Pakistan J. Zool., pp 1-11, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230225020230

Blood Transcriptome Analysis Indicates Age-Associated Differences in Immune-Related Gene Expression in Captive South China Tigers (*Panthera tigris amoynesis*)

Qunxiu Liu[1], Yingying Wang[2] and Yaohua Yuan[2]*

[1]*Shanghai Urban Construction Vocational College, Shanghai, China*
[2]*Shanghai Zoological Park, Shanghai, China*

Article Information
Received 25 February 2023
Revised 25 March 2023
Accepted 17 April 2023
Available online 27 October 2023
(early access)

Authors' Contribution
LQ carried out the experimental data analysis and wrote all of the manuscript text. WY was responsible for the collection of blood samples and the laboratory procedure. YY was the project manager and supported the funding and coordination of the study.

Key words
Transcriptome, South China tiger, Immune-related genes, Age

ABSTRACT

The South China tiger (SCT) is the most endangered tiger subspecies. Considered to be extinct in the wild, the remaining individuals in captivity may represent the only hope of saving this species. Blood samples of 9 SCTs at different ages were collected and transcriptome analysis was conducted to explore the changes in immune-related genes and pathways expressed with increasing age. A total of 493 million raw paired-end reads and 478 million high-quality reads were obtained. Compared with cubs, adult tigers showed a total of 918 differentially expressed genes (DEGs) (218 up-regulated and 700 down-regulated) and old tigers showed 210 DEGs (82 up-regulated and 128 down-regulated). For adult and old SCTs, the up-regulated DEGs functions were mainly enriched in immune system process (GO: 0002376), immune response (GO: 0006955) and inflammatory response functions (GO: 0006954). LOC102949930, CD86, C3, ANXA1, etc. were closely related to these functions. The cubs expressed more genes in B cell activation/proliferation, DNA replication and mitotic cell cycle process. LOC102965597, LOC107180566 and MZB1 were closely related to B cell activation/proliferation. This is the first study on the immune-related gene expression of SCTs, and the results suggest that cub SCTs do not have fully developed immune systems, and the aging immune systems develop and maintain both a sistentlyactivated innate immune response and adaptive immune function.

INTRODUCTION

Age, both chronological and biological, is the dominant risk factor affecting the immune system (Stanner and Denny, 2009). Changes in the immune system with increasing age are reflected in the susceptibility to infectious diseases (Castelo-Branco and Soveral, 2014), and much research has been carried out across many species to discover the mechanism involved (Lopez *et al*., 2006). There is a strong association between life-history parameters and gene expression across the mammalian lifespan (Fushan *et al*., 2015), and it is crucial to reveal the mechanisms and fully understand the molecular signatures of the normal aging process. Immune system changes with age appear to be universal in mammals and have been reported in many species, including humans (*Homo sapiens*) (Peters *et al*., 2015), wolves (*Canis lupus*) (Charruau *et al*., 2016) and giant pandas (*Ailuropoda melanoleuca*) (Du *et al*., 2019). One of the most characteristic age-related changes in gene expression is an overexpression of the inflammation and immune response genes (de Magalhaes *et al*., 2009).

Transcriptome analysis of tissues and organs provides valuable information on how gene expression changes with age. For the Giant panda, analysis of the blood transcriptome revealed the best represented functional categories of pathways were signal transduction and immune system (Du *et al*., 2015). Further intensive researches on Giant panda indicate that the genes up-regulated with age mainly involved in innate immune response, while those down-regulated with age were mainly related to B cell activation (Du *et al*., 2019). According to Huang *et al*. (2020), the immune function of giant panda improves gradually with age, and changes in the methylation profile are involved in the effects of age on immune and metabolic functions. However, very

* Corresponding author: yuanyaohua2020@qq.com
0030-9923/2023/0001-0001 $ 9.00/0

few transcriptomic signatures of genes associate with aging were identified (Yang *et al*., 2015; Genomes Project Consortium, 2015), and how the transcriptional features related to physiology and pathology changes with age remains unclear (de Magalhaes *et al*., 2009; Yang *et al*., 2015; Peters *et al*., 2015).

The south China tiger (SCT) is the most endangered tiger subspecies and is endemic to China. Ithas long been regarded as extinct in the wild and is classified as critically endangered by the IUCN (Nyhus, 2008), promoting an urgent need for rigorous actions for its conservation (Yuan *et al*., 2021). The captive population originated from six wild founders collected in 1958, with no supplementations from the wild for many decades. The captive SCT population kept a steady growth in past decades, and by the end of 2021, a total of 220 SCT individuals were kept in 17 institutions (zoos and breeding facilities) (Yin, 2021). This captive population constitutes a genetic reservoir, and represents the only hope of saving this species (Luo *et al*., 2010). Based on the current condition of SCT individuals, population management project was carried out to promote the development of captive population, and SCT conservation and coordination meeting will be hold annually to discuss the breeding and allocation plans in next year. In addition, blood sample of each newborn SCT cub was collected for genetic analysis to avoid hybridization. An increasing number of conservation efforts are focused on SCT conservation, yet there is still a lack of information about the biological process and gene mechanism of captive SCTs, especially at the molecular level. Xu *et al*. (2007) assessed the sustainability of the SCT population, and suggested that the captive SCT population is currently suffering an inbreeding depression and declining genetic diversity (Xu *et al*., 2007). Zhang *et al*. (2019) conducted an explicit genetic analysis on the genetic background of 92 SCTs and indicated a moderate level of genetic diversity of this small population. Yuan *et al*. (2021) carried out a population viability analysis of the captive SCT population, and reported a probability of extinction of 1% within the next 100 years (Yuan *et al*., 2021). Guangzhou Zoo, China carried out whole genome sequencing of SCT on chromosome level and shared the data in a public database (https://ngdc.cncb.ac.cn/, number: PRJCA006384). However, no transcriptomic analyses were carried out to examine the relationship between SCT gene expression and biological processes, cellular responses and diseases. In this study, we performed blood transcriptome analyses of nine SCTs to explore their gene expression characteristics at different ages. We focused on one scientific problem. How does gene expression correlate to changes in inflammation/immune responses in SCTs of different ages? We aimed to identity the transcriptional changes related to aging and immune functions, to deeply understand the mechanism of immune process at molecular level. These researches will be much helpful for and conservation of SCT and the physiological functions revealed could serve as the subjects of future studies.

MATERIALS AND METHODS

Laboratory animals and sample collection

Blood samples were collected from nine SCTs in different ages at the Shanghai Zoological Park, Shanghai, China, during a routine examination. Based on the age of SCTs, the samples were categorized into: The cub group (3-4 months old: C1, C2, C3), the adult group (6-7 years old: A1, A2, A3) and the old group (15-16 years old: O1, O2, O3). The blood samples were immediately stored in ACD anticoagulant produced by Sango Biotech Co. (Shanghai, China, product number: B541008).

RNA extraction and sequencing

Total RNA was extracted using a total RNA extractor kit (TRIzol) (B511311, Sangon Biotech Co.) according to the manufacturer's protocol, and treated with RNase-free DNase I to remove genomic DNA contamination. RNA integrity was evaluated with a 1.0% agarose gel. Thereafter, the quality and quantity of RNA were assessed using a Qubit® RNA Assay Kit in Qubit®2.0 Flurometer (Life Technologies, CA, USA). Paired-end sequencing of the library was performed on the HiSeq XTen sequencers (Illumina, San Diego, CA). The genome sequence of Bengal tiger was select as reference genome (SRX5416719).

Data assessment and quality control

FastQC (0.11.2) was used to evaluate the quality of the sequenced data. Raw reads were filtered using Trimmomatic (0.36) including: (1) removing any included adaptor sequences; (2) removing low quality bases from reads 3 to 5 ($Q < 20$); (3) removing low quality bases from reads 5 to 3 ($Q < 20$); (4) using a sliding window method to remove base values of less than 20 reads from the tail (with a window size of 5 bp); and (5) removing reads, and their pairs, with a read length less than 35 nt. The remaining clean data were used for further analysis.

Clean reads were mapped to the reference genome using HISAT2 (v. 2.0) with the default parameters. RSeQC (v. 2.6.1) was used to statistically analyze the results. The Qualimap (v. 2.2.1) was used to check the homogeneity of the data distribution and genome structures. BED Tools (v. 2.26.0) was used to statistical analysis the gene coverage ratio.

Analysis of differentially expressed genes (DEGs) analysis

Gene expression values of the transcripts were computed using StringTie (v. 1.3.3b). Principal component and principal co-ordinates analyses were performed to analyze the distance and differences between samples. The number of transcripts per million (TPM) was used to remove the influence of gene lengths and sequencing discrepancies and enable direct comparisons of gene expression between samples. DESeq2 (v. 1.12.4) was used to determine the DEGs between two samples. Genes were considered to be significant differentially expressed if the Q-value <0.001 and the |foldchange| >2. When the normalized expression of a gene was zero between two samples, its expression value was adjusted to 0.01 (because 0 cannot be plotted on a log plot). If the normalized expressions of a certain gene in two libraries were both lower than 1, further differential expression analysis was conducted without this gene. Gene expression differences were visualized in scatter, MA and volcano plots.

Functional analysis of DEGs

Gene functions were annotated against the Gene Ontology (GO) (http://www.geneontology.org), Kyoto Encyclopedia of Genes And Genomes (KEGG) (http://www.genome.jp/kegg/pathway) and Cluster of Orthologous Groups (COG) databases (http://www.ncbi.nlm.nih.gov/COG). Enrichment analyses were performed to further understand the biological functions of DEGs, based on these three databases.

Function-gene interaction network analysis

Protein-protein interaction networks were constructed for the DEGs using the R package I graph. Cytoscape was used to visualize the function-gene interaction network for DEGs and search for closely related genes.

RESULTS

Transcriptome sequencing

A total of 493 million raw paired-end reads were obtained and 478 million high-quality reads remained after removing adaptor sequences and low quality reads. The total sequence data were about 38.1 Gb. Each of the short-read libraries was aligned onto the Bengal tiger reference genome, and an average of 47.40 million high quality reads (89.75%) could be mapped per sample (Table I). A total of 408 million reads were uniquely mapped with an average mapping ratio of 85.94 %. A total of 26237 reads were identified as potentially novel isoforms (Supplementary Table I).

Identification of DEGs

Based on the TPM values of gene expression, the cub samples clustered into single group (Fig. 1A, B). Compared with the cub group, a total of 918 DEGs were found in the adult group (218 up-regulated and 700 down-regulated) and 210 were found in the old group (82 up-regulated and 128 down-regulated). In the old group, a total of 21 DEGs (seven genes up-regulated and 14 down-regulated) were identified compared with the adult group (Fig. 1C). Compared with the cubs, the adult and the old groups shared more DEGs in common (Fig. 1D).

GO enrichment analysis of DEGs

GO enrichment analyses were performed to determine the biological functions of DEGs. GO annotation and GO

Table I. Summary of sequencing of transcriptome of different blood samples.

Sample	C1	C2	C3	A1	A2	A3	O1	O2	O3
Age	1	1	1	6	6	6	16	15	15
Group	Young	Young	Young	Adult	Adult	Adult	Old	Old	Old
Gender	M	F	F	F	M	M	F	M	M
Average read length(bp)	150	150	150	150	150	150	150	150	150
Total raw reads	57921220	52577350	47697162	56092310	54109982	55629074	56676352	62065578	50558800
Total bases (Gb)	86.88	78.87	71.55	84.14	81.16	83.44	85.01	93.10	75.84
Clean reads	55517140	50331644	46110346	53687352	52194546	53738710	54437416	60271944	48827238
Mapped read pairs	48532442	43914380	41112768	47552222	46883689	50108056	49306191	54050446	44959038
% of mapped reads	87.42	87.25	89.16	88.57	89.82	93.24	90.57	89.68	92.08
Uniquely mapped read pairs	47272869	42695387	39965341	46053321	46051899	45154496	45803722	51724831	43367307
% of uniquely read pairs	85.15	84.83	86.67	85.78	88.23	84.03	84.14	85.82	88.82

C1, C2, C3, cub groups; A1, A2, A3, adult group; O1,O2, O3, old group.

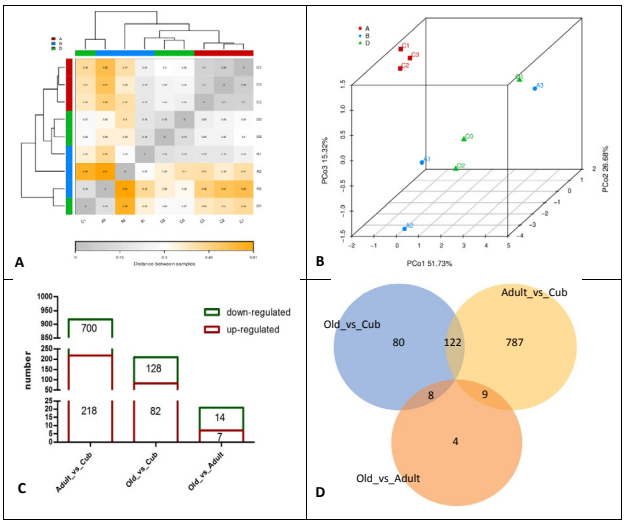

Fig. 1. Profile of the blood transcriptome of SCT. (A) Distance heatmap of all samples based on gene TPM value. (B) PCoA of all samples based on TPM value. (C) Histogram of DEGs in each group (the red box represents up-regulated DEGs, and the green box represents down-regulated DEGs. (D) Venn diagram of the number of DEGs among 3 groups.

categories were examined separately. The expression of immune-related genes was significantly higher in both adult and old groups compared with the cub group, and immune system process was the most significant term in the GO function annotations (Fig. 2A, B). Regarding the up-regulated DEGs in the adult group, of the top 30 enriched terms, 16 were associated with the immune system, such as immune response (GO: 0006955), inflammatory response (GO: 0006954), immune system process (GO: 0002376), innate immune response (GO: 0045087), defense response (GO: 0006952) and regulation of interleukin-8 production (GO:0032677) (Table II). For the old group, the immune related GO terms of the top 30 up-regulated genes were mainly annotated as immune system process (GO:0002376), immue response (GO: 0006955, GO:0045087), response to biotic stimulus (GO:0043207, GO:0009607) and response to other organism (GO: 0051707) (Fig. 3A, B, Table III). Compared with old tigers, adults showed more significant higher gene expression in defense response (GO: 0006952), response to external stimulus (GO: 0043207, GO: 0009605), innate immune response (GO: 0045087) and regulation of immune response (GO: 0050776).

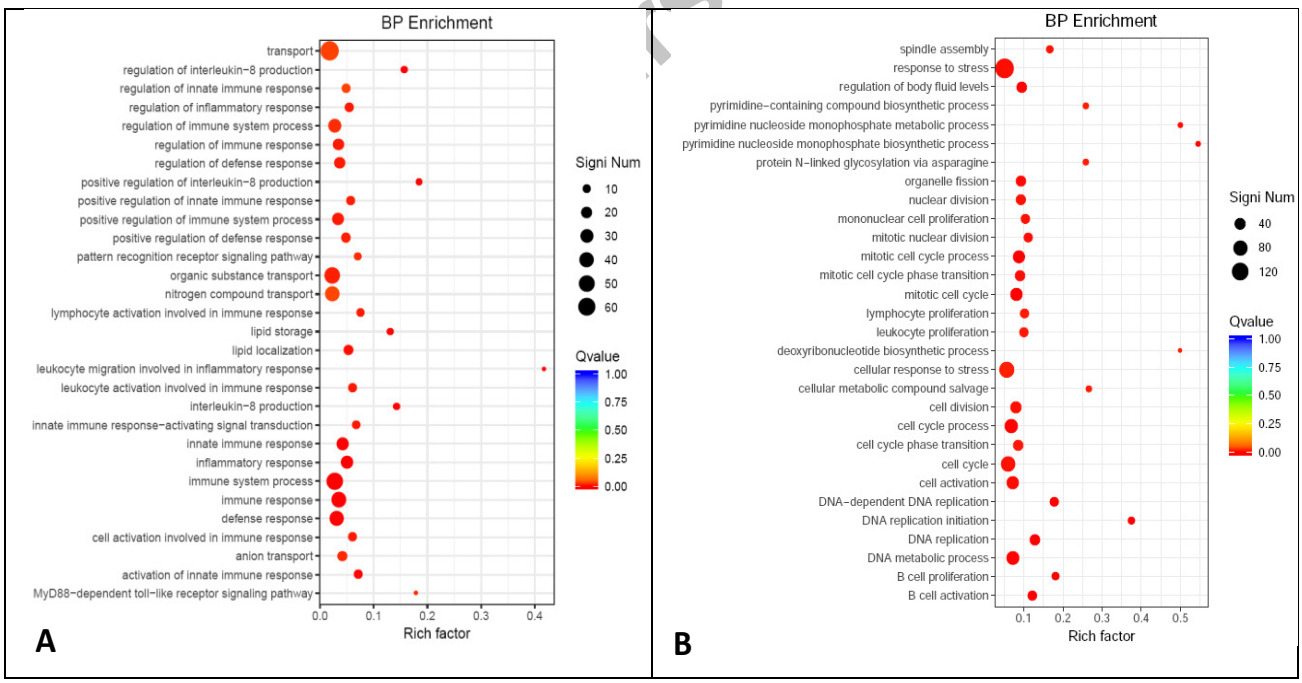

Fig. 2. Enriched functions in BP category of GO annotation for up-regulated (A) and down-regulated (B) DEGs of adult SCTs as compared with cubs.
Notes: The Y-axis represents the function annotation information and the X-axis represents the Rich factor corresponding to the function (The number of DEGs annotated to the function divided by the total number of genes annotated to the function). The Qvalue is represented by the dot color (The closer the color to red, the smaller the Qvalue is). The number of DEGs included in each function is represented by the size of the dot.

Table II. Top 30 enriched GO terms of up-regulated DEGs in adult SCTs as compared with cubs.

GO. ID	Term	Ontology	Significance	Annotated	P value	Q value
GO:0031982	vesicle	CC	79/195	3522/16962	2.30E-10	4.04E-07
GO:0005576	extracellular region	CC	82/195	3877/16962	1.60E-09	1.40E-06
GO:0006955	immune response	BP	42/184	1204/15146	3.00E-10	2.97E-06
GO:0006954	inflammatory response	BP	27/184	541/15146	4.20E-10	2.97E-06
GO:0005615	extracellular space	CC	69/195	3157/16962	1.90E-08	1.11E-05
GO:0044421	extracellular region part	CC	70/195	3288/16962	4.30E-08	1.29E-05
GO:0000323	lytic vacuole	CC	21/195	444/16962	4.40E-08	1.29E-05
GO:0005764	Lysosome	CC	21/195	444/16962	4.40E-08	1.29E-05
GO:0002376	immune system process	BP	55/184	2037/15146	4.10E-09	1.93E-05
GO:1903561	extracellular vesicle	CC	56/195	2447/16962	1.70E-07	3.73E-05
GO:0043230	extracellular organelle	CC	56/195	2449/16962	1.70E-07	3.73E-05
GO:0045087	innate immune response	BP	26/184	610/15146	2.40E-08	8.49E-05
GO:0070062	extracellular exosome	CC	54/195	2433/16962	8.30E-07	0.000162
GO:0005773	Vacuole	CC	21/195	536/16962	1.00E-06	0.000176
GO:0006952	defense response	BP	38/184	1236/15146	7.60E-08	0.000215
GO:0032677	regulation of interleukin-8 production	BP	8/184	51/15146	1.70E-07	0.000384
GO:0002523	leukocyte migration involved in inflammatory response	BP	5/184	12/15146	1.90E-07	0.000384
GO:0031410	cytoplasmic vesicle	CC	37/195	1446/16962	2.90E-06	0.000439
GO:0097708	intracellular vesicle	CC	37/195	1448/16962	3.00E-06	0.000439
GO:0032757	positive regulation of interleukin-8 production	BP	7/184	38/15146	3.20E-07	0.00055
GO:0032637	interleukin-8 production	BP	8/184	56/15146	3.50E-07	0.00055
GO:0019915	lipid storage	BP	8/184	61/15146	6.90E-07	0.000977
GO:0002218	activation of innate immune response	BP	12/184	169/15146	1.00E-06	0.001287
GO:0010876	lipid localization	BP	15/184	282/15146	1.80E-06	0.002123
GO:0002758	innate immune response-activating signal transduction	BP	11/184	163/15146	4.90E-06	0.005156
GO:0002285	lymphocyte activation involved in immune response	BP	10/184	133/15146	5.10E-06	0.005156
GO:0002366	leukocyte activation involved in immune response	BP	12/184	198/15146	5.50E-06	0.00519
GO:0002263	cell activation involved in immune response	BP	12/184	200/15146	6.10E-06	0.005397
GO:0031347	regulation of defense response	BP	21/184	574/15146	6.70E-06	0.005505
GO:0002684	positive regulation of immune system process	BP	24/184	721/15146	7.00E-06	0.005505

KEGG pathway enrichment analysis of DEGs

The KEGG pathway enrichment analysis showed that, the Toll-like receptor signaling pathway (ko04620), Cytokine-cytokine receptor interaction (ko04060), and NF-kappa B signaling pathway (ko04064) were up-regulated in the adult group compared with the cub group (Fig. 4A), while B cell receptor signal pathway was enriched in cubs (Fig. 4B). The Intestinal immune network for IgA production (ko04672) was up-regulated in the old group.

KOG enrichment analysis of DEGs

The KOG enrichment analysis indicated the up-regulated genes enriched in lipid transport and metabolism (I) and defense mechanisms (V).

Function-gene interaction network analysis

For the adult and old groups, the DEGs functions were mainly enriched in immune system process, immune response and inflammatory response. LOC102949930, CD86, C3, ANXA1, etc. were closely related to these

functions (Fig. 5). Two main functions, B cell activation/proliferation and mitotic cell cycle, were enriched in the cub group. The highly expressed genes closely related to B cell activation/proliferation contained LOC102965597, LOC107180566, and MZB1, and the genes related to DNA replication and mitotic cell cycle process contained CDK1, MCM5, and so on.

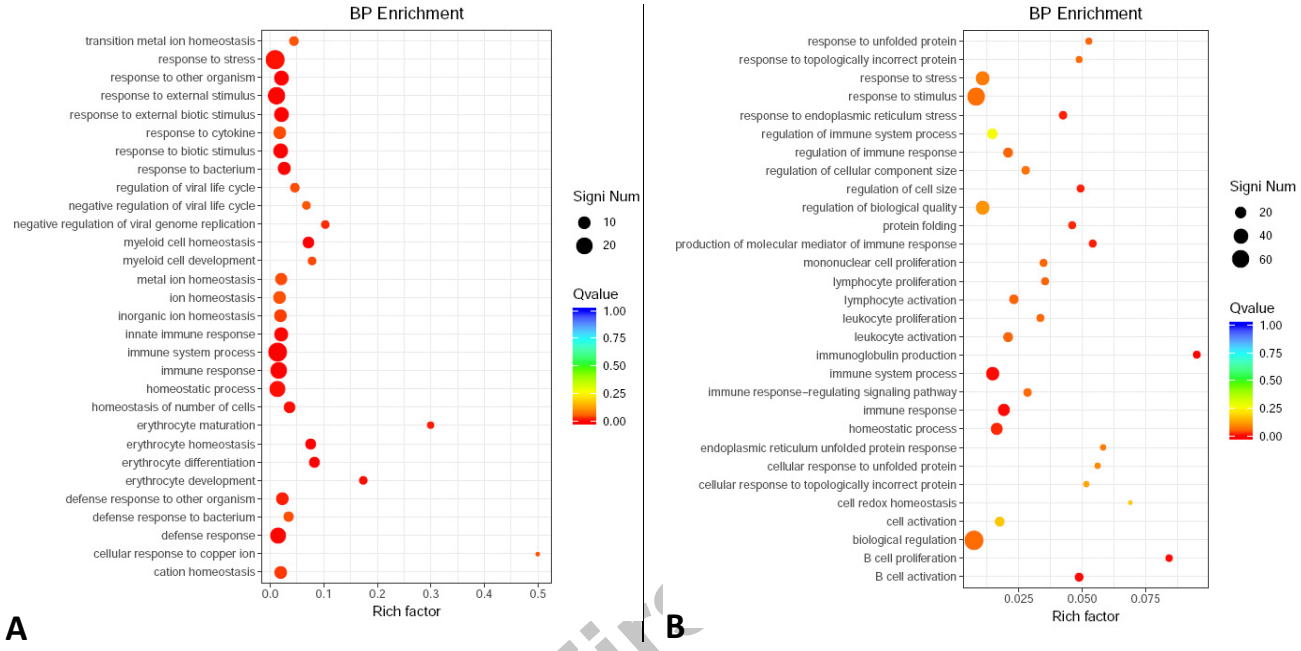

Fig. 3. Enriched functions in BP category of GO annotation for up-regulated (A) and down-regulated (B) DEGs of old SCTs as compared with cubs.
For details of X-axis, Y-axis, Q value and DEGs, see Fig. 2.

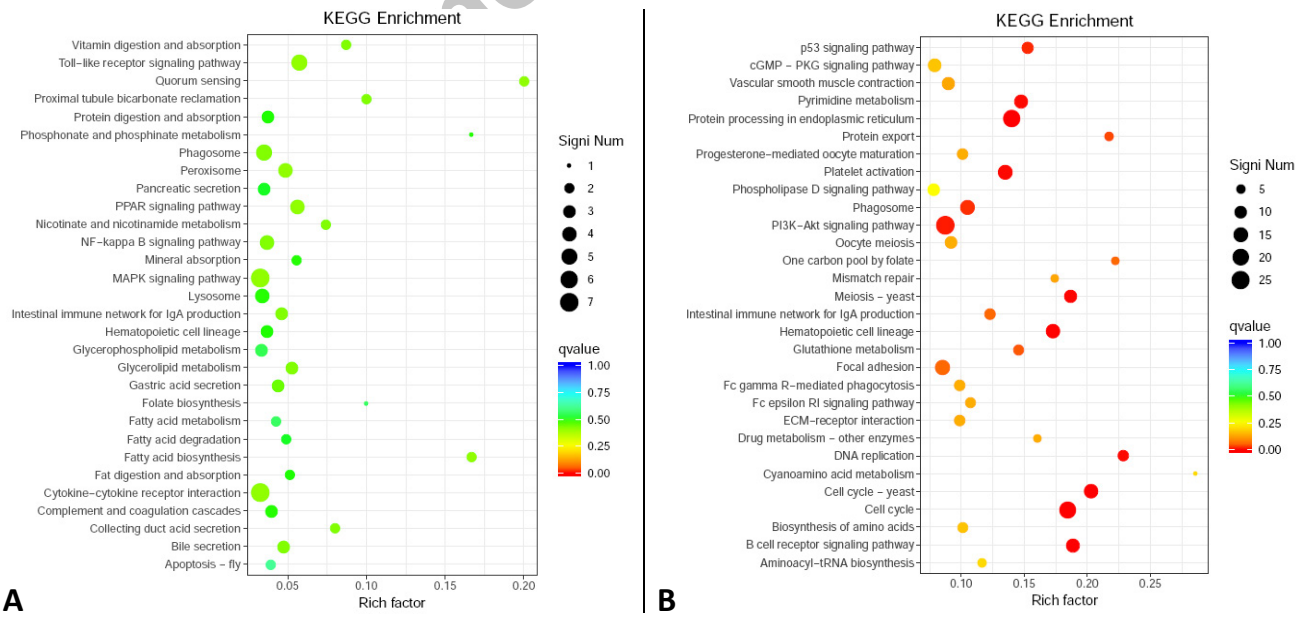

Fig. 4. Enriched functions of KEGG annotation for up-regulated (A) and down-regulated DEGs in adult SCTs as compared with cubs.
For details of X-axis, Y-axis, Q value and DEGs, see Fig. 2.

Table III. Top 30 enriched terms of up-regulated DEGs in old SCTs as compared with cubs.

GO. ID	Term	Ontology	Significant	Annotated	P value	Q value
GO:0005833	hemoglobin complex	CC	7/72	8/16962	1.50E-16	2.63E-13
GO:0005344	oxygen carrier activity	MF	7/64	12/13968	2.40E-14	9.84E-11
GO:0140104	molecular carrier activity	MF	8/64	24/13968	8.60E-14	1.76E-10
GO:0019825	oxygen binding	MF	7/64	25/13968	1.40E-11	1.91E-08
GO:0005506	iron ion binding	MF	9/64	153/13968	3.00E-08	3.08E-05
GO:0002376	immune system process	BP	27/60	2037/15146	2.50E-09	3.54E-05
GO:0002262	myeloid cell homeostasis	BP	8/60	113/15146	1.40E-08	8.02E-05
GO:0006955	immune response	BP	20/60	1204/15146	1.70E-08	8.02E-05
GO:0030218	erythrocyte differentiation	BP	7/60	85/15146	4.10E-08	0.000145
GO:0034101	erythrocyte homeostasis	BP	7/60	93/15146	7.80E-08	0.000194
GO:0043207	response to external biotic stimulus	BP	15/60	724/15146	9.60E-08	0.000194
GO:0051707	response to other organism	BP	15/60	724/15146	9.60E-08	0.000194
GO:0009607	response to biotic stimulus	BP	15/60	761/15146	1.80E-07	0.000318
GO:0009617	response to bacterium	BP	11/60	413/15146	5.60E-07	0.000785
GO:0045087	innate immune response	BP	13/60	610/15146	5.80E-07	0.000785
GO:0009605	response to external stimulus	BP	22/60	1798/15146	6.10E-07	0.000785
GO:0020037	heme binding	MF	7/64	117/13968	1.00E-06	0.00082
GO:0006952	defense response	BP	18/60	1236/15146	7.70E-07	0.000908
GO:0046906	tetrapyrrole binding	MF	7/64	126/13968	1.70E-06	0.001055
GO:0050542	icosanoid binding	MF	3/64	6/13968	1.80E-06	0.001055
GO:0048821	erythrocyte development	BP	4/60	23/15146	1.90E-06	0.002069
GO:0048872	homeostasis of number of cells	BP	8/60	219/15146	2.30E-06	0.002325
GO:0042592	homeostatic process	BP	18/60	1354/15146	2.90E-06	0.002737
GO:0006950	response to stress	BP	28/60	3065/15146	3.70E-06	0.003273
GO:0036041	long-chain fatty acid binding	MF	3/64	10/13968	1.10E-05	0.005639
GO:0043249	erythrocyte maturation	BP	3/60	10/15146	7.00E-06	0.005829
GO:0098542	defense response to other organism	BP	10/60	442/15146	8.30E-06	0.006527
GO:0045071	negative regulation of viral genome replication	BP	4/60	39/15146	1.60E-05	0.01192
GO:0055080	cation homeostasis	BP	10/60	506/15146	2.70E-05	0.019109
GO:0098771	inorganic ion homeostasis	BP	10/60	520/15146	3.40E-05	0.022918

DISCUSSION

This is the first study to assess on the biological process of immune-related gene expression on tigers. Transcriptome sequencing is a powerful tool to identify the DEGs in different samples and the functional difference in gene expression in different tissues and organs (Li *et al*., 2014). Here we provided the peripheral blood transcriptome sequences of SCTs of various ages and these will be useful in annotating the SCT genome in future studies. A total of 1774 DEGs were identified in our study suggesting a species-specific gene expression higher than the 210 DEGs found in the giant panda and 1497 DEGs found in humans (Du *et al*., 2019). Both GO and KEGG pathway enrichment analyses showed that, compared with cubs, the up-regulated genes of adult SCTs were mainly involved in inflammatory and/or immune processes. In contrast, the cubs showed up-regulated genes related to B cell activation/proliferation, and enriched B cell receptor signaling pathway compared with adults.

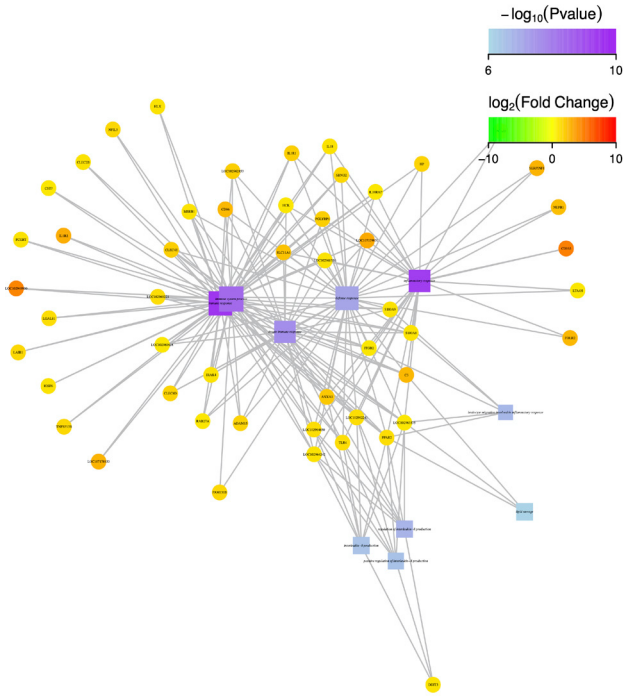

Fig. 5. Function-gene interaction network diagram on the genes related with immune system/process.
Notes: In this figure, square nodes represent enriched functions, circular nodes represent genes, and edges represent the association between genes and functions. The size of the node is proportional to the connectivity (degree), that is, more edges connected to the node, the larger the node; the color of circular node represents the degree of difference in gene expression of samples (logFC value). Green represents down-regulation, red represents up-regulation, and the color depth represents the degree of difference in regulation. The color of the square node represents the enrichment degree of the function (the darker of square node means lower P value and higher enrichment degree). The larger the area of the square node, the more DEGs involved in it, and the greater the contribution to biological phenomena (Top 10 enriched functions and related gene were selected for mapping).

Similar results were reported in wolf blood transcriptomes with increasing age; genes related to B cells were down-regulated and genes associated with innate immunity were significantly up-regulated in adults (Charruau et al., 2016). This suggested that cub SCT cubs do not fully develop their immune systems until they are grow into adults, and that innate immunity is relatively well preserved in aged SCTs. In addition, the increased ability of the adaptive immune system appeared to be closely related to the ability to produce naive lymphocytes and to accumulate functional memory in the lymphocytes (Weng, 2006). This work provided insight into age-related changes in gene expression and provides a foundation for future studies on the molecular mechanisms underlying immune system changes associated with ageing.

The life-histories and traits of mammals vary significantly because of the influence of complex interactions between genetics and environment (Fushan *et al*., 2015). It is well established that immune/inflammatory capacity increases with age and that inflammatory reactions are are associated with various age-related diseases (Bruunsgaard *et al*., 2001; Clements, 2017). Because the immune system is systemic in nature, it is reasonable that immune process changes with age could be detected at the molecular level (deMagalhães *et al*., 2009). According to the Function-gene interaction network analysis, the up-regulated genes related to immune process/response contain LOC102949930 (TNFSF13), CD86, C3 and so on. LOC102949930 (TNFSF13) is a member of the tumor necrosis factor (TNF) ligand family, and this protein and its receptor are both important for B cell development. CD86 encodes a type I membrane protein belonging to the immunoglobulin superfamily, and may play a critical role in the early stages of T-cell activation and costimulation of naive T-cells. Presence of C3 gene indicated a biochemical pathway involved in both innate and adaptive immune responses, and plays an important role in the complement activation pathway. C3 gene was treated as a signature feature of mammalian ageing (deMagalhães *et al*., 2009). TLR4 and TLR9, are pattern recognition receptors involved in the innate immune response to various microorganisms and other exogenous and endogenous stress factors (Kiechl *et al*., 2003) and mediate the production of cytokines necessary for the development of effective immunity (Zhang *et al*., 2013; Evans *et al*., 2010). Adaptive immune responses depend on the ability of lymphocytes to undergo cell division in response to antigenic challenge. For adult SCTs, the adaptive immune system adjusts to age-associated changes and protects the body against most pathogens for nearly all of adult life. However, lymphocytes have a finite replicative lifespan, aging exposes to many antigens lead to the depletion of immusystem (Weng, 2006). Therefore, the functional decline of old tigers may lead to increased susceptibility to diseases. As for the cub tigers in our study, are still partly depended on lactation. Breast milk contains immune competent cells and cytokines, which could partly serve the function of immune regulation to viral resistance. The immune system of cub is relatively immature, and must evolve through exposure to multiple external antigens during young and mature adulthood, including pregnancy. The enriched functions of immune process/system in

adults and initiate immune process in cubs indicated the possibility that the aging immune system maintains a persistently both activated innate immune response and adaptive immune function (Sharma *et al*., 2009), and that adaptive response seems to be more affected by age-related changes than the initiate immune process (Castelo-Branco and Soveral, 2014).

Our results also suggest enriched cell cycle regulation and DNA replication are active in SCT cubs, and that the related up-regulated genes contain CDK1, MCM5, CDC6, TP73 and CDC4. Cyclin-dependent kinase 1 (CDK1) is crucial for cell cycle progression and cell division and has a unique role in cell cycle regulation. CDK1 is an important modulator of various mitotic processes, including cytoskeletal reorganization, chromosome segregation, and daughter cell formation and isolation. De-regulation of CDK1 activity leads to serious defects in these processes (Song *et al*., 2017). CDC6, one of the pre-RC components, initiates DNA replication by recruiting MCM2-7 helicase to the onset of DNA replication dependent on its ATPase activity (Herbig *et al*., 1999). MCM5 plays a critical role in DNA replication and is expressed in any cell capable of proliferation (Stockley *et al*., 2020). The tp73 encodes a member of the p53 family of transcription factors involved in cellular responses to stress and development, and is thought to contain multiple tumor suppressor genes. SCT cubs showed high regulated gene expressions in DNA replication and cell division. This maybe a species-specific aging process which may relate with the rapid growth of individuals (Tomasetti *et al*., 2019). Our study provided insight into the changes in gene expression associated with aging in SCTs and provided a foundation for future studies on the molecular mechanisms underlying immune system changes based on sequencing of the blood transcriptome.

ACKNOWLEDGEMENT

We appreciate the support from GUI Jianfeng and YU Jianyi of the Veterinary Department of the Shanghai Zoological Park for blood collection. We also thank the Shanghai Sango Company for transcriptome sequencing.

Funding

This project was funded by Shanghai Landscaping and City Appearance Administrative Bureau (Project number: G200404)

IRB approval

Institutional Review Board of Shanghai Zoological Park provided IBR approval for the study.

Ethical approval

The samples collection process was approved by the animal welfare committee of the Shanghai Zoological Park (Document Number: SZP006).

Data availability

The raw sequencing data supporting the findings of this study are available in the NCBI BioProject database (Accession no. PRJNA917069)

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20230225020230

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Bruunsgaard, H., Pedersen, M. and Pedersen, B.K., 2001. Aging and proinflammatory cytokines. *Curr. Opin. Hematol*., **8**: 131-136. https://doi.org/10.1097/00062752-200105000-00001

Castelo-Branco, C. and Soveral, I., 2014. The immune system and aging: A review. *Gynecol. Endocrinol.*, **30**: 16-22. https://doi.org/10.3109/09513590.2013.852531

Charruau, P., Johnston, R.A., Stahler, D.R., Lea, A., Snyder-Mackler, N., Smith, D.W., vonHoldt, B.M., Cole, S.W., Tung, J. and Wayne, R.K., 2016. Pervasive effects of aging on gene expression in wild wolves. *Mol. Biol. Evol*., **33**: 1967-1978. https://doi.org/10.1093/molbev/msw072

Clements, S.J., 2017. *The impact of diet on immunosenescence*. Thesis of the University of East Anglia. Norwich

de-Magalhães, J.P., Curado, J. and Church, G.M., 2009. Meta-analysis of age-related gene expression profiles identifies common signatures of aging. *Bioinformatics*, **25**: 875-881. https://doi.org/10.1093/bioinformatics/btp073

Du, L.M., Li, W.J. and Fan, Z.X. 2015. First insights into the giant panda (*Ailuropoda melanoleuca*) blood transcriptome: A resource for novel gene loci and immune genetics. *Mol. Ecol. Resour*., **15**: 1001-1013. https://doi.org/10.1111/1755-0998.12367

Du, L.M., Liu, Q., Shen, F.J., Fan, Z.X., Hou, R., Yue, B.S. and Zhang, X.Y., 2019. Transcriptome analysis reveals immune-related gene expression changes with age in giant panda (*Ailuropoda*

melanoleuca) blood. *Aging*, **11**: 249-262. https://doi.org/10.18632/aging.101747

Evans, D.T., Serra-Moreno, R., Singh, R.K. and Guatelli, J.C., 2010. BST-2/tetherin: A new component of the innate immune response to enveloped viruses. *Trends Microbiol*., **18**: 388-396. https://doi.org/10.1016/j.tim.2010.06.010

Fushan, A.A., Turanov, A.A., Lee, S., Kim, E.B., Lobanov, A.V., Yim, S.H., Buffenstein, R., Lee, S., Rhee, H., Kim, J., Yang, K. and Gladyshev, V.N., 2015. Gene expression defines natural changes in mammalian lifespan. *Aging Cell*, **14**: 352-365. https://doi.org/10.1111/acel.12283

Herbig, U., Marlar, C.A. and Fanning, E., 1999. The Cdc6 nucleotide-binding site regulates its activity in DNA replication in human cells. *Mol. Biol. Cell*, **10**: 2631-2645. https://doi.org/10.1091/mbc.10.8.2631

Huang, X.Y., Ouyang, Q.Y. and Ran, M.X., 2020. The immune and metabolic changes with age in Giant panda blood by combined transcriptome and DNA methylation analysis. *Aging*, **12**: 21777-21797. https://doi.org/10.18632/aging.103990

Kiechl, S., Wiedermann, C.J. and Willeit, J., 2003. Toll-like receptor 4 and atherogenesis. *Annls Med*., **35**: 164-171. https://doi.org/10.1080/07853890310008215

Li, M., Wang, F., Jiang, Q., Ma, J. and Xiong, A., 2014. Identification of SSRs and differentially expressed genes in two cultivars of celery (*Apium graveolens* L.) by deep transcriptome sequencing. *Hortic. Res*., **10**: 1-9. https://doi.org/10.1038/hortres.2014.10

Lopez, A.D., Mathers, C.D., Ezzati, M., Jamison, D.T. and Murray, C.J., 2006. Global and regional burden of disease and risk factors, 2001: Systematic analysis of population health data. *Lancet*, **367**: 1747–1757. https://doi.org/10.1016/S0140-6736(06)68770-9

Luo, S.J., Johnson, W.E. and O'Brien, S.J., 2010. Applying molecular genetic tools to tiger conservation. *Integr. Zool*., **5**: 351-362. https://doi.org/10.1111/j.1749-4877.2010.00222.x

Nyhus, P., 2008. Panthera tigris *ssp.* amoyensis. *The IUCN red list of threatened species 2008*: e.T15965A5334628.

Peters, M.J., Joehanes, R. and Rilling, L.C., 2015. The transcriptional landscape of age in human peripheral blood. *Nat. Commun*., **6**: 8570.

Sharma, G., Hanania, N.A. and Shim, Y.M., 2009. The aging immune system and its relationship to the development of chronic obstructive pulmonary disease. *Proc. Am. Thorac. Soc*., **6**: 573-580. https://doi.org/10.1513/pats.200904-022RM

Song, Z., Pan, Y., Ling, G., Wang, S., Huang, M., Jiang, X. and Ke, Y., 2017. Escape of U251 glioma cells from temozolomide-induced senescence was modulated by CDK1/survivin signaling. *Am. J. Transl. Res*., **9**: 2163-2180.

Stanner, S. and Denny, A., 2009. Healthy ageing: The role of nutrition and lifestyle. A new British nutrition foundation task force report. *Nutr. Bull*., **34**: 58-63. https://doi.org/10.1111/j.1467-3010.2008.01734.x

Stockley, J., Akhand, R., Kennedy, A., Nyberg, C., Crosbie, E.J. and Edmondson, R.J., 2020. Detection of MCM5 as a novel non-invasive aid for the diagnosis of endometrial and ovarian tumours. *BMC Cancer*, **20**: 1000. https://doi.org/10.1186/s12885-020-07468-y

The 1000 Genomes Project Consortium, 2015. A global reference for human genetic variation. *Nature*, **526**: 68-74.

Tomasetti, C., Poling, J. and Roberts, N.J., 2019. Cell division rates decrease with age, providing a potential explanation for the age-dependent deceleration in cancer incidence. *Biol. Sci.*, **116**: 20482-20488. https://doi.org/10.1073/pnas.1905722116

Weng, N.P., 2006. Aging of the immune system: How much can the adaptive immune system adapt? *Immunity*, **24**: 495-499. https://doi.org/10.1016/j.immuni.2006.05.001

Xu, Y.C., Fang, S.G. and Li, Z.K., 2007. Sustainability of the South China tiger: Implications of inbreeding depression and introgression. *Conserv. Genet*., **8**: 1199-1207. https://doi.org/10.1007/s10592-006-9276-9

Yang, J.J., Huang, T., Petralia, F., Long, Q., Zhang, B., Argmann, C., Zhao, Y., Mobbs, C.V., Schadt, E.E., Zhu, J., Tu, Z.D. and Consortium, G., 2015. Synchronized age-related gene expression changes across multiple tissues in human and the link to complex diseases. *Sci. Rep*., **5**: 15145.

Yin, Y.Z., 2021. *International South China Tiger* (Panthera tigris amoyensis) *studbook*. Chongqing Zoological Garden.

Yuan, Y.H., Yin, Y.Z. and Liu, Q.X., 2021. Inbreeding depression and population viability analysis of the South China tigers (*Panthera tigris amoyensis*) in captivity. *Mammal. Biol.*, **101**: 803-809. https://doi.org/10.1007/s42991-021-00113-6

Zhang, B., Liu, X., Chen, W. and Chen, L., 2013. IFIT5 potentiates antiviral response through enhancing

innate immune signaling pathways. *Acta biochim. biophys. Sin.*, **45**: 867-874. https://doi.org/10.1093/abbs/gmt088

Zhang, W.P., Xu, X., Yue, B.S., Hou, R., Xie, J.J., Zou, Z.T., Han, Y., Shen, F.J., Zhang, L., Xie, Z., Yuan, Y.H., Yin, Y.Z., Fu, W.Y., Chen, D.Q., Huang, W., Liu, Z.Y., Tang, Y., Zhao, B., Zhang, Q., Chen, W., Zhang, R.Y., Chen, J., Luo, S.J. and Zhang ZH., 2019. Sorting out the genetic background of the last surviving South China tigers. *J. Hered.*, **110**: 641-650. https://doi.org/10.1093/jhered/esz034

Pakistan J. Zool., pp 1-11, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230225020230

Supplementary Material

Blood Transcriptome Analysis Indicates Age-Associated Differences in Immune-Related Gene Expression in Captive South China Tigers (*Panthera tigris amoynesis*)

Qunxiu Liu[1], Yingying Wang[2] and Yaohua Yuan[2]*

[1]*Shanghai Urban Construction Vocational College, Shanghai, China*
[2]*Shanghai Zoological Park, Shanghai, China*

Supplementary Table I.

Cuffcompare code	Number of transcripts	% of transcripts	Description
u	6022	22.95	Unknown, intergenic transcript
j	16177	61.66	Potentially novel isoform (fragment): at least one splice junction is shared with a reference transcript
i	1664	6.34	A transfrag falling entirely within a reference intron
c	1612	6.14	Contained
o	762	2.91	Generic exonic overlap with a reference transcript
Total	26237	100	

* Corresponding author: yuanyaohua2020@qq.com
0030-9923/2023/0001-0001 $ 9.00/0