Identification of aberrantly expressed circRNAs in subgroup J avian leucosis virus induced tumor livers by RNA sequencing

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ABSTRACT

ALV-J (subgroup J avian leucosis virus) is a kind of oncogenic exogenous retrovirus and diseases associated with ALV-J have caused severe reproduction problems in the poultry industry worldwide. However, the pathogenesis of ALV-J-induced tumor formation is still unclear. In recent years, circRNAs are validated to be related to cancer, which prompts us to explore whether aberrant expression of circRNAs exists in ALV-J-infected tumor samples. In this study, a total of 2822 differentially expressed circRNAs were detected by RNA sequencing in the tumor livers of ALV-J-susceptible chickens (*n* = 3) and the normal liver samples of ALV-J-resistant chickens (*n* = 3), among which 2808 circRNAs in the tumor liver samples were detected as unregulated compared to those in the normal liver samples. As circRNAs are considered as miRNA sponge, we predicted top 5 miRNA targets for 24 selected upregulated circRNAs in the ALV-J-induced tumor livers. CircRNA-miRNA-mRNA regulatory axes were also constructed to provide clear interaction relationship among them. Furthermore, Gene Ontology and KEGG pathway analyses were performed for miRNA target genes, the result of which implied that metabolism plays a key role in circRNAs alterations in ALV-J-induced tumor livers. In summary, we identified that circRNAs are involved in ALV-J-induced tumor formation in chickens. However, the pathogenicity mechanism still needs to be further explored.

INTRODUCTION

Circular RNA (circRNA) is a novel kind of endogenous noncoding RNA that form circular structures without 5′-3′ polarities and polyadenylated tails [1]. In 2017, two reports identified that circRNAs had the translation function [2, 3]. CircRNAs are produced from pre-mRNA back-splicing and according to the circRNA biogenesis, intronic circRNA, exonic circRNA, sense overlapping circRNA, antisense circRNA and intergenic circRNA are the five types that have been reported [4–7]. CircRNAs are enriched, conserved and stable in mammalian cells [7, 8]. Recently, it was reported that circRNAs function as microRNA (miRNA) sponges that naturally sequester and competitively suppress miRNA activity [9]. It was demonstrated that circRNAs are involved in the development of several types of diseases, such as atherosclerosis, myocardial Infarction and neuron injury [10–12]. Indeed, there has been a special outpouring of concern for immense amounts

of research shown that circRNAs are associated with many human cancers, such as hepatocellular carcinoma [13], esophageal squamous cell carcinoma [14], human gastric cancer [15], colorectal cancer [16]. Our previous study showed that circRNA alterations are involved in resistance to avian leucosis virus subgroup-J-induced tumor formation in chickens [17], however, no reports showed that circRNAs play a key role in chicken cancer which was induced by avian leucosis virus subgroup-J (ALV-J).

The avian leucosis virus belongs to the Alpharetrovirus genus of the Retrvoviridae family and induces sarcoma and leucosis in chickens [18]. Up to now, there are seven subgroups of ALV in chickens: ALV-A, -B, -C, -D, -E, -J and –K [19, 20]. ALV-J was first detected in meat-type chickens in 1987 as a new subgroup [21] and has spread to many countries such as America [22], UK [23], China [24] and so on. Clinical infection is associated with immune tolerance, high mortality, delayed growth, inducing different kinds of tumors [25, 26]. ALV-J can be transmitted vertically and horizontally, and horizontal transmission of ALV-J is more efficient than for other ALV subgroups [27]. ALV-J has caused great economic losses in the poultry industry worldwide [28]. Developed countries and China have made efforts to eradicate ALV-J from chicken breeding flocks in the poultry industry, unfortunately, an effective vaccine against ALV-J infection is not currently available [29, 30]. Breeding ALV-J-resistance chickens may be a new technique to reduce the severe economic cost which is caused by ALV-J and we have successfully cultivated ALV-J-resistance chickens [31].

Recently, we have identified that circRNAs alterations are involved in resistance to ALV-J-induced tumor formation in chickens [17], however, no reports showed that circRNAs aberrant expression also plays a key role in ALV-J-induced tumor formation. High-throughput RNA sequencing (RNA-seq) has been widely used to infer host gene expression in response to virus infection [32]. Herein, we confirm that enriched and differentially expressed circRNAs by RNA-seq in tumor liver samples of ALV-J-susceptible chickens. Further biological function prediction analysis was also conducted to develop a better understanding of the circRNAs function in ALV-J-induced tumor chickens.

RESULTS

Identification of differentially upregulated circRNAs in tumor liver samples of ALV-J-susceptible chickens

CircRNAs were sequenced in three tumor liver samples of ALV-J-susceptible chickens and three normal liver samples of ALV-J-resistance chickens. A total of 13419 circRNAs were detected after RNA-seq. Among those 13419 circRNAs, 2822 differentially expressed circRNAs (fold change ≥ 2, $P \leq 0.05$, FDR < 0.05) were detected in both tumor liver samples and normal liver samples by Volcano Plot, including 2808 differentially upregulated circRNAs that were identified in tumor liver samples (Figure 1A). Scatter plot analysis made the variation of circRNAs expressions of two groups be visualized in these plots (Figure 1B). Hierarchical clustering analysis of randomly selected 24 differentially expressed circRNAs clearly screened them into two groups (Figure 2). After comparison with the genome sequence of chicken, total five types of circRNAs including exonic, intronic, sense overlapping, antisense and intergenic circRNAs were found, comprising 40.62%, 6.86%, 21.36%, 13.62% and 17.53%, respectively (Figure 3).

Validation of differentially expressed circRNAs using qRT-PCR

To validate the accuracy of RNA-seq data, a total of 4 differentially expressed circRNAs (chr21:4172114-4172476-, chr19:6585504-6585731+, chr9:22282452-22289593- and chr13:15110945-15122920-) were randomly selected and the quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was carried out in 3 tumor liver samples and 3 normal liver samples. QRT-PCR results of those selected differentially expressed circRNAs were consisted with RNA-seq data (Figure 4).

MiRNA response elements analysis of differentially upregulated circRNAs in tumor liver samples

For better understanding the detailed original information of circRNAs, 24 upregulated circRNAs in tumor liver samples of ALV-J-susceptible chickens were selected and ranked by fold change (FC), the 24 circRNAs were annotated in the gallus genome and the names of the best-predicted transcript for each of this 24 circRNAs were shown in Table 1.

The homology of chicken circRNAs

Comparing chicken circRNAs with those of mouse and humans, homology analysis results showed that 8564 (63.82%) chicken circRNAs were homologous with humans, 8278 (61.69%) were homologous with the mouse. Importantly, a total of 8024 chicken circRNAs (59.80%) showed homology with mouse and humans (Figure 5).

MiRNA binding sites prediction and circRNA-targeted miRNA-gene network identification

Because that circRNAs as an active miRNA sponge regulator, we then listed top 5 possible miRNA targets for each circRNA which was up-regulated in tumor

liver samples of ALV-J-susceptible chickens in Table 2. CircRNA-targeted miRNA-gene network was constructed using Targetscan and miRanda. CircRNAs contain many miRNA response elements (MREs) [33], we then selected the top 5 predicted miRNA targets of 3 randomly differentially upregulated circRNAs (chr5: 51521674-51524828, chr3: 3137166-3138170 +, chr4: 59505289-59510641-) in tumor liver samples, meanwhile, the top 10 target genes for each miRNA were collected. Finally, the integrated circRNA-targeted miRNA-gene network was determined (Figure 6).

Functional annotations for miRNA target genes

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the predicted miRNA target genes which were predicted to be targeted by the 24 differentially upregulated circRNAs in tumor liver samples of ALV-J-susceptible chickens showed that those miRNA target genes were involved in cysteine and methionine metabolism pathway (Figure 7). Furthermore, Gene Ontology (GO) annotation of biological process (BP) of those miRNA target genes participated in mRNA metabolic process, cell development, mRNA catabolic process, phospholipid metabolic process, regulation of cell projection organization, regulation of cellular component size, regulation of cellular component organization, regulation of transmembrane transporter activity, regulation of cell projection assembly and mRNA processing (Figure 8A). GO annotation of cellular component (CC) of those miRNA target genes participated in cell junction and collagen trimer (Figure 8B), while for GO annotation of molecular function (MF) of those miRNA target genes participated in nucleoside phosphate binding, nucleotide binding, small molecule binding, DNA helicase activity, adenyl nucleotide binding, adenyl ribonucleotide binding, binding, cytoskeletal protein binding, ATP binding and RNA binding (Figure 8C).

DISCUSSION

CircRNA expression has the cell-type-specific, tissue-specific or developmental-stage specific manner [34, 35]. To date, several studies have proved that circRNAs may regulate gene expression by manipulating miRNAs through its miRNA sponges function [36], and circRNAs also compete with mRNAs for miRNA binding in the cytoplasm and interfere in gene regulation [8]. MiRNAs control a large set of biological processes through direct interaction with their target mRNA, so circRNA sponge activity will also affect these pathways [37, 38]. Importantly, circRNAs have become the new markers for diseases including cancers [10, 39].

RNA-seq was used for discovering new transcript and genes expression levels including circRNAs expression in many cancer research [40, 41, 42]. ALV-J mainly induces myelocytomatosis in chickens and can cause other reproduction problems in the poultry industry worldwide [43]. In the present study, we want to explore

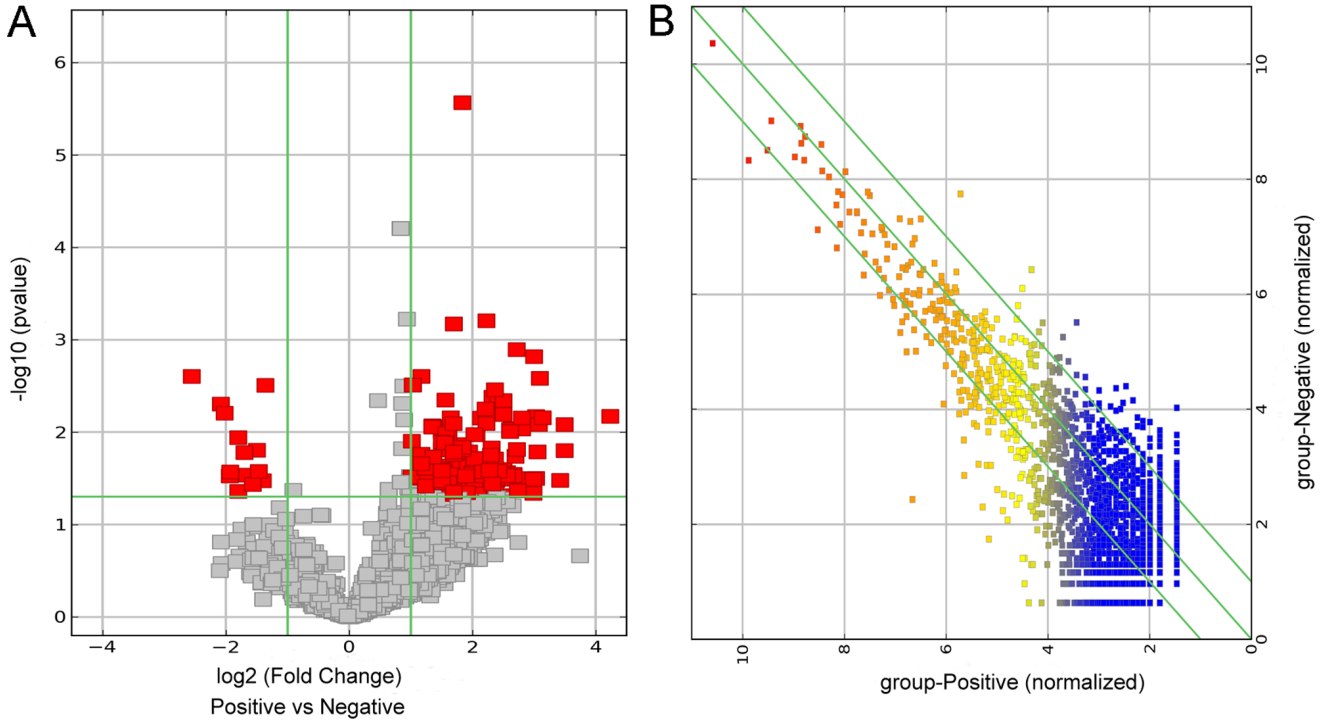

Figure 1: Bioinformatics analysis of differentially expressed circRNAs in tumor livers of ALV-J-susceptible chickens and normal livers of ALV-J-resistance chickens. (**A**) Volcano plot of differentially expressed circRNAs. (**B**) Scatter plot of differentially expressed circRNAs.

whether circRNAs are involved in ALV-J-induced tumor formation or not. Interestingly, totally 13419 circRNAs were detected using RNA-seq in tumor liver samples of ALV-J-susceptible chickens and normal liver samples of ALV-J-resistance chickens, totally 2822 differentially expressed circRNAs (fold change ≥ 2, $P \leq 0.05$, FDR < 0.05) were detected in both of tumor liver samples and normal liver samples, while 2808 differentially upregulated circRNAs were identified in tumor liver samples, the different expression patterns implied circRNAs may play a key role in tumor formation which is induced by ALV-J. QRT-PCR was a necessary tool to valid the RNA-seq data in many studies [44, 45]. Here, in our study, we confirmed the differentially expressed circRNAs from RNA-seq data by qRT-PCR. As expected, qRT-PCR results of randomly selected 4 differentially expressed circRNAs had the same expression level trend compared with that from RNA-seq data, then we were sure that the RNA-seq results were reliable.

For better understanding the function of miRNA target genes, the additional functional analysis was conducted using the GO annotation and KEGG pathway. Statistical analysis of miRNA target genes was used to determine which GO terms or KEGG pathways were significantly

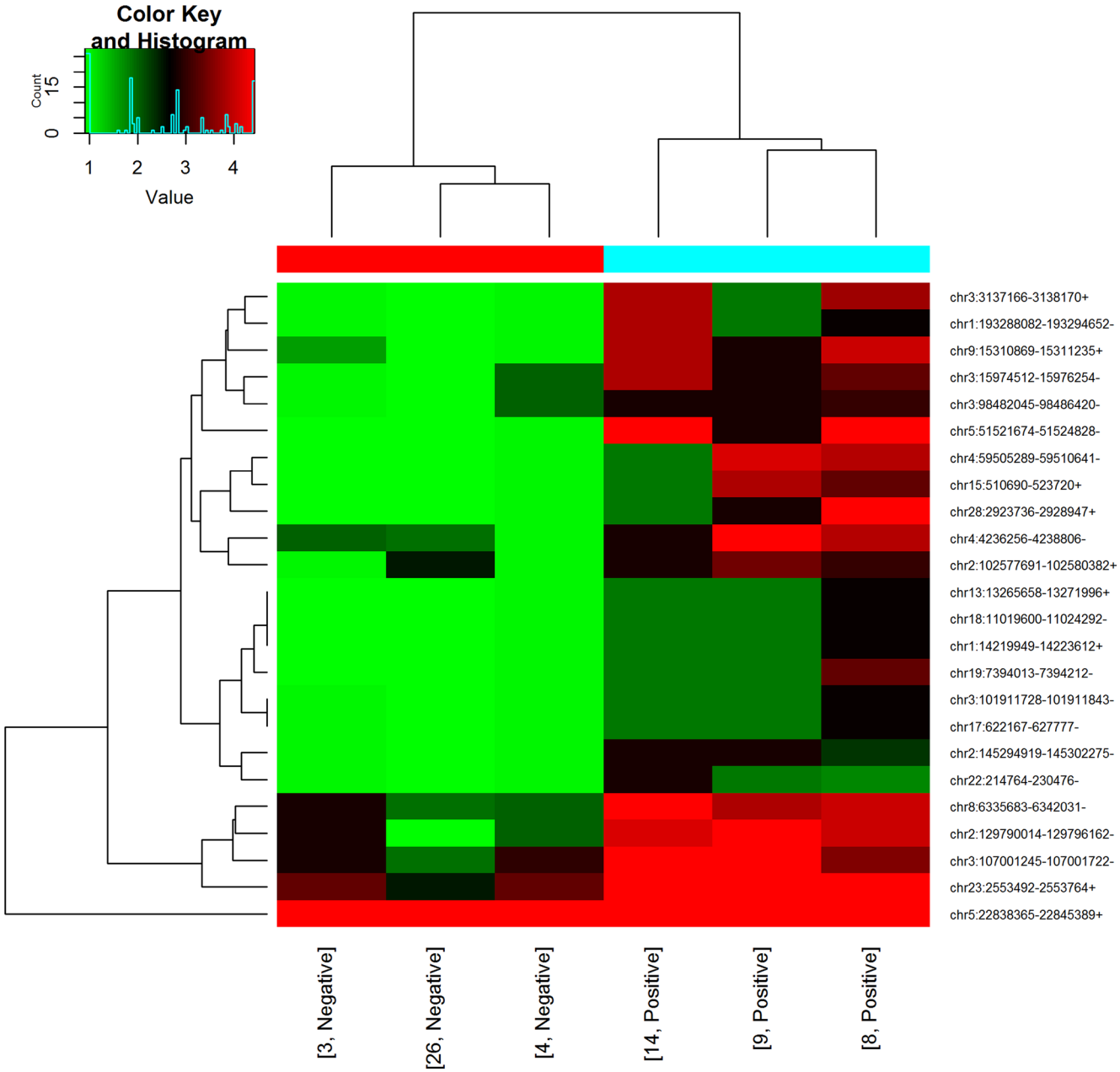

Figure 2: Hierarchical cluster analysis of circRNAs. circRNA expression profile of six RNA-seq samples was showed in red (upregulated expression) and green (downregulated expression) scale. The dendrogram shows the relationships among the expression levels of samples.

enriched in the tumor livers samples as compared to the normal livers samples. These enriched categories along with the number of miRNA target genes identified for biological process, cellular component, molecular function three aspects. Interestingly, GO term of biological process and KEGG pathway analysis both showed that miRNA target genes are involved in the metabolic process, that implied metabolism make an important function in tumor liver formation induced by ALV-J. We suspected that maybe those up-regulated circRNAs changed the status and then caused the physical disorder and finally result in tumor formation. We also conducted homology analysis of circRNAs among chickens, mouse and humans to explore whether differentially expressed circRNAs of tumorigenesis in chickens may also involve in tumorigenesis in humans. It is worth mentioning that homology analysis results showed that 63.82% and 61.69% of chicken circRNAs were homologous with humans and mouse respectively, while a total of 59.80% chicken circRNAs showed homology with mouse and humans, that implies those differentially expressed circRNAs of tumor livers in chickens may also involve in tumor formation in humans.

In recent years, circRNA-miRNA-gene regulatory axes were confirmed that involved in pathogenic processes or cancer [46, 47]. In order to show a more intuitionistic result, three up-regulated circRNAs (chr5: 51521674-51524828-, chr3: 3137166-3138170+, chr4: 59505289-59510641-) in tumor liver samples of ALV-J-susceptible chickens were selected and five target miRNAs and 10 miRNA target genes were showed in the circRNA-miRNA-gene network which provides a foundation for further pathogenic mechanism research.

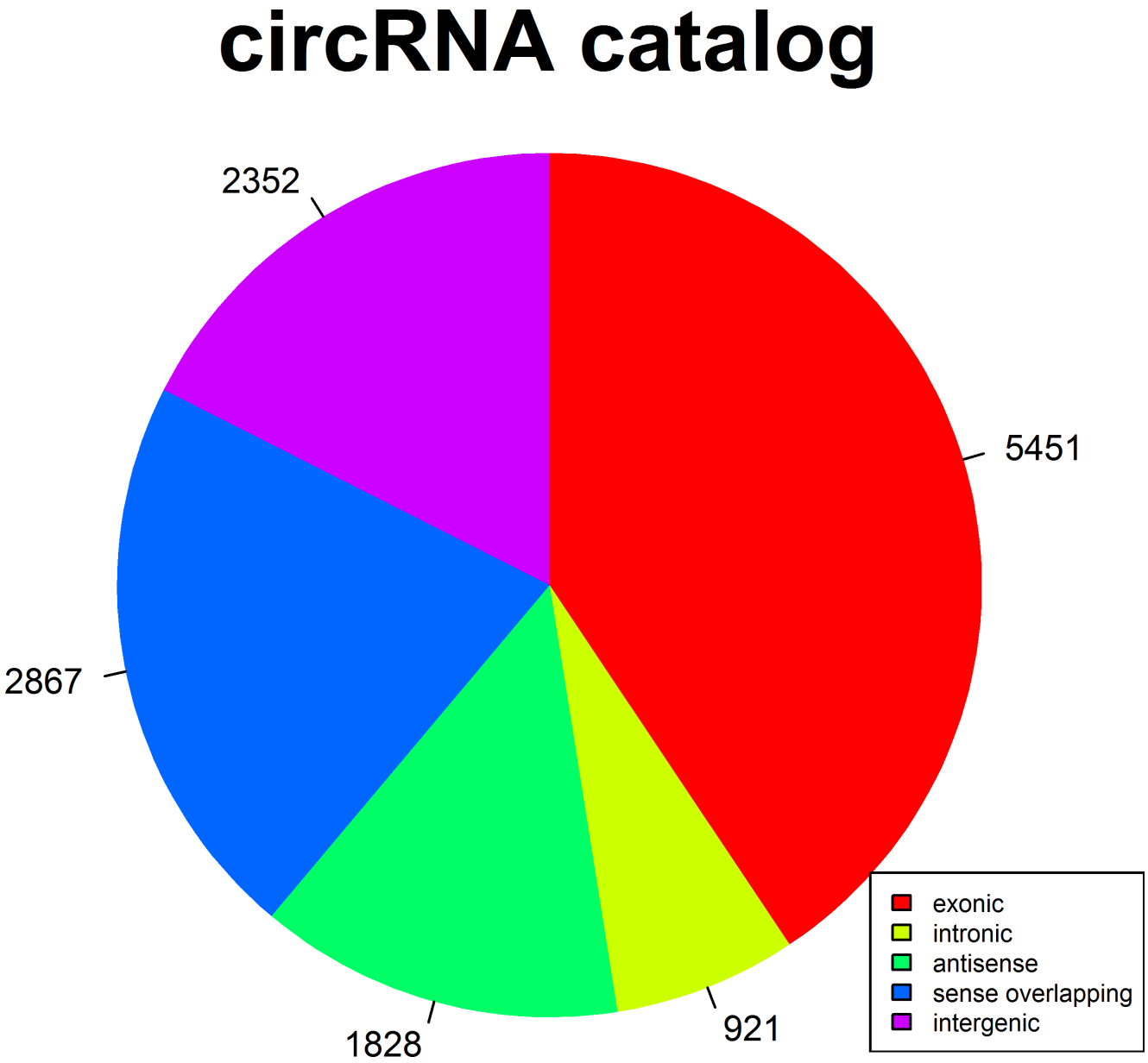

Figure 3:The percentage of five different types of circRNAs in chickens.

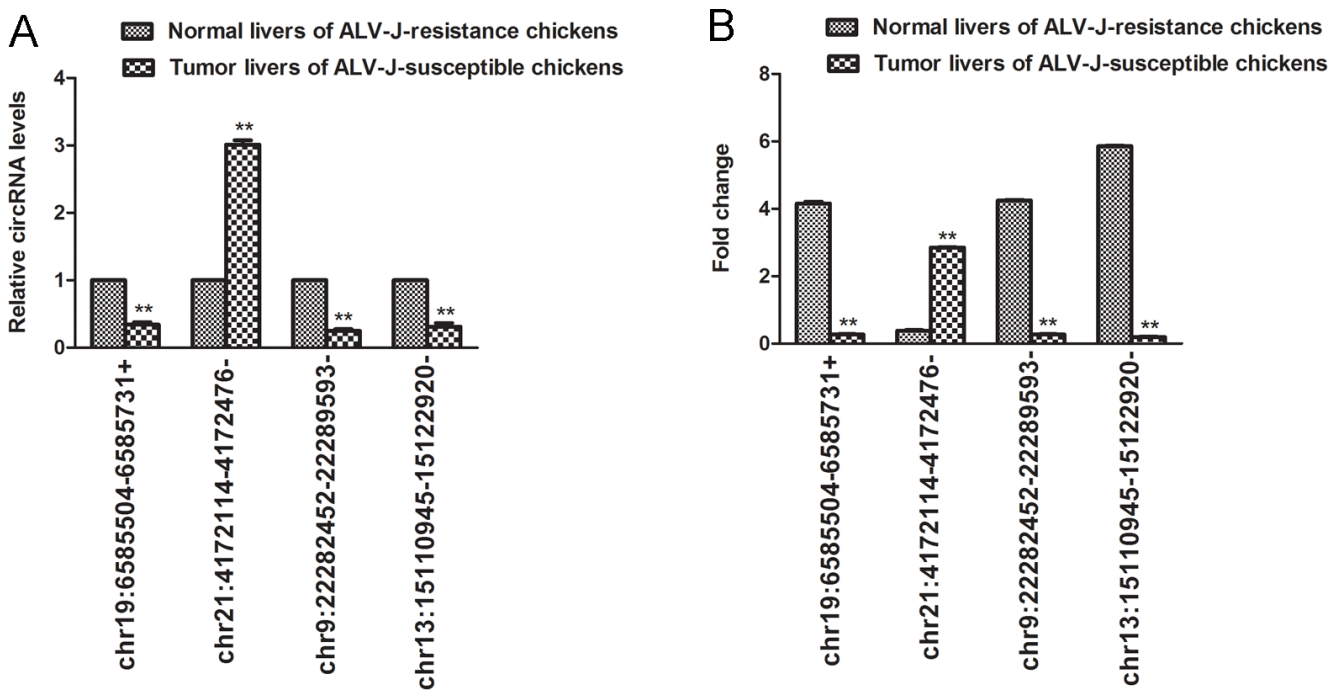

Figure 4: Validation of the expression level of four selected differentially expressed circRNAs by qRT-PCR. (**A**) Relative circRNA levels as indicated by RNA-seq; (**B**) Fold change for circRNA levels as indicated by qRT-PCR. Bars are mean ± SD (n = 3); $^{**}P < 0.05$.

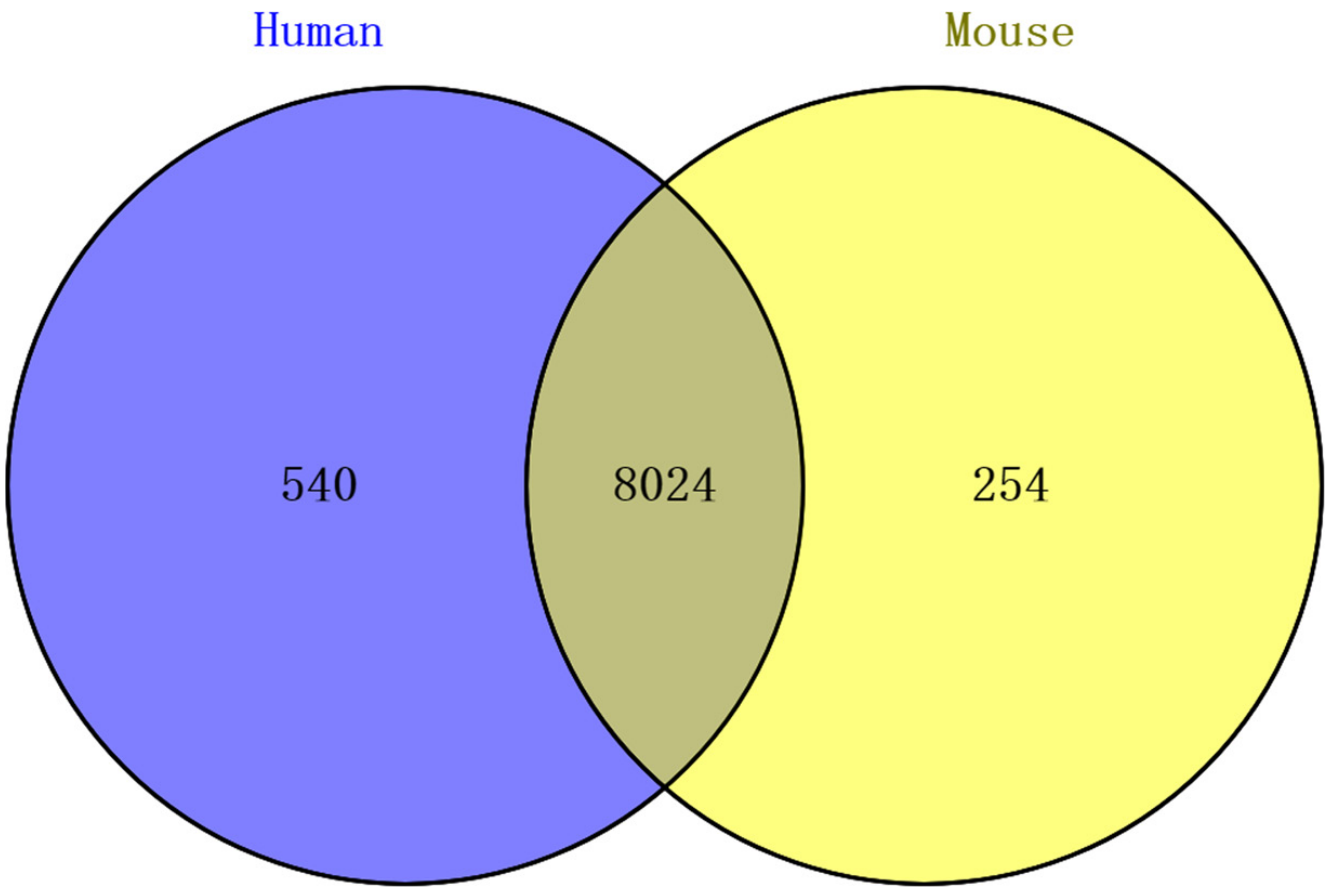

Figure 5: Chicken circRNAs shared homology with mouse and humans.

In conclusion, our study for the first time reveals that circRNAs are involved in ALV-J-induced tumor formation in chickens. We totally identified 2808 differentially expressed upregulated circRNAs in tumor livers of ALV-J-susceptible chickens. Based on miRNA sponge function, top 5 miRNA targets for 24 selected upregulated circRNAs in ALV-J-induced tumor livers were listed according to the match score. We also constructed the circRNA-miRNA-gene network, furthermore, GO terms and KEGG pathway analyses to point out that metabolism plays a key role in circRNAs alterations of ALV-J-induced tumor livers. Homology analysis results showed that totally 59.80% chicken circRNAs showed homology with mouse and humans, that opens a scenario for the investigation of the role of those differentially expressed chicken circRNA in human tumors. However, more underlying mechanism research still needs to be done to reveal which circRNAs alteration are directly involved in ALV-J-induced tumor formation in chickens.

MATERIALS AND METHODS

Ethics statement

All experiments were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The use of animals in this study was approved by South China Agricultural University Committee of Animal Experiments (approval ID: 201004152).

RNA-seq specimens

Tumor livers of ALV-J-susceptible chickens (the model of ALV-J-induced tumor chickens) and normal livers of ALV-J-resistance chickens (the model of ALV-J-infected negative chickens) in the F3 generation at 20 weeks after challenge with ALV-J NX0101 strain which induced myeloid tumors were collected for RNA-seq [31]. In each group, three livers were collected as three duplicates.

RNA library synthesis and RNA-seq

Total RNAs were extracted from tumor livers and normal livers using TRizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The purity and concentration of the RNA samples were determined by assessing the OD260/280 using a NanoDrop ND-2000 instrument (Thermo, Waltham, MA, USA). To enrich circRNAs, RNase R (Epicentre, Madison, WI) was used to digest the extracted total RNAs to remove linear

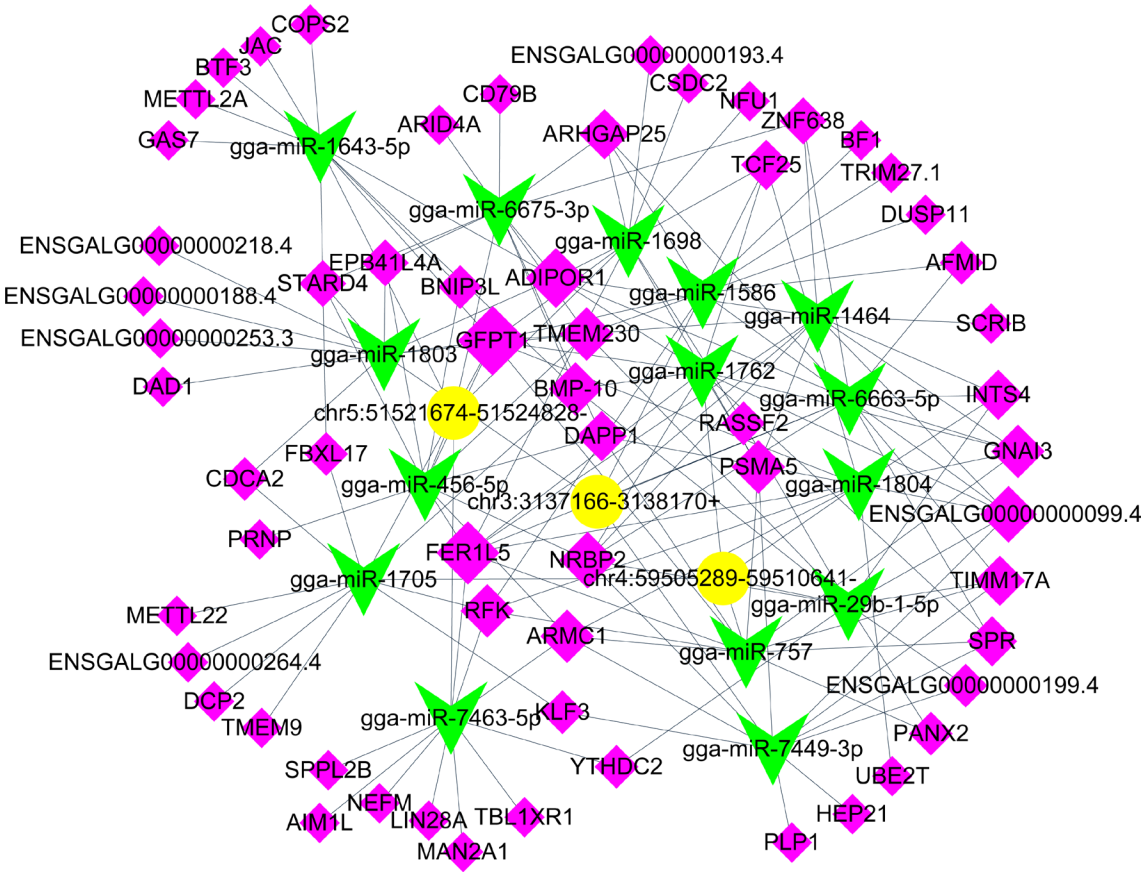

Figure 6: circRNA-miRNA-gene network for three selected upregulated circRNAs in tumor livers of ALV-J-susceptible chickens. Yellow represents circRNAs, green represents miRNAs and pink represents gene.

RNAs [48]. RNA libraries were constructed from the treated RNAs using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Libraries were controlled for quality and quantity according to the BioAnalyzer 2100 system (Agilent Technologies, Inc., Santa Clara, CA, USA) and then was subjected to paired-end sequencing for 150 cycles by HiSeq 4000 Sequencer according to the manufacturer's instructions (Illumina, SanDiego, CA). The paired-end reads were harvested and subject to quality control using Q30 [49]. After 3′ adaptor-trimming and the removal of low quality reads using Cutadapt software [50], the high quality trimmed reads were used for analysis of circRNAs. The differential expressions of circRNAs between the two groups were detected through fold-change filtering and Student's *t*-test. CircRNAs exhibiting fold changes ≥ 2.0 and *P*-values ≤ 0.05 were selected as significantly differentially expressed circRNAs.

Quantitative real-time PCR

QRT-PCR was achieved using PrimeScript RT Reagent Kit (Perfect Real Time; TaKaRa, Osaka, Japan) on a Mx3005P real-time PCR System (Stratagene, La Jolla, CA) with SYBR Green qPCR SMix (ROX; Roche) following the manufacturer's instructions. Divergent primers, rather

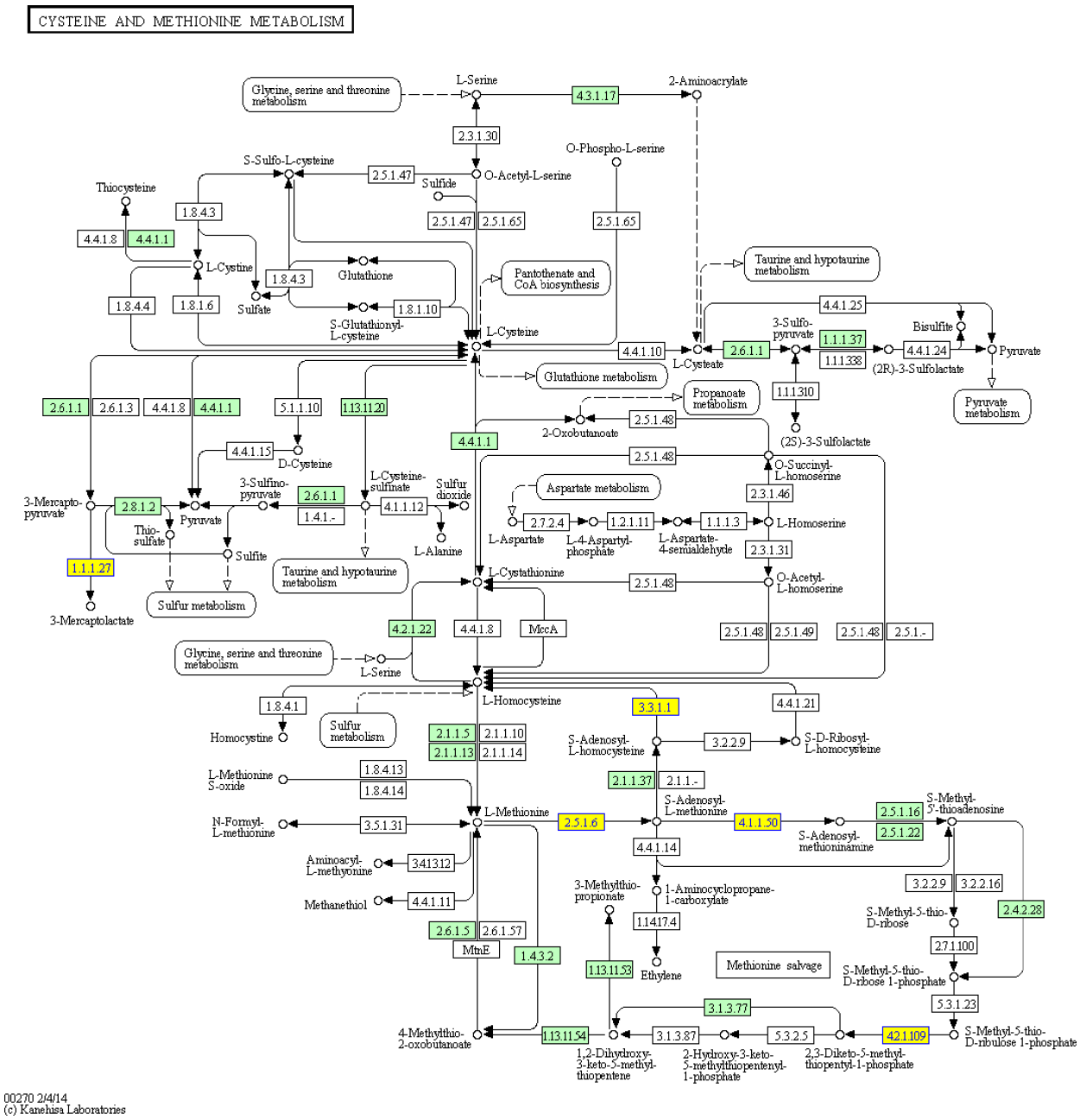

Figure 7: KEGG pathway analysis for the miRNA target genes of 24 upregulated circRNAs in tumor livers of ALV-J-susceptible chickens. The yellow boxes indicate the genes targeted by differentially expressed circRNAs.

Table 1: The 24 significantly upregulated circRNAs in tumor livers of ALV-J-susceptible chickens

CircRNA ID	Fold Change	*P*-value	txStart	txEnd riginal	chrom	Parent Gene
chr15:510690-523720+	5.14	0.026	510689	523720	Chr15	MAPK1
chr5:51521674-51524828-	11.26	0.008	51521673	51524828	Chr5	AKT1
chr28:2923736-2928947+	5.54	0.048	2923735	2928947	Chr28	STk11
chr4:4236256-4238806-	4.25	0.022	4236255	4238806	Chr4	MAP7D3
chr18:11019600-11024292-	2.83	0.026	11019599	11024292	Chr18	GRB2
chr3:3137166-3138170+	4.49	0.028	3137165	3138170	Chr3	MERTK
chr1:14219949-14223612+	2.83	0.026	14219948	14223612	Chr1	PIK3CG
chr2:129790014-129796162-	4.95	0.015	129790013	129796162	Chr2	LRP12
chr19:73940137394212-	3.24	0.044	7394012	7394212	Chr19	RPS6KB1
chr4:59505289-59510641-	6.34	0.030	59505288	59510641	Chr4	EIF4E
chr23:2553492-2553764+	5.00	0.004	2553491	2553764	Chr23	PTPRU
chr9:15310869-15311235+	5.33	0.005	15310868	15311235	Chr9	AP2M1
chr17:622167-627777-	2.25	0.017	622166	627777	Chr17	RABL6
chr3:101911728-101911843-	2.25	0.017	101911727	101911843	Chr3	APOB
chr22:214764-230476-	2.25	0.029	214763	230476	Chr22	ANTXR1
chr5:2283836522845389+	2.84	0.026	22815788	22883775	Chr5	AMBRA1
chr3:98482045-98486420-	3.05	0.011	98482044	98486420	Chr3	NBAS
chr2:102577691-102580382+	3.05	0.038	102577690	102580382	Chr2	CABLES1
chr3:107001245-107001722-	3.10	0.027	107001244	107001722	Chr3	CTSB
chr13:13265658-13271996+	2.83	0.026	13265657	13271996	Chr13	MAPK9
chr2:145294919-145302275-	3.24	0.001	145294918	145302275	Chr2	PTK2
chr1:193288082193294652-	3.57	0.033	193288081	193294652	Chr1	UVRAG
chr3:15974512-15976254-	4.08	0.011	15974511	15976254	Chr3	SOS1
chr8:6335683-6342031-	6.60	0.001	6350257	6368257	Chr8	RALGPS2

than the more commonly used convergent primers, of chr21:4172114-4172476-, chr19:6585504-6585731+, chr9:22282452-22289593- and chr13:15110945-15122920- were designed for further test the accuracy of RNA-seq. Primers for above four randomly selected differentially expressed circRNAs and β-actin were synthesized by Sangon

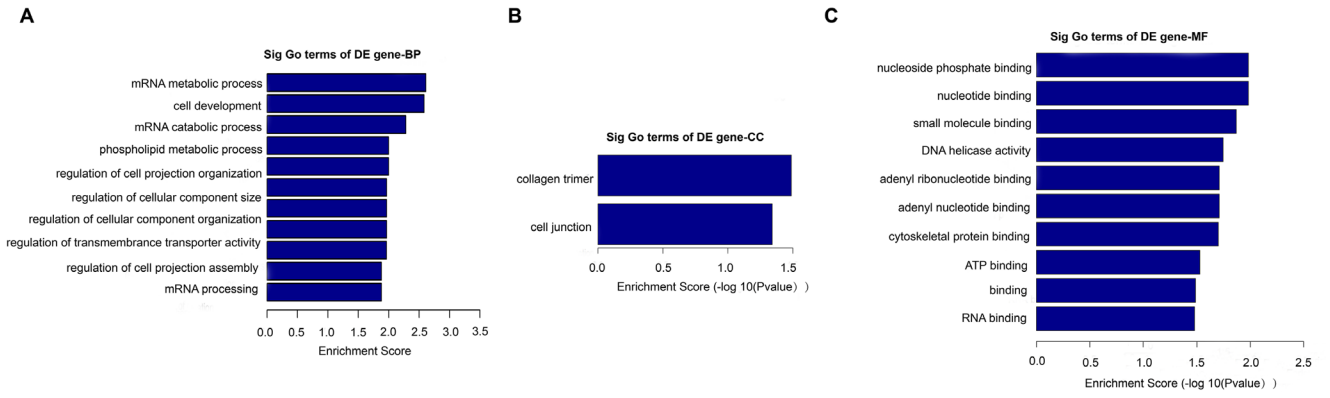

Figure 8: Go analysis of the predicted miRNA target genes. Go analysis of the predicted miRNA target genes which were predicted to be targeted by those 24 upregulated circRNAs in tumor liver samples of ALV-J-susceptible chickens according to the values in the enrichment score under the theme of BP, CC and MF.

Table 2: Predicted miRNAs for 24 differentially upregulated circRNAs in tumor livers of ALV-J-susceptible chickens

CircRNA ID	miRNA	miRNA	miRNA	miRNA	miRNA
chr15:510690-523720+	gga-miR-6640-5p	gga-miR-106-3p	gga-miR-1551-5p	gga-miR-1688	gga-miR-1793
chr5:51521674-51524828-	gga-miR-6675-3p	gga-miR-7463-5p	gga-miR-1643-5p	gga-miR-1698	gga-miR-456-5p
chr28:2923736-2928947+	gga-miR-20a-3p	gga-miR-6665-5p	gga-miR-1730-3p	gga-miR-6646-3p	gga-miR-6623-3p
chr4:4236256-4238806-	gga-miR-1651-3p	gga-miR-7450-5p	gga-miR-1600	gga-miR-1748	gga-miR-1641
chr18:11019600-11024292-	gga-miR-6573-5p	gga-miR-6619-5p	gga-miR-1573	gga-miR-187-3p	gga-miR-1785
chr3:3137166-3138170+	gga-miR-7449-3p	gga-miR-6663-5p	gga-miR-1586	gga-miR-1803	gga-miR-1464
chr1:14219949-14223612+	gga-miR-1627-5p	gga-miR-1799	gga-miR-6545-5p	gga-miR-1753	gga-miR-6552-3p
chr2:129790014-129796162-	gga-miR-1806	gga-miR-6678-3p	gga-miR-1565	gga-miR-211	gga-miR-204
chr19:7394013-7394212-	gga-miR-2128	gga-miR-1662	gga-miR-1811	gga-miR-184-5p	gga-miR-1598
chr4:59505289-59510641-	gga-miR-757	gga-miR-29b-1-5p	gga-miR-1705	gga-miR-1804	gga-miR-1762
chr23:2553492-2553764+	gga-miR-6698-3p	gga-miR-20b-5p	gga-miR-17-5p	gga-miR-106-5p	gga-miR-6587-5p
chr9:15310869-15311235+	gga-miR-6578-3p	gga-miR-6700-3p	gga-miR-6552-5p	gga-miR-7473-5p	gga-miR-6582-3p
chr17:622167-627777-	gga-miR-140-5p	gga-miR-1744-3p	gga-miR-1654	gga-miR-302b-5p	gga-miR-1815
chr3:101911728-101911843-	gga-miR-6551-3p	gga-miR-7450-3p	gga-miR-30e-5p	gga-miR-30c-5p	gga-miR-30b-5p
chr22:214764-230476-	gga-miR-148a-5p	gga-miR-6584-5p	gga-miR-1611	gga-miR-205b	gga-miR-1626-3p
chr5:22838365-22845389+	gga-miR-7448-5p	gga-miR-6679-5p	gga-miR-4732-5p	gga-miR-1762	gga-miR-200b-3p
chr3:98482045-98486420-	gga-miR-7450-5p	gga-miR-6673-3p	gga-miR-6655-5p	gga-miR-6577-5p	gga-miR-7464-5p
chr2:102577691-102580382+	gga-miR-1754-5p	gga-miR-6673-3p	gga-miR-148a-5p	gga-miR-7470-5p	gga-miR-1662
chr3:107001245-107001722-	gga-miR-383-3p	gga-miR-6607-5p	gga-miR-1756b	gga-miR-1665	gga-miR-1608
chr13:13265658-13271996+	gga-miR-190a-5p	gga-miR-365-1-5p	gga-miR-7483-5p	gga-miR-217-3p	gga-miR-146b-3p
chr2:145294919-145302275-	gga-miR-29b-1-5p	gga-miR-29b-2-5p	gga-miR-1656	gga-miR-1716	gga-miR-9-5p
chr1:193288082-193294652-	gga-miR-1564-3p	gga-miR-7479-3p	gga-miR-1416-3p	gga-miR-1731-5p	gga-miR-9-5p
chr3:15974512-15976254-	gga-miR-19b-5p	gga-miR-19a-5p	gga-miR-23b-5p	gga-miR-1710	gga-miR-6577-5p
chr8:6335683-6342031-	gga-miR-7481-3p	gga-miR-1685-5p	gga-miR-7462-5p	gga-miR-1645	gga-miR-7483-3p

Table 3: The primers were used for qRT-PCR in this study

CircRNA	Primers
chr21:4172114-4172476-	F: GGGCTTGTTTCTGGTTTGGGTTAG R: GAGACTGTGGTTTTACTTTGTCCTTGAATC
chr19:6585504-6585731+	F: GGTGGACTCCTTCTGGATGTTGTAGT R: GTTGAACCCAGTGACACCATTGAGA
chr9:22282452-22289593-	F: CTGATCCCGAGACATCTAATGTTTCCT R: GACTCTGGTGGCTGATACGGTTGA
chr13:15110945-15122920-	F: CAGGGGTCTGCCAGCACATAGT R: GCTCAAGGAGATGGGTTTTCCG
β-actin	R: GCTCAAGGAGATGGGTTTTCCG F: GAAGTACCCCATTGAACACGG R: AGGCATACAGGGACAGCACA

Biotech (Shanghai, China) and were listed in Table 3. The $2^{-\Delta\Delta Ct}$ method was used to analyze the qRT-PCR results [46].

Bioinformatics analysis

CircRNA and miRNA interactions were predicted using customized Arraystar miRNA target prediction software based on TargetScan and miRanda [51, 52]; the top five putative target miRNAs were identified for upregulated circRNAs in tumor liver samples of ALV-J-susceptible chickens. The putative target genes of these miRNAs were identified using Targetscan [51]. Cytoscape software was used to construct the circRNA-miRNA-gene networks [53]. Homology analysis of chicken circRNA was conducted using blastn (v2.2.27) from the alignment between human and mouse [54]. Gene ontology and KEGG pathway analysis were performed for the differentially expressed circRNA-associated genes [55, 56].

Statistical analysis

Data were processed using GraphPad Prism (version 5.0) and are expressed as the mean ± SE. The Student's *t*-test was used to assess differences among groups, where $P \leq 0.05$ was considered to show significant differences between the groups.

Abbreviations

circRNA, circular RNA; RNA sequencing, RNA-seq; ALV-J, subgroup J avian leucosis virus; miRNA, microRNA; qRT-PCR, quantitative real-time polymerase chain reaction; FC, fold change; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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