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Association study of polymorphisms in miRNA-1687 with growth traits of chickens

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Abstract

Polymorphisms within microRNAs can lead to phenotypic variations in organisms. The purpose of this research was to investigate the potential impact of the pre-miR-1687 single nucleotide polymorphism (SNP) on the economic characteristics of weight and body size in chickens. The SNP was genotyped using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. A linear mixed model was utilized to analyse the associations between the SNP and chicken body size and growth traits. The SNP in the *pre-miR-1687* gene was correlated with F2 chicken body weight (BW) at birth and at 4, 6, 8, and 10 weeks of age. The SNP in the *gga-miR-1687* gene was correlated with shank length, shank girth, pectoral angle (at 4 weeks), and pelvic breadth (at 8 weeks). Different BW genotypes were observed in the studied flocks. The changes in the secondary structure of pre-miR-1687 and in the free energy values were estimated using online M-fold software. The results serve as a helpful resource for subsequent research on the mechanisms and functions of miRNAs. In addition, the study provides a credible basis for the application of biomolecular technology in poultry breeding.

Keywords: correlation analysis; F2 chicken population; growth trait; pre-miR-1687; single nucleotide polymorphism
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Introduction

MicroRNAs (miRNAs), which are widely expressed in many organisms and tissues in animals and humans, are a novel group of highly conserved, small noncoding RNAs (ncRNAs) that are approximately 18 to 22 nucleotides (nt) in length and act as master regulators of gene expression at the posttranscriptional stage via RNA interference pathways (Kabekkodu *et al*., 2018; Rani and Sengar, 2022; Salim *et al*., 2022). They are major posttranscriptional regulators and perform various functions within cells, including modulation of gene expression. MiRNAs can regulate and control one-third of all protein-coding genes by base-pairing to target mRNAs complementary to the 3′ untranslated region (3′ UTR), resulting in mRNA degradation and inhibition of mRNA translation (Sand *et al*., 2009; Salim *et al*., 2022). MicroRNAs are transcription products derived from endogenous DNA transcripts, and the biogenesis of miRNAs begins at internuclear primary transcripts that may or may not code proteins and are present in either intergenic regions or overlapping genes. In the cell nucleus, almost all primary transcripts are biologically processed into hairpin-like structures, partially duplexed pre-miRNAs (precursor miRNAs), by the RNase III Drosha; pre-miRNAs are usually 55 nt to 80 nt in length and are sent to the cytoplasm by Exportin-5 (a shuttle protein) with the aid of the G-protein, Ran, at the time of bioprocessing. The pre-miRNAs are identified and cleaved by Dicer RNaseIII. They are precisely processed into approximately 18- to 22-nt-long miRNA/miRNA* double strands and bind to the RNA-binding protein (RBP) of the regulatory factor transactivation response element (TRE) in the cytoplasm.

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The RNA-induced silencing complex (RISC) promotes the conjugation of one chain of the miRNA double strand as a mature miRNA to match the matching mRNA sequence in the 3' UTR of a target gene; the other chain of the double strand is degraded (Wang *et al*., 2013; Li *et al*., 2014).

Accumulating evidence confirms that miRNAs are associated with an extremely wide range of biological processes, for example, sperm motility (Kumar *et al*., 2015), embryonic development (Collignon, 2007; Yuan *et al*., 2016), haemopoiesis (Lazare *et al*., 2014; Kotaki *et al*., 2017; Rasko & Wong, 2017), fat deposition (Zhang *et al*., 2016; Qiang *et al*., 2018), cell differentiation (Martin *et al*., 2016; Zhao *et al*., 2020), cell proliferation (Wang *et al*., 2017; Du *et al*., 2020), oocyte maturation (Song *et al*., 2016; Zhang *et al*., 2019a), ovarian follicular development (Imbar and Eisenberg, 2014; Maalouf *et al*., 2016; Zhang *et al*., 2019b), metabolism (Vienberg *et al*., 2017; Alamoudi *et al*., 2018), apoptosis (Fan *et al*., 2016; Akkafa *et al*., 2018), stem cell maintenance (Mehta *et al*., 2015; Luinenburg & de Haan, 2020), and skeletal muscle growth (Diniz and Wang, 2016; Wang *et al*., 2018).

Single-nucleotide polymorphisms (SNPs) located on miRNA-binding sites (miR-SNPs) have an effect on gene expression. Normally, SNPs influence miRNA function by altering miRNA expression levels and relevant targets. Common SNPs occurring in miRNAs can regulate the transcription of pri-miRNA transcripts and the stability or biological processing of pre-miRNAs or pri-miRNAs. The sites of miRNA targets and processing machinery might potentially affect the expression of many genes and pathways and might profoundly interfere with the function of miRNAs, resulting in either an increase or a decrease in mature miRNA levels, including individual phenotypic variations and disease susceptibility (Duan *et al*., 2007; Jazdzewski, 2008; Mencia *et al*., 2009).

Chickens are among the most economically, culturally, and socially important poultry species. Previous research has demonstrated the significant effectiveness of a SNP (AB604331, g.420 C>A) occurring in the gene of the cholecystokinin type A receptor for improving the growth characteristics of Amakusa × Daioh cross chickens (Momoi *et al*., 2021). The mutation, c.-652 C>T, in the promoter region of the ubiquitin carboxyl-terminal hydrolase-L1 gene was identified as being relevant to goose growth performance (Wang *et al*., 2021). A research report found that the SNPs, G244A and A239G, in exon 2 of the growth hormone secretagogue receptor were strongly correlated with feed intake, growth characteristics, and expression of the growth hormone secretagogue receptor gene, growth hormone, and the endogenous growth hormone receptor ghrelin (GHRL) mRNAs (El-Magd *et al*., 2016). SNPs (g.69307744 C>T, g.69340192 G>A, and g.69355665 T>C) had a marked impact on carcass characteristics, such as carcass and semi-eviscerated weight. This evidence showed that the SNP in the *TBC1D1* gene was strongly associated with carcass traits in poultry flocks (Wang, 2014). The growth traits of chickens are among their most important economically important traits.

In the present study, we screened and identified a novel SNP located in the precursor of *Gallus gallus miR-1687* (*gga-miR-1687*) and researched the correlation between the SNP and growth traits, including body weight (BW) and body indices, at multiple growth and developmental stages of chickens of the Gushi × Anka F2 reference family. The influence of polymorphisms in the *pre-miR-1687* gene on the secondary structure and energy of *pre-miRNA* was also predicted and analysed. In general, the data identified the potential application value of *miR-1687* in regulating chicken growth.

Materials and Methods

The related poultry experiments were conducted according to the rules and requirements of the Animal Care & Use Committee of Henan Agricultural University, China (approval ID: 11-0085).

The study design was adapted from Han *et al*. (2011). Briefly, a Gushi × Anka F2 resource population was used that included a total of 860 chickens. The study population previously established by the researchers of the Henan Innovative Engineering Research Center of Poultry Germplasm Resource (Han *et al*., 2012; Shi and Sun, 2017) was used as the source of experimental subjects for research. Specifically, the resource population of F2 chickens was derived by crossing Gushi chickens and Anka broiler chickens. Gushi chickens, a typical, slow-growing, native Chinese chicken breed, exhibit excellent meat characteristics. In contrast, Anka broiler chickens are large-bodied, fast-growing broiler chickens. In other words, a slow-growing breed (Gushi chickens) was crossed with a fast-growing breed (Anka broiler chickens). The F0 chickens (42 grandparents) were derived from two groups: one group (two reciprocal cross families) was generated by mating two Gushi roosters with 12 Anka broiler hens, and the other group (four cross families) was generated by mating four Anka broiler roosters with 24 Gushi hens. As noted earlier, this F2 resource population was established by intercrossing 63 F1 parental hens and seven F1 parental cockerels.

All the experimental chickens experienced identical breeding conditions and were raised in cages until 12 weeks of age, with free access to food and water. The 860 F2 chickens were all humanely slaughtered at 84 days of age. All animal experimental procedures used complied with the related animal care protocol.

The BW and body size traits of each chicken were measured accurately and recorded every two weeks from week 0 (hatching) to 12 weeks of age (i.e., weeks 0, 2, 4, 6, 8, 10, and 12). The primary chicken body size parameters measured at every different stage of growth and development (4, 8 and 12 weeks) were slanting body length, shank length, shank girth, chest depth, chest breadth, breast-bone length, pectoral angle, and pelvic breadth.

A total of 860 genomic DNA samples of the Gushi × Anka F2 chicken reference family were extracted from venous blood by adding the anticoagulant ethylenediaminetetraacetic acid (EDTA) using the phenol–chloroform method with a commercial DNA extraction kit (Tiangen Biotechnology Co. Ltd., Beijing). Considering the standards for genotyping using mass array matrix–assisted laser desorption and ionization/time-of-flight mass spectrometry (MALDI–TOF MS), the exact concentrations of the prepared DNA samples were measured, and the samples were stored at -80 °C for subsequent research. One hundred genomic DNA samples from individual F2 chickens were chosen at will, and equivalent amounts of the samples of each genomic DNA with the same working concentration were pooled. The sequence of the chicken *pre-miR-1687* gene (GenBank: MI0007421), which was located on chromosome 20, was acquired from the online miRBase database (version 19.0). The pooled DNA for the F2 resource population was sequenced by Shanghai Sangon Biotechnology Company to scan for gene polymorphisms. A pair of PCR amplification primers (F: 5′- GCTGATGGTGTCGCGGTGAGC -3′; R: 5′- CTGCATAAAGATGGGAGAAG -3′) for sequencing was designed on the basis of the location in the genome (NC_006107.3) to detect SNPs in the precursor region of *miR-1687*. The primers were applied to amplify the gene sequence, including that of *pre-miR-1687*. The DNA samples were delivered to a reputable testing company for detecting SNPs by mass array MALDI-TOF MS (Sequenom Inc., USA).

In the Gushi × Anka F2 chicken population, the rs15179830 (+ 90 bp T>G) SNP within *pre-miR-1687* was genotyped using a MassARRAY-iPLEX Gold system. SNP genotyping was performed using a pair of specific primers for amplified sequences and one single-base extension primer designed by Professional Assay Design software (version 3.1). The upstream primer sequence for the pre-miR-1687 SNP was 5′-ACGTTGGATGTGAACAGCAACACAGCTAGG-3′; the downstream PCR amplification primer sequence for the SNP was 5′-ACGTTGGATGAGCAACTTCTTTGCTGGCTG-3′; and the single-base extension primer was 5′-AGTGACTGCAGCATAAAAA-3′. The SNP was genotyped by MALDI–TOF MS as described in the operation manual. The genotyping data for the peak area and call rate were collected. The alleles were designated automatically by the software package offered by the product manufacturer.

In chickens, due to the rs15179830 SNP with T>G alleles in *pre-miR-1687*, the most stable secondary structure of *pre-mir-1687* with the lowest free energy was computed by the online M-fold web server. The absolute value of the free energy difference between the two different alleles (T/G) of *miRNA-1687* was calculated as a parameter to evaluate the effect on the secondary structure of *gga-miR-1687*.

A statistical data analysis software program (SPSS 20.0) was applied for correlation analysis of the relevant data for the SNP of *pre-miR-1687* and the growth and development traits of the Gushi × Anka F2 resource population birds. The association between gene polymorphisms and the F2 chicken population-associated economic characteristics was analysed by using a biostatistical program with an established linear mixed model. The model was applied to analyse and evaluate the correlation between SNPs and growth traits. In this data analysis, the mixed linear model equation was:

$$Y_{ijkl} = \mu + G_i + S_j + H_k + f_l + e_{ijklm}$$

Y_{ijkl} in the equation represents the measured value, μ represents the overall average, G_i represents the fixed effects of the three genotypes (i = 3: TT, TG, GG), f_l represents the random effect of the resource population (l = 7, 7 reference families), s_j represents the fixed effect of sex (j = 2), H_k represents the hatch fixed effect (k = 2), and e_{ijklm} represents the random error. A *P* value <0.05 was recognized as statistically significant.

Results

The results for the polymorphism were confirmed using PCR amplification-based sequencing by comparison with the released chicken genome DNA sequence, which indicated the existence of a T>G mutation at +90 bp in *pre-miR-1687* in the F2 resource population (Figure 1). The obtained mass spectrograms of three variant genotypes, namely, GG, GT, and TT, are shown in Figure 2. In this F2 resource population, the allele frequencies within the SNP in the *pre-miR-1687* gene were 0.685 for G and 0.315 for T. Similarly, the genotype frequencies were 0.417, 0.537, and 0.046 for TT, GT, and GG, respectively.

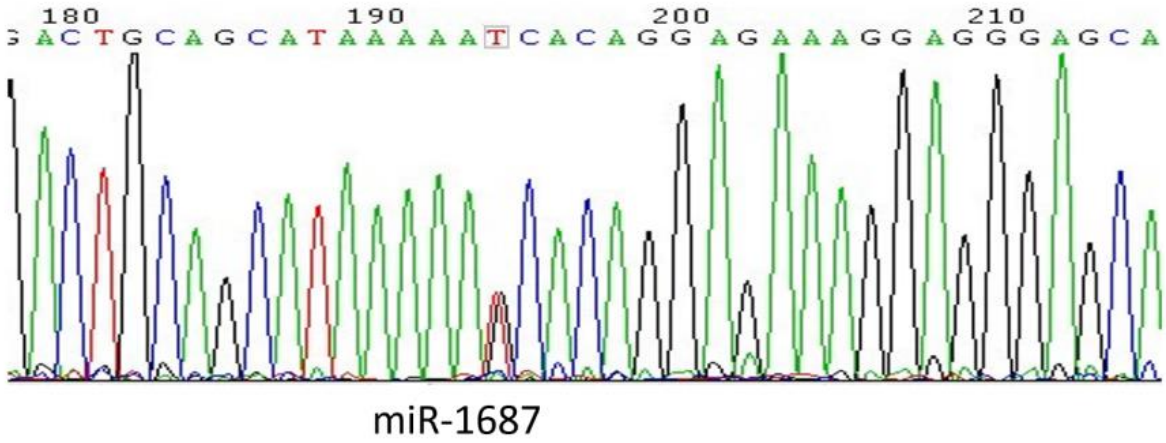

Figure 1 Analysis of PCR sequencing results for *miRNA-1687*. The base marked with a box in the figure indicates the mutation site in the gene.

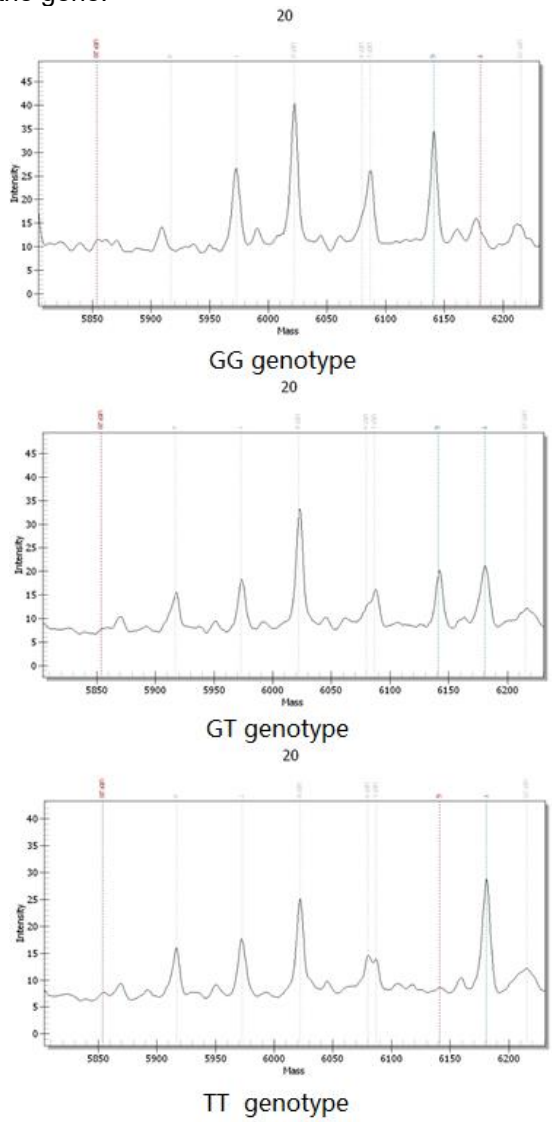

Figure 2 Mass spectrometry analysis of the GG, GT, and TT polymorphisms of T > G in *miRNA-1687*

The alleles of the *pre-miR-1687* gene SNP can give rise to changes in the secondary structure of the miR-1687 RNA, as predicted by the online software program, M-fold. The prediction result showed that the SNP (+90 bp T>G) variation located in the precursor domain of *pre-miR-1687* could generate one base pairing mismatch, leading to the production of a novel RNA ring-like protrusion in the

secondary structure of the base mutant, along with a concurrent change in free energy (Figure 3).

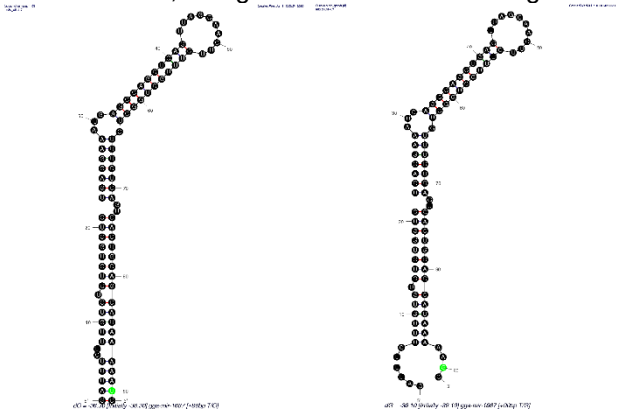

Figure 3 Prediction of the secondary structure of different alleles (T/G) of *miRNA-1687*

To study the effect of the rs15179830 SNP in *pre-miR-1687* on chicken phenotypic traits, an association analysis of the new SNP with the weight of chickens at different growth times (at birth and at 2 w, 4 w, 6 w, 8 w, 10 w, and 12 w of age) (Table 1). The *pre-miR-1687* SNP gene had an impact (P <0.05) on BW at 0, 2, 4, 6, 8, and 10 weeks of age in the Gushi × Anka F2 chicken resource population. The F2 chickens with the TT genotype were the heaviest in terms of BW from 0–12 w. The results of the association analysis of different rs15179830 genotypes with chicken body size are presented in Table 2. The new mutation had an indirect influence on the economic characteristics (mainly including weight and body size) of the chickens at different growth and developmental stages.

Table 1 Association analysis of rs15179830 (+90 T>G) genotypes with chicken growth traits (mean ± SE)

Body weight	**GG genotype**	**GT genotype**	**TT genotype**	***P* value**
0BW (g)	30.27±0.18[a]	31.04±0.16[b]	31.95±0 .54[b]	0.002
2BW (g)	118.85±1.27[a]	125.73±1.14[b]	130.26± 3.75[b]	0.000
4BW (g)	315.96± 3.04[a]	327.92± 2.74[b]	342.54± 9.00[b]	0.004
6BW (g)	550.19±5.75[a]	572.59± 5.18[b]	600.09±17.03[b]	0.004
8BW (g)	800.56+8.50[a]	834.18 ±7.65[b]	854.98± 25.18[b]	0.015
10BW (g)	1098.02±10.71[a]	1133.49±9.64[b]	1182.05±31.72[b]	0.012
12BW (g)	1341.87±12.94	1368.77±11.65	1424.14±38.32	0.114

Note: 0, 2, 4, 6, 8, 10, 12BW = body weight at 0 days and 2, 4, 6, 8, 10, and 12 weeks of age, respectively. [a, b] Means within a row with no common superscript differ significantly (P <0.05)

Discussion

Single-stranded RNAs are short ncRNA molecules that regulate genes in a specific sequence. MicroRNAs perform vital functions in regulating gene expression by promoting the degradation of mRNA and inhibiting transcription. SNPs in miRNA genes can change the characteristics of miRNAs by changing the expression level of miRNAs or affecting the maturity of miRNAs. An increasing number of studies have focused on the functions of microRNAs in biological processes in recent years. Increasing evidence suggests that SNPs occurring in miRNA genes or their targets might affect the economic characteristics of livestock and poultry (Li *et al*., 2015). For example, more than twenty SNPs were screened for their association with days to 100 kg and birth weight in 600 Yorkshire pigs. One locus (location: 46 226 512 bp) located on SSC12 was assessed to affect both birth weight and age at 100 kg. According to their roles, the *DOCK7* gene and *NSRP1* gene are considered to be the most promising candidate genes associated with growth characteristics (Wu, 2021). In Hu sheep, one new SNP (g. 4819 A>G) in LIPE was found in intron 4 of the noncoding region of the fragmented gene, and

this SNP was strongly related to the intramuscular fat content. The expression level of LIPE in the longissimus dorsi muscle was negatively correlated with intramuscular fat content. With increasing LIPE expression, the intramuscular fat content decreased.

Table 2 Association analysis of rs15179830 (+90 T > G) genotypes with chicken body size traits (mean ± SE)

Age	Body size traits	GG genotype	GT genotype	TT genotype	*P* value
4 weeks	SL (cm)	5.34±0.05^{a}	5.56±0.04^{b}	5.68±0.14^{b}	0.001
	SG (cm)	2.67±0.01^{a}	2.71±0.01^{b}	2.71±0.04^{ab}	0.046
	CD (cm)	4.83±0.04	4.85±0.03	4.87±0.11	0.975
	CB (cm)	4.07±0.03	4.08±0.03	4.13±0.09	0.389
	BBL (cm)	6.17±0.03	6.24±0.03	6.26±0.10	0.390
	PA (°)	74.51±0.25^{a}	74.04±0.22^{b}	73.11±0.74^{c}	0.026
	BSL (cm)	11.32±0.05	11.42±0.05	11.42±0.15	0.457
	PB (cm)	5.09±0.03	5.18±0.03	5.26±0.08	0.050
8 weeks	SL (cm)	7.85±0.04	7.93±0.04	7.94±0.12	0.228
	SG (cm)	3.40±0.01	3.41±0.01	3.44±0.04	0.424
	CD (cm)	6.50±0.06	6.53±0.05	6.46±0.16	0.845
	CB (cm)	5.64±0.04	5.72±0.03	5.69±0.11	0.389
	BBL (cm)	8.86±0.04	8.94±0.04	9.11±0.13	0.102
	PA (°)	76.61±0.32	76.37±0.29	75.56±0.98	0.283
	BSL (cm)	16.21±0.07	16.26±0.06	16.41±0.21	0.803
	PB (cm)	6.89±0.04^{a}	6.82±0.04^{a}	7.17±0.12^{b}	0.033
12 weeks	SL (cm)	9.43±0.04	9.40±0.04	9.46±0.12	0.632
	SG (cm)	3.85±0.02	3.84±0.01	3.84±0.05	0.291
	CD (cm)	7.90±0.05	7.91±0.05	7.92±0.15	0.716
	CB (cm)	6.40±0.04	6.38±0.04	6.29±0.12	0.711
	BBL (cm)	11.01±0.05	11.03±0.04	10.94±0.14	0.716
	PA (°)	79.13±0.28	79.03±0.24	78.36±0.83	0.854
	BSL (cm)	19.81±0.07	19.84±0.06	19.83±0.20	0.686
	PB (cm)	8.75±0.05	8.62±0.05	8.69±0.16	0.134

Note: 0, 4, 8, and 12 weeks. SL=shank length; SG=shank girth, CD=chest depth; CB=chest breadth; BBL=breastbone length, PA=pectoral angle; BSL= slanting body length, PB=pelvic breadth. Means with different superscripts in the same line indicate significant differences; a small letter indicates P <0.05

These results showed that the SNP (g.4819 A>G) in the *LIPE* gene was promising as a molecular genetic marker for breeding Hu sheep with better intramuscular fat content (Kong *et al*., 2022). The missense SNP in HSD17B12 predicted to affect protein function was correlated with earlier submission for seasonal breeding, perhaps on account of earlier resumption of cyclicity postpartum in dairy cows (Juengel *et al*., 2022). In the cross of Nellore beef × Bos Taurus cattle, the SNP (g. 98 535 683 A>G) located in the *BTAU7 calpastatin* gene had an effect on beef quality traits. These results showed its application value for genetic breeding improvement of beef cattle for meat quality traits (Enriquez-Valencia *et al*., 2017). In Landrace and Yorkshire pigs, four notable SNPs (SSC6.149876737, 149876507, SSC8.54567459, SSC11.33043081) were discovered to be closely associated with growth performance. In addition, *DOCK7*, a functional candidate gene with potential application value, was recommended for selective breeding programs for BW and backfat thickness in Landrace and Yorkshire pigs (Yang *et al*., 2019). Analysis of the differences in expression at the transcriptional level for the SNP (g. 265 T>C) from FASN in semimembranous muscle tissue and backfat tissue of Italian Duroc (ID) pigs and Italian Large White (ILW) pigs was performed. The results showed that the transcription level of the fatty acid synthase (*FASN*) gene could be regarded as a molecular marker to identify the level of fat deposition in the ID pig breed (Braglia *et al*., 2014). One SNP (g.358 A>T) in the intronic location of the cluster-of-differentiation antigen-9 (CD9) molecule of Frieswal (HF × Sahiwal) crossbred bulls was strongly correlated with sperm content and sperm motility percentage (Kumar *et al*., 2015). One SNP (c. *7750 G>A) occurring in the miRNA-binding site of the epidermal growth factor receptor (*EGFR*) gene 3′ UTR was related to egg production. The expression level of the *EGFR* gene may affect egg productivity in the Yangzhou goose breed (Alsiddig *et al*., 2022). The increase in BW and body size, which are important economic traits, is a result of the influence on production performance in chickens. The growth traits of chickens have a strong genetic background (Zerehdaran *et al*., 2004). Growth

characteristics are important economic characteristics in poultry breeding.

The main purpose of the current study was to screen and evaluate the SNPs in *pre-miR-1687* by making use of the Gushi × Anka F2 chicken resource population to determine if the SNPs were related to growth performance. The test method consisted of sequencing pre-miR-1687 by using a set of DNA samples from different F2 chicken individuals to identify the SNPs. We predicted the secondary structure and the free energy values of different alleles (T/G) of *pre-miR-1687* by using online M-fold software. The SNPs altered the secondary structure and free energy of *pre-miR-1687*. The polymorphism, rs15179830, was identified in the *pre-miR-1687* gene and was related to BW and pectoral angle in the chicken population. Correlation analysis was carried out for the SNP and phenotypic traits (growth traits) in F2 chickens. The Bonferroni-corrected model was used to decrease incorrect associations in the data analysis. The SNP was demonstrated to have a strong influence on the BW of chicks at 4, 6, 8, and 10 weeks and the pectoral angle at 4 w. In summary, the findings provide hard evidence that the gene polymorphism in pre-miR-1687 is associated with growth performance and is an available candidate gene molecular marker for breeding to effectively improve economic performance. We evaluated the effects of *pre-miR-1687* in the Gushi × Anka F2 resource chicken population; correlation analyses of the SNP with growth characteristics have not been previously reported. In the present research, the results of the analysis confirmed that rs15179830 was strongly associated with birth weight, BW, at 4, 6, 8, and 10 weeks and PA at 4 w. The SNPs were strongly associated with diverse genotypes and growth traits.

Moreover, the genotypes GG, GT and TT were strongly related to BW in the order TT>GT>GG; T was a superior allele. The results showed that the T allele promoted the increase in BW, while the G allele was unfavourable for the increase in BW. The main aim of the current research was to identify the influence of the *pre-miR-1687* SNP on chicken growth characteristics, and the findings enrich the knowledge and understanding of poultry genetic breeding technology, facilitating the development of improved genetic breeding schemes (Li *et al*., 2012). Therefore, the TT genotype of the SNP of the *pre-miR-1687* gene was selected as the molecular genetic marker for improving BW in marker-assisted selection for poultry breeding.

Conclusion

Our data suggest that *pre-miR-1687* might participate in regulating growth and development, implying that *pre-miR-1687* may be a candidate gene correlated with chicken growth performance. Therefore, the SNP (rs15179830 T>G) of *pre-miR-1687* is a potential, novel, molecular genetic marker that can be used for the selection of excellent growth and development traits in chickens. The results will be a helpful resource for further work on miRNA biological processes and functions and provide reference data for the application of molecular breeding technology in poultry genetics and breeding.

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Author Contributions

Conceptualization, G.R. Sun; methodology, G.R. Sun and J.Z. Shi; software, Y.W. Wang; validation, J.Z. Shi, T.J. Wang, and J. Ji; formal analysis, J.Z. Shi; investigation, G.R. Sun; resources, G.R. Sun; data curation, G.R. Sun; writing—original draft preparation, J.Z. Shi; writing—review and editing, J. Ji; visualization, J.Z. Shi; supervision, L.G. Yao; project administration, L.G. Yao; funding acquisition, L.G. Yao. All authors have read and agreed to the published version of the manuscript.

Conflict of interest declaration

All authors jointly declare that there is no conflict of interest in this study.

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