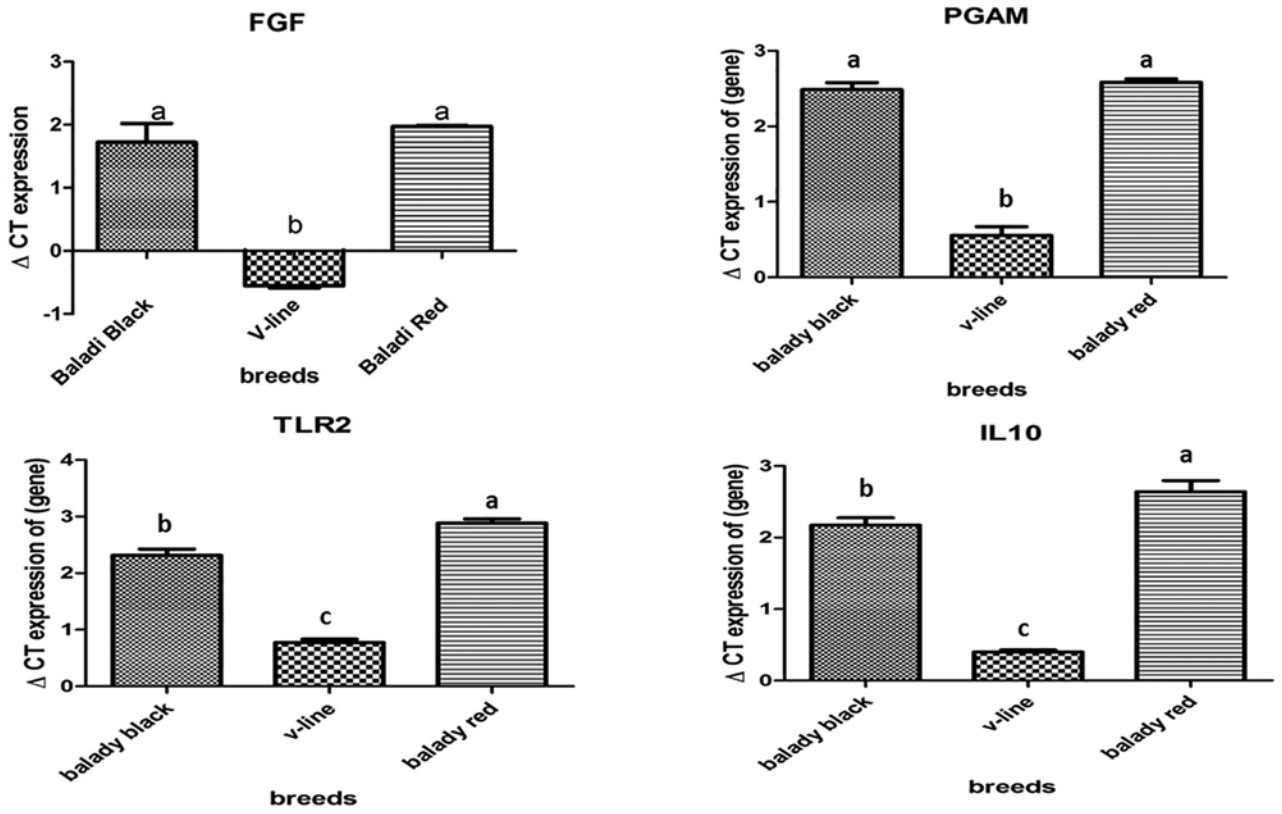

Figure 1. Relative *FGF5*, *PGAM2*, *TLR2* and *IL10* genes expression in different rabbit breeds. β-actin gene was used as a reference gene to normalize data and shown as ΔCT ± SE. Lower ΔCT values indicate increased expressions.

> T, c.195C > T, and c.414+17C > T. The c.195C > T SNP was also genotyped by PCR-RFLP in a total of 222 rabbits. In our study, DNA sequencing of 488-bp of *PGAM2* gene revealed a monomorphic pattern in the 3 breeds (submitted to GenBank with accession number gb|MT675101|) with 100% identity as compared with matched reference sequence (XM_002713845.3). The differences between our study and pervious work may be attributed to the genetic background differences between the studied animals. Another possibility may be that genetic polymorphisms were investigated on different fragments of *PGAM2* gene by PCR-DNA sequencing assessment.

Similarly, another group has previously sequenced the coding regions of the *TLR2* gene in 18 individual rabbits of 5 breeds.[103] The authors reported 11 SNPs in the *TLR2* gene with 8 novel SNPs as compared with a matched reference sequence (NM_001082781.1). However, our study found 25 novel SNPs in the coding region of *TLR2* gene (submitted to GenBank with accession numbers gb|MT664824|, gb|MT664825|, gb|MT664826|, gb|MT664827|, gb|MT664828|, gb|MT664829|, and gb|MT664830|) as compared with a matched rabbit *TLR2* reference sequence. For *IL10*, SNPs on 5 exons of the *IL-10* gene were genotyped in 204 healthy rabbits via PCR-SSCP and DNA sequencing.[91] Two SNPs (A1435G and G1519A, both synonymous mutations) and 6 genotypes (AA, BB, CC, AB, AC and BC) were found on exon 3 and one SNP (T base insertion between loci 2532 and 2533, which caused a frameshift mutation), and 3 genotypes (OO, TT and TO) were present on exon 4.[91] Our study found 63 SNPs by DNA sequencing of *IL10* gene (submitted to GenBank with accession number gb|MT684773|). Comparison of the SNPs

Table 7. Gene expression pattern and rabbit breed interaction effect. Data shown as ΔCT ± SEM. Lower ΔCT values indicate increased expressions

	Mean ± SE.		
Gene / breed	Baladi black	V-line	Baladi red
PGAM	$2.49^{bc} \pm 0.09$	$0.56^{fg} \pm 0.11$	$2.58^{ab} \pm 0.04$
FGF	$1.72^{e} \pm 0.30$	$-0.56^{h} \pm 0.03$	$1.98^{de} \pm 0.01$
TLR2	$2.32^{bcd} \pm 0.11$	$0.77^{f} \pm 0.06$	$2.89^{a} \pm 0.07$
IL10	$2.17^{cd} \pm 0.10$	$0.40^{g} \pm 0.03$	$2.64^{ab} \pm 0.16$

Means of different levels having different superscript are significantly different ($P < 0.05$).

Table 8. Discriminant analysis for classification of rabbit breed based on gene expression pattern

Classification results						
			Predicted group membership			
	Breed		Baladi black	V line	Baladi red	Total
Original	Count	Baladi Black	11	0	9	20
		V line	0	20	0	20
		Baladi Red	7	0	13	20
	%	Baladi Black	55	0	45	100
		V line	0	100	0	100
		Baladi Red	35	0	65	100

73% of original grouped cases correctly classified.

with reference sequences available in GenBank (DQ437508.2) revealed that our sequence was novel.

In our study, PCR was carried out for the amplification of fragments of exon 3 of *FGF* (255-bp), exon 1 of *PGAM2* (525-bp), part of coding sequence (CDS) of *TLR2* (683-bp), and exon 4 of *IL10* (255-bp) genes. We found a monomorphic pattern of DNA sequence for the *PGAM2* gene in the 3 breeds. Our data also revealed common SNPs between the 3 rabbit breeds. The monomorphic pattern of *PGAM2* gene, similarity in the remaining part of the amplified fragments in *FGF5*, *TLR2* and *IL10* genes and the common SNPs between the 3 breeds may be attributed to conducting PCR-DNA sequencing on a conserved region that is an exon of investigated genes, allows accurate molecular characterization of genes, and reveals possible physiologic differences between breeds.[35] Other causes may be the single origin of domestication in wild populations, such that the majority of polymorphisms shared and thus transferable among breeds.[7,14]

In the present study, real time PCR was carried out to quantify mRNA levels of *FGF5, PGAM2, TLR2* and *IL10* in the 3 rabbit breeds. Our findings revealed that the expression pattern was higher in V-line breed as compared with Baladi Black or Baladi Red breeds, as shown by the lower values of ΔCT in the v-line breed. LDA revealed that the gene expression pattern and type of gene were variable loadings for classification of breed. Our study is the first reported polymorphism detection of *FGF5, PGAM2, TLR2* and *IL10* mRNA levels in healthy rabbit breeds using real time PCR. Previous studies have explored *FGF5, PGAM2, TLR2* and *IL10* polymorphisms using other genetic markers as RFLP and SSCP.[51,75,83,87] However, our study was designed to overcome the limitations of previous work by investigating polymorphism in gene using SNP genetic marker and gene expression. Our results allow us to accept the alternative Hypothesis (H_A) which stated that genetic variation characterizes the 3 breeds and thus reject null H_o hypothesis. Consequently, *FGF5, PGAM2, TLR2* and *IL10* regulation mechanisms are better understood in the 3 rabbit breeds.

Most FGF family members are glycosaminoglycan binding proteins that have broad mitogenic and cell survival activities.[43] FGF family members are also involved in a variety of biologic processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion.[62] Other reports have identified *PGAM2* as one of the candidate genes affecting body weight and average daily weight gain in rabbits.[97] Therefore, variations in nucleotide sequence in *FGF5* gene as well as muscle mRNA levels of *FGF5* and *PGAM2* genes may account for phenotypic variations in growth traits between the 3 breeds. Our results could explain findings of previous studies that compared growth performance of V-line to Baladi Black and Baladi Red and found that the V-line breed had better growth performance.[2,27,75] However, another study reported a negative association between *FGF5* exon 3 and body weight in rabbits.[64] Genetic polymorphisms of *FGF5* gene were also associated with hair length in different animals.[22,24,44,50,55,56,57] Recently, CRISPR modification of goats to artificially knock out the *FGF5* gene was shown to result in a higher wool yield, without any loss of fertility or other negative effects on the animals.[92]

The ectodomains of TLR molecules consist mainly of LRR domains, which are essential structures that distinguish PAMPs.[11] Comparisons of SNP distribution in *TLR* coding sequences in different animals revealed that sequences encoding LRR domains are particularly rich in nonsynonymous SNPs.[78,83,94] Nonsynonymous SNPs in LRR domains may dramatically alter the ability of the molecule to identify extracellular pathogens.[31] Furthermore, polymorphisms in the sequences encoding ectodomains that are involved in pattern recognition could improve recognition of various kinds of PAMPs originating from rapidly evolving pathogens.[16,53] Amino acid substitutions that alter amino acid polarity may also have an impact on host immune responses and resistance to disease.[78]

In the same regard, previous studies found that *IL10* SNPs directly affect *in vivo IL10* expression and thus are closely related to many diseases.[33,47,56] For example, one study showed that *IL10* knockout mice were susceptible to spontaneous inflammatory bowel disease,[95] indicating that *IL10* expression maintained immune homeostasis. Thus, we speculate that *IL10* levels in healthy individuals may affect immunity. Consequently, we can suggest that SNPs of *TLR2* and *IL10* genes with their subsequent alterations in amino acids and variations in spleen mRNA levels of *TLR2* and *IL10* genes may account for phenotypic variations in the immune traits between the 3 breeds, with V-line rabbits potentially having favorable immune traits as compared with Baladi Black and Baladi Red. Our findings could explain the results of another study[75] showing that V-line rabbits had a better blood immune profile than did New Zealand White and Gabali rabbits.

Conclusions

Our PCR-DNA sequencing of *FGF5, TLR2 and IL10* genes revealed nucleotide sequence variations in SNPs between V-line, Baladi Black and Baladi Red breeds of rabbits. However, the *PGAM2* gene showed no variations between breeds. The variable expression pattern of *FGF5, PGAM2, TLR2 and IL10* genes in rabbit breeds was also studied using real time PCR analysis, with the V-line breed having higher gene expression than did Baladi Black and Baladi Red breeds. These findings suggest that variation in these genes could provide proxy biomarkers for characterization of rabbit breeds and may allow a rapid and

rigorous selection within and between these breeds. The variability of these markers makes also allows assessment of the predisposition of animals to a specific type of a desired trait. In addition, variation in the expression patterns could be a useful biomarker for studying the growth and immune status of rabbits, allowing prediction of the most susceptible risk time for growth related problems and disease occurrence and building an effective management approach to improving rabbit health through good breeding and vaccination regimens.

Supplementary Materials

Figure S1. Representative DNA sequence alignment of *FGF5* gene (288-bp) among V-line, Baladi Black and Baladi Red rabbit and reference sequence available in GenBank gb | KP682502.1 | . Asterisks represent similarity. V is V-line, BB is Baladi Black and BR is Baladi Red.

Figure S2. Representative DNA sequence alignment of *PGAM2* gene (488-bp) among V-line, Baladi Black and Baladi Red rabbit and reference sequence available in GenBank gb | XM_002713845.3 | . Asterisks represent similarity. V is V-line, BB is Baladi Black and BR is Baladi Red.

Figure S3. Representative DNA sequence alignment of *TLR2* gene (683-bp) among V-line, Baladi Black and Baladi Red rabbit and reference sequence available in GenBank gb | NM_001082781.1 | . Asterisks represent similarity. V is V-line, BB is Baladi Black and BR is Baladi Red.

Figure S4. Representative DNA sequence alignment of *IL10* gene (255-bp) among V-line, Baladi Black and Baladi Red rabbit and reference sequence available in GenBank gb | DQ437508.2L | . Asterisks represent similarity. V is V-line, BB is Baladi Black and BR is Baladi Red.

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