Molecular Biology Reports
https://doi.org/10.1007/s11033-018-4534-8

ORIGINAL ARTICLE

The comparative analysis of phenotypic and whole transcriptome gene expression data of ascites susceptible versus ascites resistant chickens

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Received: 10 January 2018 / Accepted: 28 November 2018

Abstract

Ascites syndrome (AS) is a metabolic disorder that mainly occurs at later ages of meat-type chickens. Despite many research, there is no consensus about the origin of this syndrome. Our main purpose were to investigate the syndrome using both phenotypic and RNA-Seq data to elucidate the most causative factors predisposing the birds to AS. Phenotypic data analysis showed that AS indicator traits (AITs) were moderate to high heritable. Inexistence of consistent direct genetic correlation between AITs and growth related traits, indicated that neither faster growth rate nor heavier body weight is the most causative factor affecting the susceptibility of broilers to AS. However, respiratory capacity was revealed to be the most probable factor predisposing the birds to AS, as both lung weight and lung percentage were negatively correlated with AITs. Transcriptomic data analysis revealed 125 differentially expressed genes (DEGs) between the ascitic and healthy groups. Up-regulated genes in ascitic group enriched mainly in gas transport biological process, while down-regulated genes involved in defense response to bacteria, biological adhesion, cell adhesion, killing of cells of another organism and cell division. Genetic association of the DEGs with human cardiovascular diseases suggested excessive heart problems of the ascitic chicks. Heart is, probably, the first tissue suffering from the incompetence of small respiratory system of the AS-susceptible chickens. In other word, tissue hypoxia, that causes free radicals to concentrate in heart cells, may be the commencement of events that finally result to heart failure, suffocation and death of chicks due to the AS.

Keywords Gene expression profile · Ascites · RNA-seq · Ascites indicator traits

Introduction

Ascites syndrome (AS), also called pulmonary hypertension syndrome, is a multifactorial metabolic disorder in meat-type chickens that has raised a considerable economic concern to the poultry industry worldwide, since it was monitored in high altitude in 1970th [1]. Due to its wider occurrence at high altitudes, lower partial O_2 pressure was considered as the most probable cause of AS. However, several research revealed that the lower O_2 pressure in venous blood could make no significant direct influence on the commencement of the AS [2]. Since venous blood is inherently hypoxic [2]. On the contrary, higher venous partial pressure of CO_2 was introduced to have an indirect but significant effect on the incidence of AS [3, 4]. Further studies showed that AS widely occurs at sea level as well. Therefore, selection for higher body weight or faster growth rate seemed to be the most probable factor affecting the incidence of AS [5] as it was truly more prevalent in heavier chicks when

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11033-018-4534-8) contains supplementary material, which is available to authorized users.

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Published online: 05 December 2018

rearing under normal breeding conditions [6]. Although earlier findings suggested a direct influence of the growth rate or heavier body weight on the incidence of AS [5, 6], later results demonstrated that these factors have no significant influence on the occurrence of AS [7]. It was hypothesized that AS can occur in all susceptible individuals regardless of their growth potential, when exposed to severe AS stimulating stressors [8]. Based on this hypothesis, the incidence of AS would probably, be due to unproportioned development of skeletal muscles and internal organs such as heart and lungs [9]. If so, the cardiopulmonary system could no longer support the increasing O_2 demand of rapid growing broilers and thus AS would occur eventually. To date, numerous research have been conducted to elucidate the etiology of AS and to study the physiological mechanisms that are altered due to AS or influenced by the occurrence of AS [6, 10–13]. Also, other researchers surveyed the molecular modifications that occur prior or after the commencement of AS [14–17]. However, molecular modifications in biological mechanisms under AS have not yet been comprehensively studied. Recently, progress in some technologies including nanotechnology, biotechnology and computer sciences has emerged a new field of research called "omic" in which the transcriptomics hold a great promise in studying the gene expression profile of different tissues or diseased cases as well as in discovering the eQTL related to certain diseases or phenotypes. Therefore, the main goals of the current work were (1) to study the genetic relationship of AITs with GRTs as well as with carcass related traits (CRTs) and (2) to investigate the whole transcriptome gene expression profile of ascitic chicks in comparison with their healthy counterparts, in order to assess the influence of smaller lung capacity of ascitic chicks (as was discovered in the step 1) on the gene expression profile of heart right ventricular tissue.

Materials and methods

Rearing protocol

Chickens from a pure AS-susceptible sire line were used in the current study. All chicks were wing-banded for pedigree identification at hatchery. The rearing protocol has previously been described [18]. Briefly, 1458 1-day-old chicks (offspring of 68 sires and 501 dams) were reared together in a house with normal commercial condition (NCC) until day 21 when a total of 464 chicks were transferred to another house with lower than standard temperature in order to stimulate the occurrence of AS using cool temperature as AS inducing condition (AIC). At NCC, the house temperature was kept 33–35 °C during days 1 and 2 and was decreased by 1 °C every other day until 21 °C was achieved on day 21 and kept constant until the end. At AIC, temperature was kept around 15 to 18 °C during daytime and 10 to 15 °C during nighttime until the end [19]. To induce AS in the majority of AS-susceptible chicks, a mild wind stream was generated using water coolers. Drinking water was also supplemented with higher levels of salt (1200 mg/l) as was previously described elsewhere [20, 21]. In addition, the chicks were provided a three-phase diet with higher-than-catalogue energy levels (approximately 100 to 120 units). The characteristics of the used diets are shown in Supplementary Table 1. The remaining rearing protocols were as described previously [18].

Animal ethics statement

The ethical guidelines of the Department of Animal Science of Ferdowsi University of Mashhad, Iran were followed with respect to animal care and use. Birds were checked daily and all moribund chicks were euthanized immediately. Birds with early-stage ascites do not show evident signs of discomfort. However, at late stages of ascites, the ascetic birds can be identifiable from their healthy counterparts when handling and palpation of their abdominal region for accumulated fluid. This is not applicable, however, at practice since the handling of birds may be stressful for the stock. Therefore, handling of the chicks kept minimal at all rearing periods.

Measurement and data collection

Body weight was measured individually once a week after 2 h of starvation. The recording process was carried out in as short time as possible. Growth rate by weekly intervals was calculated as was previously described [20]. Furthermore, growth rate at the first 21 days of age ($GR_{1–21}$), second 21 days of age ($GR_{21–42}$), and the entire of the standard rearing period ($GR_{1–42}$) were also calculated. Traits related to body weight and growth rate at different intervals are referred as growth related traits (GRTs) hereafter.

After the slaughter, carcass weight (C) was measured and the internal organs and legs were removed. Internal organs including lung weight (Lu), liver weight (L), and heart weight (H) were also measured. Moreover, in order to calculate the ratio of right ventricular (RV) to total ventricular (TV) weight (referred as RV/TV hereafter), atrium was precisely removed and the RV were separated from the left ventricular (LV) and weighed afterward. The internal organs weights were further calculated as percentage of live weight. All of the traits related to carcass or the internal organs will be called CRTs hereafter.

For diagnosis of AS, all of the chicks died after the commencement of AIC were necropsied and examined to determine the cause of death. Chicks with ascitic fluid in the abdominal cavity or hydropericardium were considered as

died from AS and therefore recorded as water-belly positive. Moreover, all of the birds that survived until the end of rearing period were examined for AS symptoms after killing by cervical dislocation. Those with above-mentioned signs of AS were also recorded as water-belly positive, while the remaining were considered as water-belly negative. Hereafter, water-belly and RV/TV will be called as AS indicator traits (AITs).

Quantitative genetic analysis

AS was more prevalent at AIC and a number of chicks died before the end of rearing period. Therefore, recording the weight of internal organs from the dead chicks was biased due to the age factor. Water-belly and RV/TV were, however, less biased due to the age factor. Therefore, we utilized the records of AITs which collected from the AIC, while the records of GRTs and CRTs that were collected from NCC in the analyses.

Water-belly is a binomial trait with a discrete distribution as 1 for water-belly positive chicks and 0 for healthy chicks. The genetic relationship between the water-belly and other traits was assessed using Thrgibbs1F90 software [22]. The Gibbs sampler was run for 100,000 rounds, and the first 20,000 rounds were discarded as a warming-up period. A thinning interval of ten rounds was used to retain sampled values that reduced lag correlation among thinned samples. The posterior mean of variance components was calculated using Postgibbs program [22], and those expected values for heritability and genetic correlations of the analyzed traits were calculated, afterward. RV/TV is a quantitative trait with normal distribution. Therefore, genetic relationship of RV/TV with other traits was analyzed using the AI-REML algorithm of WOMBAT software [23]. All of the analyses were based on the bivariate analyses with the following equation;

$$y_{ij} = \mu + sex_j + A_i + e_{ij}$$

where sex is the fixed effect of sex (1 male and 2 female) and A is the random effect of bird.

Sample collection for RNA isolation

At age 39, almost 65 chicks were chosen from those rearing under AIC and were killed by cervical dislocation. Chicks with high hematocrit values (measured at day 21), RV/TV ratio above 0.29, and water-belly positive were considered as diseased while those without the aforementioned signs of AS were categorized as healthy. The RV tissues of 12 healthy and 12 diseased chicks with extreme signature of AS were used for RNA extraction. Total RNA was extracted using BIOZOL reagent according to the manufacturer instructions and was treated with DNase to remove any potential genomic DNA contamination, afterward. The quality of RNA was monitored by 1% agarose gel electrophoresis and the purity and concentration of RNA was checked using a Nanodrop. Samples with 260/280 and 230/260 ratios lower than 2 were re-prepared. After RNA extraction, equal amount of RNA from 6 chicks (3 males and 3 females) were pooled to have four pooled samples (two healthy and two diseased). Each of the pooled samples was considered as a biological replicate and sent to the BGI Company for sequencing.

mRNA sequencing

Samples with RNA integrity number above 7.5 were used for library preparation. Magnetic beads with Oligo dT were used to isolate mRNA from the other types of RNA. The mRNA is fragmented into short fragments using fragmentation buffer and size selection was accomplished afterward. Fragments with average ~ 160 bp in length were selected for cDNA synthesis. Short fragments were purified, end repaired and single nucleotide A (adenine) added. After that, the short fragments were connected with adapters. Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used for assessment of quantification and qualification of the sample libraries. At last, the libraries were sequenced using Illumina HiSeq^{TM}2000 sequencer machines. Raw data were submitted to gene expression omnibus in the National Center for Biotechnology Information with the accession number GSE122745.

Differential gene expression analysis

Raw reads were preprocessed using softwares including FastQC [24], PRINSEQ [25] and Trimmomatic [26] and thus clean reads were acquired for subsequent analyses. Tuxedo software suite was employed for differential expression (DE) analyses. Briefly, bowtie2 [27] was used for building the genome index as is required for Tophat [28] to align clean reads against the ensembl Gallus gallus Build 4.0 reference genome (https://asia.ensembl.org/Gallus_gallus/info/index). It is allowed for only one hit per read as was enabled with option --max-multihits 1. The --read-realign-edit-dist was set to zero as was suggested strongly by developer of the software in order to eliminate the issues might be resulted from the pseudogenes. In addition, the option of -G was enabled to use the annotation file as guidance for splice junction search. After alignment, RseQC software [29] was utilized for post-alignment quality control. Cufflinks, cuffmerge and cuffdiff softwares [30] were utilized for assembly, merging the transcripts created in each samples, and DE analysis, respectively. The bias correction (--frag-bias-correct) option of cufflinks was used in all cufflinks, cuffdiff and cuffmerge analyses. Transcript abundances were measured in fragments per kilo base of exon per million fragments mapped

(FPKM) [31]. The R package CummeRbund [32] was finally employed for visualization of the results.

GO and pathway analysis

List of significantly differentially expressed genes (DEGs) was subjected to gene ontology (GO) and pathway enrichment analysis using DAVID web-tool [33] (available at: http://david.abcc.ncifcrf.gov) in order to determine the functional enrichment of the DEGs and to test for over-representation of the GO categories. Other web-tools such as PANTHER [34] (available at: http://www.pantherdb.org) and geneontology [35] (available at: http://geneontology.org) were also used, but their results were as same as the DAVID's. Therefore, only the results of DAVID web-tool were reported in the current manuscript.

Results

Genetic association between AITs and economic traits

Ascites was highly prevalent, with occurrence of nearly 30% at cold condition and more than 3% at normal condition, as was previously reported elsewhere [18]. The estimated heritability (SE) of RV/TV was 0.23 (0.14) and the estimated heritability (PSD) of water belly was 0.42 (0.09). The genetic correlations of AITs with GRTs at early ages of the chicks were negligibly positive while were considerably negative at later ages. The genetic correlations between AITs and GRTs are reported in Table 1.

All of the CRTs, other than RV, were inversely correlated with water belly. These genetic correlations were −0.70, −0.36, −0.52, −0.76, −0.79, and −0.52 between water belly and C, H, L, Lu, LV and TV traits, respectively. RV was directly correlated to water belly (r = 0.69; SE = 0.25). Genetic correlations between the RV/TV and CRTs were as similar as genetic correlations of water belly with CRTs. The genetic correlations between AITs and CRTs are presented in Table 2.

Although CRTs were negatively correlated to AITs when measured as absolute values, they did not show, however, the similar trend when measured as percentage of body weight. Other than the genetic correlation between AITs and the lung weight, the remaining genetic correlations were extremely affected by the effect of body weight. Genetic correlations of the Lu with the RV/TV and water belly were −0.30 and −0.76, respectively. Similarly, the genetic correlations of the Lu% with the two mentioned AITs were −0.14 and −0.94, respectively. The direction and magnitude of the remaining CRTs' correlations when measured as percentage of body weight were not as similar as when measured as absolute values. The results of genetic correlations between AITs and CRTs when measured as percentage of body weight are presented in Table 3.

Table 1 Genetic correlations between ascites indicator traits (RV/TV and water-belly) and growth related traits (GRTs)

Traits[a]	RV/TV	Water belly
BW_1	NC	NC[b]
BW_7	0.06	0.22
BW_{14}	−0.32	NC
BW_{21}	−0.18	0.17
BW_{28}	−0.26	0.21
BW_{35}	−0.40	−0.12
BW_{42}	−0.45	−0.06
$GR_{1–7}$	−0.10	0.10
$GR_{7–14}$	−0.39	0.12
$GR_{14–21}$	−0.19	0.17
$GR_{21–28}$	0.06	0.41
$GR_{28–35}$	−0.61	−0.37
$GR_{35–42}$	−0.38	−0.65
$GR_{1–21}$	−0.28	0.13
$GR_{21–42}$	−0.38	−0.32
$GR_{1–42}$	−0.43	−0.22

[a]Standard error (SE) of genetic correlations between RV/TV and GRTs varied 0.28–0.52 while posterior standard deviation (PSD) of genetic correlations between water-belly and GRTs varied 0.24–0.93

[b]Algorithm did not converge or the number of Gibbs sampling did not complete

Table 2 Genetic correlations between ascites indicator traits (RV/TV and water belly) and carcass related traits (CRTs) when measured as absolute values

Traits[a]	RV/TV	Water belly
C	NC[b]	−0.70
H	NC	−0.36
L	0.09	−0.52
Lu	−0.30	−0.76
RV	0.90	0.69
LV	−0.44	−0.79
TV	0.28	−0.52

[a]Standard error (SE) of genetic correlations between RV/TV and CRTs varied 0.01–0.07 while posterior standard deviation (PSD) of genetic correlations between water belly and CRTs varied 0.15–0.38

[b]Algorithm did not converge or the number of Gibbs sampling did not complete

Table 3 Genetic correlations between Ascites indicator traits (RV/TV and water belly) and carcass related traits when measured as percentage of body weight

Traits[a]	RV/TV	Water belly
C%	−0.29	−0.62
H%	1	0.93
L%	0.16	0.98
Lu%	−0.14	−0.94
RV%	0.99	0.65
LV%	0.94	0.19
TV%	0.99	NC[b]

[a]Standard error (SE) of genetic correlations between RV/TV and CRTs varied 0.31–0.61 while posterior standard deviation (PSD) of genetic correlations between water belly and CRTs varied 0.02–0.38

[b]Algorithm did not converge or the number of Gibbs sampling did not complete

Table 4 General statistics of next generation sequence reads (pair) pre- and post-quality control and trimming

Sample[a]	Raw reads	Clean reads
As-1	25,389,617	24,992,539
As-2	25,380,387	25,061,262
He-1	25,367,470	25,085,727
He-2	25,364,938	25,070,763
Overall	101,402,412	100,210,291

[a]As-1 and As-2 are two biological replicates of diseased group while He-1 and He-2 are two biological replicates of healthy group

Preprocessing of raw reads

In the second step of the current research, the whole transcriptome gene expression profile of AS-susceptible chicks was studied in comparison with that of non-ascitic healthy chicks using RNA-Seq technology. Almost 25 million paired-end raw reads per sample were obtained from the company. Some reads (~ 1.1 to 2.6% of the reads) failed to pass the preprocessing cutoff thresholds. First, the adapter contamination was searched using the Trimmomatics. Then, first 10 nucleotides at left side were cut. Reads with Phred quality mean value below 25 at sliding windows with 3 bases as well as with poly-A or poly-T tails were trimmed off. In addition, reads with GC content above 80% or below 20%, with unknown nucleotides (N), with length smaller than 50 nucleotides, and mean read quality less than 28 were filtered out. The general statistics of next generation sequence reads for all of the samples pre- and post-processing are presented in Table 4.

Read mapping

Read mapping was carried out using tophat2, a splice junction aware software. Almost 90% (89.7–90.5%) of the reads mapped to the reference genome with 86.9–87.6% of concordant alignments. The post-alignment quality control results showed that almost half of the reads mapped on + strand while the remaining were mapped on − strand with nearly all of the read pairs mapped properly. The distribution of reads over genome DNA strands and different gene feature positions are shown in Supplementary Figs. 1 and 2.

Differential expression analysis

For DE analysis, a total of 21,376 genes, 48,799 Isoforms, 31,806 transcript start sites (TSSs), and 16,223 coding sequences (CDS) were investigated. The results revealed that 125 genes were differentially (Q-value < 0.05) expressed between the diseased and healthy groups. The identification of significant genes was based on the q-value (adjusted p-value for false discovery rate) criterion. The expression of 79 genes (out of 125 significantly DEGs), were up-regulated in the diseased group while the expression of 46 genes were down-regulated. The volcano plot and heatmap of the DEGs are shown in Figs. 1 and 2, respectively.

Investigating the other features of these 125 DEGs showed that 62 isoforms, 85 TSS, and 40 CDS were differentially (Q-value < = 0.05) expressed between the diseased and healthy groups. Forty one out of 62 isoforms, 55 out of 85 TSS and 26 out of 40 CDS were up-regulated in the diseased group while the remaining features were down-regulated. The frequency of significantly expressed features is shown in Fig. 3. The list of DE genes, Isoforms, TSS, and CDS are also reported in Supplementary Tables 2–5, respectively. Almost 90% of the DEGs (106 genes) were from the previously known genes, with relatively good annotations. Whilst, there were no annotations for the remaining 19 loci in the databases. Visualizing these un-annotated genes with IGV genome browser revealed that almost all of them are un-ambiguously from the genes with considerable expression. Further research is needed to annotate these loci and uncover the association of them with AS. The IGV snapshot figures from some of the un-annotated genes are shown in Supplementary Figs. 3–7.

Gene ontology and KEGG pathway analysis

For GO analysis, all of the up- and down-regulated genes were submitted to DAVID database, separately. GO analysis showed that the up-regulated genes were enriched in gas transport biological process, while the down-regulated genes enriched in biological processes such as defense response to bacteria, biological adhesion, cell adhesion, killing of cells

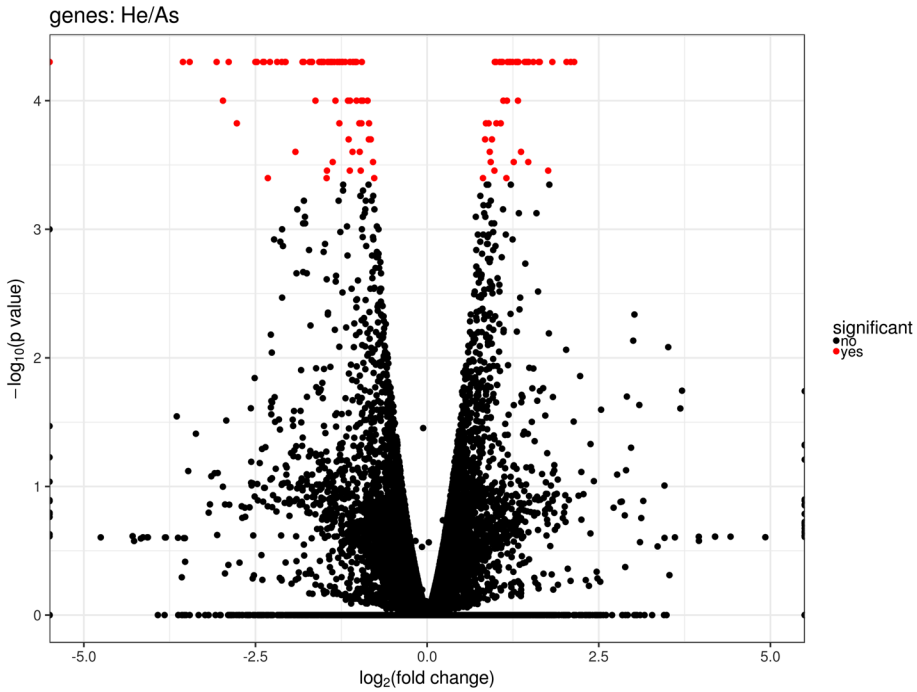

Fig. 1 Volcano plot of the expressed genes in the two ascitic and healthy groups. The red points indicate the differentially expressed genes. (Color figure online)

of another organism and cell division. The GO biological process terms of DEGs are shown in Fig. 4.

There were three pathways namely PPAR signaling, fatty acid metabolism and adipocytokine signaling which enriched with up-regulated genes. All of the three pathways are associated with lipid metabolism. Only one pathway, namely p53 signaling pathway was enriched with down-regulated genes. The significantly enriched pathways are shown in Supplementary Figs. 8–11.

Association of differentially expressed genes with genetic diseases in human

Since the information about the functionality of genes and their potential relationship with genetic disease in farm animals is negligible, we searched the association of the identified DEGs with genetic diseases in human. The results showed that the DEGs were genetically associated with human cardiovascular diseases, suggesting the excessive heart problems of the ascitic chicks.

Discussion

Phenotypic data analysis

As compared with the maternal lines with a relatively light body weight (data not shown), the higher AS frequency in the paternal line with heavier body weight that was used in the current study obliged us to design the current research to investigate the genetic association of AITs with GRTs as well as with CRTs in order to assess the key elements trigger the occurrence of AS. The results revealed that the AITs were moderate to high heritable, suggesting the feasibility of eliminating AS from this highly inbred population. The genetic association analysis showed that there were no consistently direct genetic relationship between the AITs and GRTs, indicating neither faster growth rate nor heavier body weight was the most causative factor predisposing the chicks to AS. We showed, investigating the influence of early growth related traits in our previous research, there was no direct genetic association between the occurrence of AS and either heavy body weight or faster growth rate at early ages of the chicks of the same line [20]. Similar findings have previously been reported in a different broiler line [7, 8]. In another study, we also investigated the potential relationship of the growth curve parameters with the occurrence of AS [18]. The results of that study demonstrated that, although the healthy chicks had more suitable growth curves than the ascitic chicks, neither of the growth curve related traits, however, influenced the occurrence of AS significantly. Gathering together all of the findings, it can be concluded that the heavy body weight and rapid growth rate are probably not the predisposing factors for the development of AS in the studied sire line.

It is then aimed to investigate the genetic relationship of AITs with CRTs either as absolute values or as percentages of body weight. The results showed that the genetic correlation between water belly and almost all of the CRTs (including C, H, L, Lu, LV and TV traits) were large and negative,

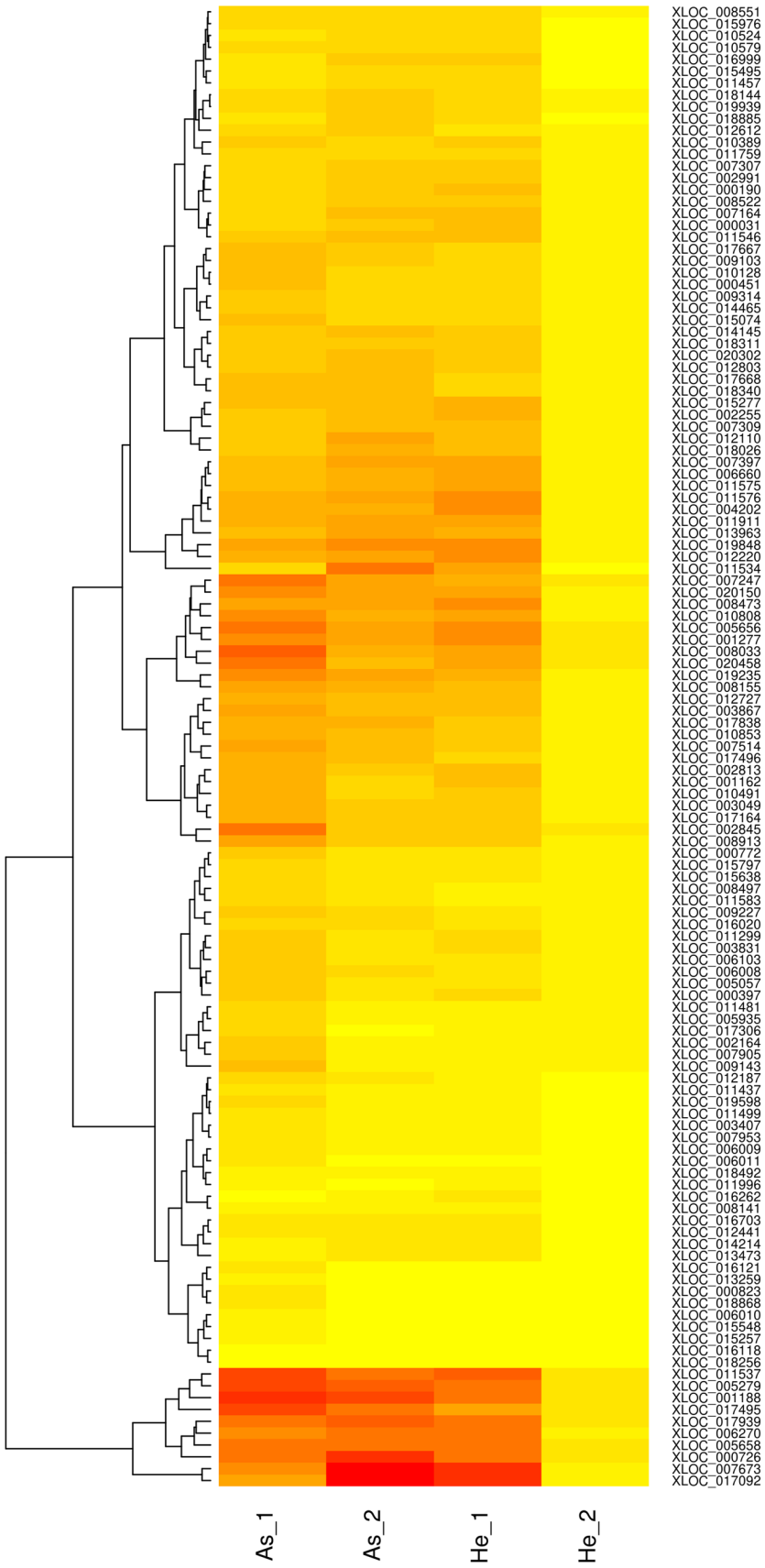

Fig. 2 The heatmap (log FPKM+1) of differentially expressed genes between the ascitic and healthy groups

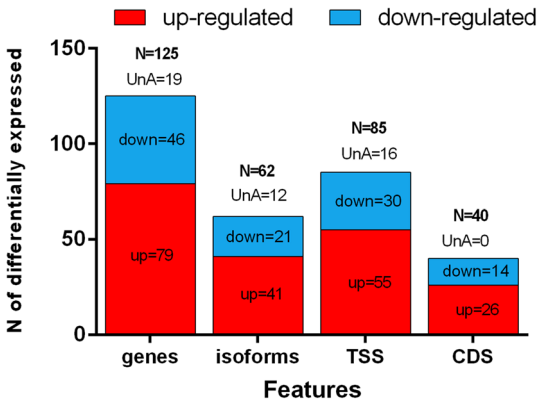

Fig. 3 The number of differentially expressed gene features between the ascitic and healthy groups. *N* Number of differentially expressed features, *UnA* Number of un-annotated novel features, *down* Number of down-regulated features in the diseased group, *up* Number of up-regulated features in the ascitic group

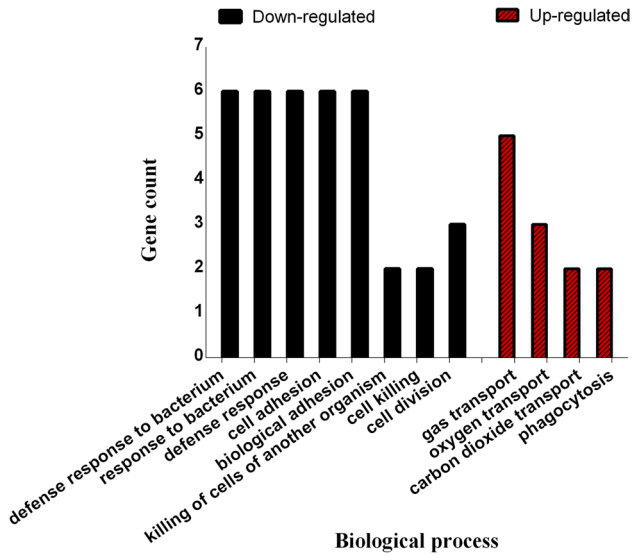

Fig. 4 Gene ontology (Biological process) of significantly up- and down-regulated genes between the ascitic and healthy groups

suggesting that the ascitic chicks had a relatively smaller internal organs than their healthy counterparts. This means that the ascitic chicks suffer from the incompetence of their internal organs in supporting their skeletal muscles' growth. These findings support the findings of Druyan et al. [19] who hypothesized that the AS can occur in all susceptible chicks regardless of their growth rate. Although we found out that the lower relative growth rate of internal organs is the probable cause of the susceptibility of the chicks to AS, however, the most potential causative factor triggering the incidence of AS was not yet identified. As all of the correlations were at the same magnitude and direction, we re-estimated the genetic correlations of AITs with CRTs albeit as percentages of body weight at this time. Here, the direction of genetic correlations between AITs and H%, L%, LV% and TV% traits changed considerably. The Lu%, however, did show the same direction as Lu, indicating that the genetic correlation of AITs with lung weight is not affected by the body weight factor. Therefore, it is concluded that the weight of lung (as an index of respiratory capacity) was, among the other studied internal organ traits, the most causative factor affecting the susceptibility of the birds to AS. This trait, along with the RV/TV, is introduced in the current manuscript as new indicator traits for indirect selection against AS susceptibility. Being the last stage of the syndrome, water belly is not a suitable indicator trait for selection against AS susceptibility. Because, it cannot be observed unless the affected chicks die due to AS. In addition, it is well known that all AS-susceptible chicks do not show the final stages of the syndrome. Whereas, the lung weight can be measured in all chicks at all stages of the syndrome. Our unpublished results (not reported here) have shown that the lung weight was moderate to high heritable and was in a direct genetic relationship with GRTs. This trait was, desirably, in inverse genetic relationship with both heart weight and RV/TV, indicating that the selection based on both higher lung weight and lower RV/TV values could facilitate the genetic improvement of this pure line for both GRTs and resistance to AS.

Transcriptomic data analysis

In addition to the phenotypic data analysis of diseased *vs*. healthy chicks, we carried out a comparative transcriptome analysis of RV tissue of the diseased *vs*. healthy chicks as well using RNA-seq data. This analysis discovered a total of 125 DEGs. Almost 63% of the DEGs were up-regulated in the diseased group while the remaining DEGs were down-regulated. It seems that the number of genes that enhance their expression due to the AS is much larger than the number of genes suppressed. The few high-throughput research that have considered the AS as their primary subjects reported similar results. Wang et al. [14] compared the liver transcriptome of diseased with that of healthy birds using microarray chips and found 178 DEGs with 128 up- and 50 down-regulated genes in the diseased group. Shi et al. [17], also investigating the liver transcriptome of AS-susceptible chicks in comparison with that of healthy controls, identified 390 DEGs, among which, 212 genes were up-regulated and 178 genes were down-regulated in the diseased group. In contrast, Yang et al. [36] identified 437 up-regulated, and 458 down-regulated genes in the pulmonary artery of the diseased chicks as compared with the healthy control.

In this study, 106 out of 125 differentially expressed loci were those already known. However, for 19 loci (13 up- and 6 down-regulated loci) there were no annotation in

the databases. These loci seem to be functional genes with considerable expression that might associated with AS. In other words, the FPKM value for some of these loci were large enough to be considered as functionally important, and therefore indicating that these loci may play key roles in the pathology of AS. Out of these 19 loci, 11 loci were on the scaffolds with their unknown chromosomal positions. Three, out of 13 up-regulated and un-annotated genes were expressed only in the diseased group without any expression in the healthy group. The FPKM values of two of them were above 2, indicating the probable relationship of them with the ascites. Further comparative genomics analysis is needed to uncover the potential relationship of these luci with the AS. In fact rapidly improving of the annotation of chicken genome in near future may help to understand the genetic relationship of these loci with the AS.

Although the identified DEGs enriched in processes (e.g. Gas transfer process) that are in accordance with the effect of factors that have traditionally been known as the most probable factors predisposing the chicks to AS, there were also some other enriched processes that complicated the conclusion of the results. Furthermore, reviewing the enriched GO terms or pathways of the previous studies [14, 17, 36] revealed that, although there were some GO terms or pathways in common between them and the current study (e.g. lipid metabolism pathway and GO term), many of the GO terms or pathways were not in common and thereby made the comparison of the results harder. Thus, relying merely on the results of GO or pathway analysis may not be rational. Because, some of the DEGs have been identified as a causal genes on PAH, a similar disease as AS in human, without any classification in none of the categories of the GO. Therefore, considering the results of GO and KEGG pathway analyses as well as investigating the DEGs individually in databases such as GeneCards (http://www.genecards.org), Medscape (http://www.medscape.com), Malcards (http://www.malcards.org), Reactome (http://www.reactome.org) and other related databases, it is found that the DEGs were generally related with processes such as gas transferring system from the respiratory system to the cell organelles, response to microbial elements, lipid metabolism, and cardiovascular diseases. Therefore, AS probably resulted from the deficiency or influence of one or some of these genes and thus, some of them are discussed in detail below.

Genes related to gas transfer system

Genes such as *RHAG, CA2, HBG1, HBAD* and *HBAA* were all overexpressed in the diseased group as compared to the healthy group. These genes, especially gamma globin genes (*HBG1* and *HBG2*), are normally expressed in the tissues that produce blood cells. Searching Reactome and GeneCards, however, showed that these genes are also expressed in heart cells and almost all of them are directly/indirectly related to gas transferring system from the lungs to organelles within the cells. Consistent with the previous findings [4, 37], we previously showed a meaningful differences in blood gas parameters between the ascitic and healthy chicks in the same line [21]. However, despite having significantly altered blood gas parameters at later ages of the ascitic chicks, as compared with that of their healthy counterparts, we rejected the usefulness of the blood gas parameters as early predictors of ascites [21]. In other words, the blood shows only a negligible amount of differences between the diseased and healthy chicks. Therefore, it seems that the blood, collected from all of the metabolically more or less active organs, may not be a good indicator of the damages probably the metabolically more active organs experience from the reduced O_2 or the increased CO_2 tension. The higher expression of genes related to gas transfer system of the heart cells may be an indication of more pronounced O_2 reduction or CO_2 enhancement in the heart cells. If so, it can be concluded that the heart cells, having much work load and energy metabolism, as compared to other organs, may suffer more from the imbalance between the O_2 supply and CO_2 removal of the gas transferring elements. In such tissue hypoxia or hypercapnia, the accumulated oxidant agents in ascetic bird's tissues [38, 39] may damage the heart cells then the right-sided heart failure can occur as a secondary manifestation of the AS. It is worth mentioned that at least part of the identified DEGs were resulted from the progressive deterioration of cardiac muscle at later stages of the syndrome (day 39) and, thereby, attributing the DEGs to merely the underlying genetic basis of AS susceptibility is not error-free.

Genes related to lipid metabolism

There were some DEGs with direct effect on the lipid metabolism. These genes enriched in three KEGG pathways, including PPAR signaling, fatty acid metabolism, and adipocytokine signaling, all of them are associated with lipid metabolism. Furthermore, it had repeatedly been observed in this study that the ascitic chicks had a negligible reservoir of abdominal fat (personal observations). Therefore, we searched the databases about the possible relationship of the DEGs related to lipid metabolism with the AS. Among the investigated genes, *THRSP, PDK4, CD36*, and *CPT1A* were deduced as more important genes. Thyroid Hormone Responsive (*THRSP*) gene plays a role in the regulation of lipogenesis and is important for the biosynthesis of triglycerides. The lower abdominal fat content of the ascitic chicks is probably as a result of long term fasting of the ascitic chicks. Thus, the up-regulation of this gene might be induced in order to

compensate the lower fat repository of the ascitic chicks. The up-regulation of two other genes that are also related to lipid metabolism namely *FABP4* and *Ex-FABP* is also due to the result of long term fasting of the ascitic chicks. Gentili et al. [40] demonstrated that the heart of chicks embryos treated with antibody against *Ex-FABP* have increased apoptotic cells and high level of fatty acids, significantly, concluding that the accumulation of fatty acid, specific ligand of *Ex-FABP*, in the cell microenvironment is responsible of heart cell death. They suggested that *Ex-FABP* may act as a survival protein by playing a role as scavenger for fatty acids. It is concluded that the lower fat content is related to the increased lipid catabolism due to the reduced feed intake and starvation of the ascitic chicks. The higher catabolism of body fat reservoirs, which is accelerated due to the lower utilization of blood glucose in the ascitic chicks, might increase the fatty acid concentration in the heart cells, which in turn might cause the right ventricular injury of the ascitic chicks' heart. Pyruvate dehydrogenase kinase 4 (*PDK4*) is another gene involved in fatty acid metabolism. *PDK4* regulates glucose and fatty acid metabolism via phosphorylation of the pyruvate dehydrogenase subunits. *PDK4* regulates metabolite flux through the Krebs cycle and down-regulates aerobic respiration and inhibits the formation of acetyl-coenzyme A from pyruvate, therefore, decreases glucose utilization and increases fat metabolism in response to prolonged fasting and starvation [41]. Perhaps the 2.5 fold higher expression of *PDK4* in the ascitic group is associated with the higher blood glucose content of the ascitic group [14–16] as well as significantly lower abdominal fat content of the ascitic chicks. *CD36* also is a gene involved in lipid metabolism. It binds long-chain fatty acids and facilitates their transport into cells, thus participating in muscle lipid utilization, adipose energy storage, and gut fat absorption. It approximately two times overexpressed in the ascitic group than in the healthy chicks. Carnitine palmitoyl transferase 1A (*CPT1A*) is a protein coding gene. The mitochondrial oxidation of long-chain fatty acids is initiated by the sequential action of *CPT I* and *CPT II*, together with a carnitine-acylcarnitine translocase. Perhaps, the enhanced tendency of the heart cells to preserve glucose but utilize fatty acid chains as energy sources have caused the expression level of *CPT1A* to be increased in the ascitic group as its expression level is 2.5 times higher in the diseased group than in the healthy group. On the contrary, *ACACB* (Acetyl-CoA carboxylase) gene, having more than 4.5 folds expression in the ascitic group, has inhibitory effect on the *CPT1A*. Therefore, it seems that the lipid metabolism of the ascitic chicks altered into a too complicated one that can be deduced based merely on gene expression experiments and therefore further research is needed to investigate the potential association of abdominal fat content with AS.

Genes related to cardiovascular or other genetic diseases

There were some DEGs such as *FOS, PLIN1, AHSG, MEF2A, PTX3, PRCP, PDK4, CPT1A, CD36, APLNR, HADHB* and *AOX1* that have been identified as genes related to cardiovascular diseases in human. Pentraxin 3 (*PTX3*) has recently been linked to obesity-associated inflammation, serving as a cardioprotective modulator against cardiovascular disease. In addition to obesity-associated complications, low cardiorespiratory fitness levels could impact exercise-induced *PTX3* elevations, thereby potentially diminishing *PTX3*'s effects of anti-inflammation and/or cardioprotection [42]. Also, *PTX3* has been shown to be elevated in systemic sclerosis patients who had also PAH. Thus, the elevated *PTX3* plasma level has been introduced as an independent parameter associated with the presence of PAH [43]. As compared with that of non-ascitic chicks, the significant (~ fivefold) over-expression of *PTX3* in the diseased chicks might accompany by the high level of *PTX3* sputum level. If so, the high PTX3 sputum level can serve as a predictor or diagnostic tools of ascites susceptible chicks. *APLNR* is related to the angiotensin receptor, inhibits adenylate cyclase and plays a counter-regulatory role against the pressure action of angiotensin II by exerting hypertensive effect, and plays a role in various processes such as heart contractility, and heart failure. *APLNR* is associated with pulmonary venoocclusive disease, which is one of the less commonly encountered causes of pulmonary hypertension that accounts for 5–20% of cases classified as idiopathic PAH in human (Medscape). An important paralog of APLNR is *AGTR1* which is a potent vasopressor hormone and a primary regulator of aldosterone secretion. It is an important effector controlling blood pressure and volume in the cardiovascular system. The effect of *AGTR1* on ascites susceptibility has recently been under wide investigations since it was suspected to be a candidate gene for resistance to AS [44–46]. In the current study, the expression of *AGTR1* was non-significantly higher (4.7 fold) in the ascitic group than in healthy group. Prolylcarboxypeptidase (*PRCP*), also called angiotensinase C, encodes an enzyme that cleaves C-terminal amino acids linked to proline in peptides such as angiotension II, III and regulates renin-angiotensin system. The importance of angiotension II, one of the substrates of this enzyme, in regulating blood pressure suggests that this gene may be related to essential hypertension. Wang et al. [47] showed that prolylcarboxypeptidase D allele coupled with chronic hypertension was associated with a significantly increased risk of preeclampsia, a disease in pregnant women with high blood pressure. Myocyte enhancer factor

2A (*MEF2A*) gene is a transcription factor that activates many muscle-specific, growth factor-induced, and stress-induced genes. Defects in this gene could be a cause of autosomal dominant coronary artery disease 1 with myocardial infarction. Among MEF2A-related pathways are MAPK targets/nuclear events mediated by MAP kinases and NFAT and Cardiac Hypertrophy. The over-expression of *MEF2A* in the heart cells may be an indicator of stress load on the heart cells. *AOX1* (Aldehyde oxidase 1) produces hydrogen peroxide and, under certain conditions, can catalyze the formation of superoxide. *AOX1* is probably involved in the regulation of reactive oxygen species homeostasis [48, 49] and may also catalyze nitric oxide (NO) production. As mentioned, the P53 signaling pathway was the only enriched pathway with down-regulated genes. This pathway is induced by a number of stress signals, among them the oxidative stress, hypoxia and NO notably highlight the significance of this pathway on the AS. The down-regulation of DEGs enriched in this pathway may mean that these genes are probably inefficient in fulfilling the resistance of heart cells of the ascitic chicks against oxidant agents. The damages that result from the oxidant agents such as reactive oxygen species on the cells may appear as various pathologies. Heart failure, which almost all of the ascitic chicks experience, could be caused by reactive oxygen species as some of the studies have demonstrated that the AS is caused due to the effect of these kind of agents [50].

Conclusion

The results of phenotypic data demonstrated that the lower respiratory capacity is probably the main factor triggering the occurrence of AS. Accordingly, the results of transcriptome analysis showed that the heart of AS-susceptible chickens was, probably, the first tissue suffering from the incompetence of small respiratory system in delivering a sufficient amount of O_2 for metabolism. This tissue hypoxia may cause free radicals to concentrate in the heart cells that, in turn, damage the mitochondria and make the electron respiratory chain uncoupled. In such condition, the energy production machine of the damaged cells would not produce sufficient energy for increasing workload of the ascitic birds' heart in pushing the gravid blood into arterioles that resist against blood flow. In the case of persistent pulmonary hypertension, the congestive heart failure would eventually occur and the bird dies from the AS.

Acknowledgements We cordially acknowledge Dr. Hamid Varnaseri, director of NDJ Co, Tehran, Iran, and staffs of the Pure Broiler Breeder Lines Co., Babolkenar, Iran for their technical assistance and for providing the pedigreed chickens for the current study.

Author contributions KH drafted the manuscript and carried out the field work. GHS advised the laboratory work. KH and GHS designed the work and did the statistical and bioinformatics analysis. MN supervised the work and proof read the final manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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