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Transcriptional Analysis of Host Responses to Marek's Disease Virus Infection in Chicken Thymus

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Key Words
Marek's disease virus · Chicken · Thymus · Microarray

Abstract
Marek's disease virus (MDV) is a cell-associated alpha-herpesvirus that causes T-cell lymphomas and nervous disorders in chickens. Different from other lymphoid organs, the thymus is the site of T-cell maturation and differentiation. However, the transcriptional response to MDV infection in the chicken thymus is still not known. In this study, we performed genome-wide expression analysis in thymus tissues of RB1B-infected chickens at different time points to investigate the molecular mechanisms of MDV pathogenesis. The number of differentially expressed genes with 2-fold or higher changes (>2) are as follows: 1,250 genes (7 dpi), 834 genes (14 dpi), 1,958 genes (21 dpi), and 2,306 genes (28 dpi). Gene ontology enrichment analysis revealed that the upregulated genes were involved in immune and inflammatory response at 7 dpi; angiogenesis, cytoskeleton organization, cell adhesion, and signal transduction showed different expressions at 21 and 28 dpi. The expression pattern of 18 randomly selected genes was confirmed by real-time RT-PCR. Several differently expressed host genes associated with tumor development are discussed. We identified the global host-gene expression pattern in the thymus of chickens that responded to MDV infection. The present data may provide groundwork for future investigation in the biology and pathogenesis of MDV.

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Introduction

Marek's disease is a lymphoproliferative disease in domestic chickens caused by a highly oncogenic, cell-associated alpha-herpesvirus termed Marek's disease virus (MDV) [1]. MDV infection in chicken induced T-cell lymphomas. The thymus, unique among lymphoid organs, is where T cells mature and differentiate. Severe atrophy of the thymus is found in MDV-infected chickens. This indicates that MDV does not destroy cell-mediated immune but certain cells of the system (e.g. CD4+ cells), leading to immune suppression [2]. A higher transcriptional response in the spleen, both in resistant and susceptible lines of chickens at 2~4 days after infection (dpi), was reported [3]. Several studies have examined host responses to MDV infection in chicken embryo fibroblasts as well as spleen and liver tissue of chicken by microarray analysis [4–7]. Little is known, however, about transcrip-

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tional responses occurring in the chicken thymus during MDV infection. We found that a latent infection was established at 14 dpi in infected thymuses in our prior research; the expression of most of the genes was reduced or shut down at this stage [8]. We speculated that genes related to immune response and tumor development may exhibit different trends in transcriptional activity in infected chicken thymuses at different time points.

The availability of the chicken genome sequence [9] and commercially available whole-genome microarrays has revolutionized our ability to evaluate host-pathogen interactions [10] and has been applied to the study of host response to MDV [11]. Earlier microarray studies were mainly performed in vitro to investigate the interactions between chicken embryo fibroblasts and HVT or the RB1B strain of MDV [5, 6]. The differential expressions of host genes were involved in antigen presentation (MHC class I, MHC class II, and β_2-microglobulin) and interferon responses in infected cells [5, 6]. Recently, some studies have focused on chicken spleen responses to the MDV. They found the expression levels of more than 22 immune-response and related genes were downregulated, while the expression levels of at least 58 genes were increased at 5 dpi (cytolytic infection), compared with age-matched control birds [12]. In comparison, of 73 immune-response and related genes, 67 genes were downregulated, with only 6 genes having higher expression levels at 15 dpi (latency infection). Many MDV-induced downregulated genes are critical for an effective antiviral immune response. Kano et al. [13] found that the expression of T-cell receptor 1-related genes was upregulated in vaccinated-challenged chickens compared to unvaccinated chickens during the latent phase of infection. However, a comprehensive gene expression in response to MDV infection in the thymuses of chickens at different stages of viral pathogenesis – including the cytolytic, latent, and transformation phases – remains unknown.

In this study, we performed a comprehensive gene expression study using Affymetrix Gene-Chip Chicken Genome Arrays to analyze the host response to infection in chicken thymus tissues at 7, 14, 21, and 28 dpi. Our results revealed a lower transcriptional response at 14 dpi than other time points during MDV infection. Gene ontology (GO) enrichment analysis showed upregulated expressed genes involved in immunity and inflammatory response at 7 dpi. Others associated with angiogenesis, cytoskeleton organization, cell adhesion, and signal transduction upregulated at 21 and 28 dpi. We experimented with several host genes (e.g. JUN, STAT3, STMN1, and OCM2) associated with tumor development.

Materials and Methods

Chickens and Virus

All chickens used in this study were 1-day-old specific pathogen-free white Leghorns obtained from Merial Vital (Laboratory Animal Technology Co., Ltd., Beijing, China). Chickens were housed in an isolation facility at the College of Veterinary Medicine, Yangzhou University. RB1B strains of very virulent MDV were maintained in the laboratory.

Experimental Design

The experimental work was performed as reported before [8, 14]. Briefly, infected and uninfected control birds were kept in separate units with similar environmental conditions. At 7, 14, 21, and 28 days after infection (dpi), 6 chickens (3 infected and uninfected control birds each) were sacrificed, and whole thymuses were rapidly excised, rinsed with ice-cold phosphate-buffered saline (pH 7.4) to remove blood contaminants, and then immediately stored in liquid nitrogen until gene expression analysis.

RNA Extraction and Purification

The thymus samples were from the same chickens used in previous studies [8, 14]. Total RNA was extracted using TRIzol Reagent (Life Technologies, Carlsbad, Calif., USA) following the manufacturer's instructions and checked for a RIN number to enable inspection of RNA integrity with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, Calif., USA). Qualified total RNA was further purified by an RNeasy Mini Kit (Qiagen GmbH, Germany) and an RNase-Free DNase Set (Qiagen).

RNA Amplification and Labeling, Array Hybridization and Data Acquisition

Total RNA was amplified, labeled, and purified using GeneChip 3′IVT Express Kit (Affymetrix, Santa Clara, Calif., USA) following the manufacturer's instructions to obtain biotin-labeled cRNA. Array hybridization and wash was performed using GeneChip® Hybridization and the Wash and Stain Kit (Affymetrix) in the Hybridization Oven 645 (Affymetrix) and Fluidics Station 450 (Affymetrix) by meticulously following the manufacturer's instructions. Slides were scanned by a GeneChip® Scanner 3000 (Affymetrix) and analyzed using Command Console Software 3.1 (Affymetrix) with default settings. Raw data were normalized using a quantile algorithm and Gene Spring Software 11.0 (Agilent Technologies). Genes in which expression was significantly different between the compared groups were chosen based on a log 2-fold change ≥2 in gene expression, t test p value <0.05, and corrected for multiple testing by the Benjamin-Hochberg method. Differentially expressed genes between control and infected groups were identified by a threshold of ≥2-fold change and $p \le 0.05$.

Bioinformatics Analysis of Microarray Data

Significantly different genes were determined using GeneSpring GX Software. Gene preliminary screening was conducted using the SBC Analysis System. To highlight the most relevant GO terms associated with a given gene list, we performed GO term enrichment analysis by GOEAST (Gene Ontology Enrichment Analysis Software Toolkit; http://omicslab.genetics.ac.cn/GOEAST/index.php), a Web-based software toolkit for GO enrichment analysis. The log-transformed expression values (with a base of 2) of normalized microarray data were conducted with gene sets and

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Table 1. Primers used for real-time PCR

Gene Symbol	Nucleotide sequence		Product size, bp	Reference/accession No.
CD74	F: R:	5′-TCAGACCCCAGGAAGACATT-3′ 5′-CTCAAAATCCTGCCAGTCCA-3′	102	NM_001001613.1
BLA	F: R:	5′-GTCATGATCGGCAACTCCAACC-3′ 5′-TATCGGCGTAGCAGATGAGGAC-3′	118	NM_001245061.1
BLB2	F: R:	5′-GCTGACTGCCACTACCTGA-3′ 5′-GGCGTTGCTGTTCCAGTAT-3′	159	NM_001044679.1
TLR2	F: R:	5′-GCAACTGGAAAATGTGGGC-3′ 5′-CGGGCGAATGAAGTCCAAAC-3′	140	NM_001161650.1
TLR4	F: R:	5′-TGACCTACCCATCGGACACT-3′ 5′-CTCAGGGCATCAAGGTCTCC-3′	171	NM_001030693.1
CD8B	F: R:	5′-AACAAACAACAGCACAGAGA-3′ 5′-CGGACTGGAAAATAGCAAGA-3′	115	NM_205247.2
CD8A	F: R:	5′-TCAGAGCCAGGAACAAGCA-3′ 5′-CAGGCATCCCATTTGGAG-3′	178	NM_205235.1
VAV3	F: R:	5′-ACCCGCAGATGTCCCAGTT-3′ 5′-CAATAGGAGTGCGGGAAAG-3′	174	NM_206863.2
GADD45A	F: R:	5′-GCTGCGAGAACGACATCA-3′ 5′-GGGGATTCGTGACCAAGAC-3′	123	NM_001044678.1
CASP6	F: R:	5′-CGTGGGACTTTGGCAGACA-3′ 5′-TGTAGTCATCCCGAGAGGC-3′	133	NM_204726.1
RB1	F: R:	5′-TGCGACCTCACAAACACAGA-3′ 5′-CAGGTCAGGATGTTCGGAG-3′	130	NM_204419.1
p21	F: R:	5′-CGAGCAGATCCAGAACGACT-3′ 5′-GCGTCTCGGTCTCGAAGTT-3′	92	NM_204396.1
VIM	F: R:	5′-CAACACGGAGTTCAAGGCGA-3′ 5′-GATGTAGTTGGCGAAGCGGT-3′	79	NM_001048076.1
NPM1	F: R:	5′-GGTTACATTAGGGGCTGG-3′ 5′-GTTGCCTTCGTAGTCCAGTG-3′	73	NM_205267.1
HSP90AA1	F: R:	5′-TTTGACTGACCCGAGCA-3′ 5′-TTGGTCATCCCTATGCCG-3′	110	NM_001109785.1
HIF1A	F: R:	5′-CGTTCCTCAGTCGTCACAGT-3′ 5′-ACCTGCCCTTTCGTGAACAT-3′	178	NM_204297.1
p53	F: R:	5′-GTTACCACGACGACGAGACC-3′ 5′-ACAGCACCGTGGTACAGTCA-3′	90	NM_205264.1
ANXA1	F: R:	5′-AAAACTGCCTGACTGCCCTT-3′ 5′-TTCCACTCCCCTTCATTGCC-3′	90	NM_206906.1
18S	F: R:	5′-TCAGATACCGTCGTAGTTCC-3′ 5′-TTCCGTCAATTCCTTTAAGTT-3′	154	[17]

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Table 2. GO enrichment analysis for upregulated gene transcripts in infected chicken thymus tissues according to biological process

GO_ID	Term	Days after infection (q)				Days after infection (p)				Days after infection (log odds ratio)			
		7	14	21	28	7	14	21	28	7	14	21	28
GO:0030036	actin cytoskeleton organization				26				1.93E-02				1.1558144
GO:0002526	acute inflammatory response	5				3.91E-02				3.02017			
GO:0002250	adaptive immune response				7				5.19E-02				2.2850974
GO:0001525	angiogenesis	12		16		1.72E-02		7.16E-03		1.8872757		1.6548605	
GO:0019882	antigen processing and presentation	8		13	14	3.12E-02		2.99E-04	3.74E-04	2.273744		2.3267311	2.1976346
GO:0065007	biological regulation	270	160	387	490	2.32E-12	1.22E-03	2.07E-12	1.01E-22	0.6842486	0.5131699	0.5561701	0.6606065
GO:0001568	blood vessel development	21		27	26	5.59E-04		6.92E-04	2.21E-02	1.716435		1.4315524	1.1410929
GO:0048514	blood vessel morphogenesis	19		23	20	4.18E-05		1.30E-04	3.01E-02	2.0960648		1.7242466	1.286601
GO:0007155	cell adhesion			55	64			2.09E-04	5.28E-05			1.0112982	0.9939267
GO:0007154	cell communication	131	88	198	237	2.06E-04	9.98E-03	6.34E-06	5.99E-08	0.6214907	0.6313081	0.5699716	0.5933465
GO:0008219	cell death	18				4.22E-02				1.3413073			
GO:0048468	cell development				46				9.97E-02				0.6975237
GO:0030154	cell differentiation	54		88	112	1.64E-02		3.85E-05	1.46E-08	0.7836848		0.8407763	0.9526878
GO:0016477	cell migration	19			27	1.45E-02			3.20E-02	1.4526299			1.0761255
GO:0048870	cell motility	21			30	4.88E-03			9.85E-03	1.499283			1.1303918
GO:0007166	cell surface receptor signaling pathway	52	103	124		5.08E-03	7.34E-04	3.43E-05		0.9096174	0.6644167	0.6961007	
GO:0001816	cytokine production	7				9.50E-03				2.799328			
GO:0007010	cytoskeleton organization				39				1.43E-02				0.9429657
GO:0006952	defense response	23		28	31	2.35E-06		6.99E-06	4.50E-06	2.1210032		1.7573435	1.6681731
GO:0007167	enzyme linked receptor protein signaling pathway			29	34			0.0202	0.0087			1.0692349	1.062705
GO:0051234	establishment of localization		99		193		6.13E-06		1.99E-02		0.879144		0.3749712
GO:0006955	immune response	44	27	47	60	3.73E-16	2.97E-08	1.38E-11	2.99E-17	2.4340575	2.3133221	1.881762	1.998052
GO:0002376	immune system process	54	32	65	79	1.28E-11	4.84E-05	1.45E-09	9.32E-13	1.7762444	1.6051657	1.396272	1.4416732
GO:0006954	inflammatory response	9		11		2.24E-02		2.88E-02		2.1853571		1.827411	
GO:0045087	innate immune response	11		11	11	3.81E-04		1.33E-02	4.90E-02	2.6242413		1.9767886	1.7407769
GO:0032609	interferon-gamma production	3				4.26E-02				4.2092038			
GO:0051179	localization	122	110		224	4.07E-02	7.64E-07		4.54E-04	0.4490701	0.8835013		0.4422233
GO:0040011	locomotion	30		34	45	2.51E-04		1.84E-02	1.65E-04	1.4521806		0.9853001	1.1536786
GO:0008152	metabolic process	301	201	499	544	2.18E-08	5.13E-05	8.46E-20	1.21E-14	0.5164533	0.5176942	0.598277	0.4868321
GO:0016310	phosphorylation			84				3.01E-03				0.6826055	
GO:0050896	response to stimulus	184	130	289	336	2.83E-09	2.17E-07	1.09E-15	6.97E-18	0.7477777	0.8303924	0.7516888	0.7330688
GO:0007165	signal transduction	121	83	187	220	1.87E-04	4.74E-03	7.16E-07	2.73E-08	0.6543613	0.6943463	0.6349398	0.6333933
GO:0050852	T-cell receptor signaling pathway	5				2.35E-02				3.1806346			
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway			26	27			9.30E-04	5.43E-03			1.436914	1.2553501
GO:0006810	transport		99		193		4.42E-06		1.43E-02		0.8902081		0.3860353
GO:0035295	tube development	21		29		1.19E-02		4.63E-03		1.3901196		1.2083305	

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Table 3. GO enrichment analysis for downregulated gene transcripts in infected chicken thymus tissues according to biological process

GO_ID	Term	Days after infection (q)				Days after infection (p values)				Days after infection (log odds ratio)			
		7	14	21	28	7	14	21	28	7	14	21	28
GO:0007049	cell cycle				41				9.86E-06				1.3733199
GO:0048468	cell development				43				4.54E-02				0.8106773
GO:0051301	cell division				21				2.41E-04				1.7706554
GO:0000902	cell morphogenesis				28				1.40E-02				1.1497734
GO:0006325	chromatin organization			38				3.46E-03				1.0862605	
GO:0051276	chromosome organization			45	38			5.45E-04	1.13E-03			1.0998049	1.1460439
GO:0007059	chromosome segregation				13				6.54E-07				3.0193992
GO:0016482	cytoplasmic transport			24	27			1.30E-02	1.16E-05			1.2825515	1.7426411
GO:0007010	cytoskeleton organization				36				8.46E-03				1.0379393
GO:0006310	DNA recombination			15	19			1.61E-02	2.28E-06			1.6610631	2.2922646
GO:0006281	DNA repair			30	37			1.93E-02	1.69E-07			1.0889819	1.6817092
GO:0006260	DNA replication				28				6.21E-07				1.9006016
GO:0051234	establishment of localization	86				1.30E-02				0.6725966			
GO:0051649	establishment of localization in cell			68	59			1.49E-03	7.98E-04			0.8249014	0.9102461
GO:0045184	establishment of protein localization			68	53			6.46E-04	1.56E-02			0.8634398	0.7940619
GO:0010467	gene expression			84				1.23E-02				0.635528	
GO:0048193	Golgi vesicle transport			12				3.07E-02				1.786594	
GO:0002520	immune system development				23				4.98E-02				1.145666
GO:0046907	intracellular transport				54				5.93E-05				1.0884641
GO:0051179	localization	91				2.99E-02				0.6064807			
GO:0000279	M phase				22				2.80E-05				1.8996974
GO:0008152	metabolic process	209	140	588	494	2.69E-06	2.27E-02	2.97E-18	1.84E-17	0.570546	0.4977052	0.5193279	0.5581873
GO:0000226	microtubule cytoskeleton organization			16	16			2.36E-02	2.15E-03			1.5400477	1.8302123
GO:0007017	microtubule-based process				32				6.55E-04				1.3073715
GO:0007067	mitosis				13				9.61E-03				1.865071
GO:0000280	nuclear division				13				9.61E-03				1.865071
GO:0051170	nuclear import				12				2.08E-03				2.1856929
GO:0051169	nuclear transport				16				4.05E-04				2.0252283
GO:0016310	phosphorylation			98				1.10E-02				0.5892725	
GO:0006457	protein folding				23				4.25E-03				1.4130134
GO:0017038	protein import				13				3.36E-03				2.0193992
GO:0006606	protein import into nucleus				12				2.08E-03				2.1856929
GO:0008104	protein localization			77				4.13E-04	7.46E-03			0.823513	0.7776284
GO:0034504	protein localization to nucleus				13				3.98E-03				1.9963156
GO:0033365	protein localization to organelle				19				4.22E-04				1.8206436
GO:0006605	protein targeting				19				3.29E-04				1.8486579
GO:0044744	protein targeting to nucleus				12				2.08E-03				2.1856929
GO:0015031	protein transport			67	53			7.37E-04	1.10E-02			0.8642051	0.8162009
GO:0050896	response to stimulus			275	224			2.39E-03	1.24E-02			0.3643254	0.3585571
GO:0006950	response to stress			86	76			8.01E-05	1.52E-05			0.8337167	0.945544
GO:0006396	RNA processing			42	41			1.77E-02	4.05E-04			0.9077031	1.1631022
GO:0006810	transport	84				2.56E-02				0.6497134			

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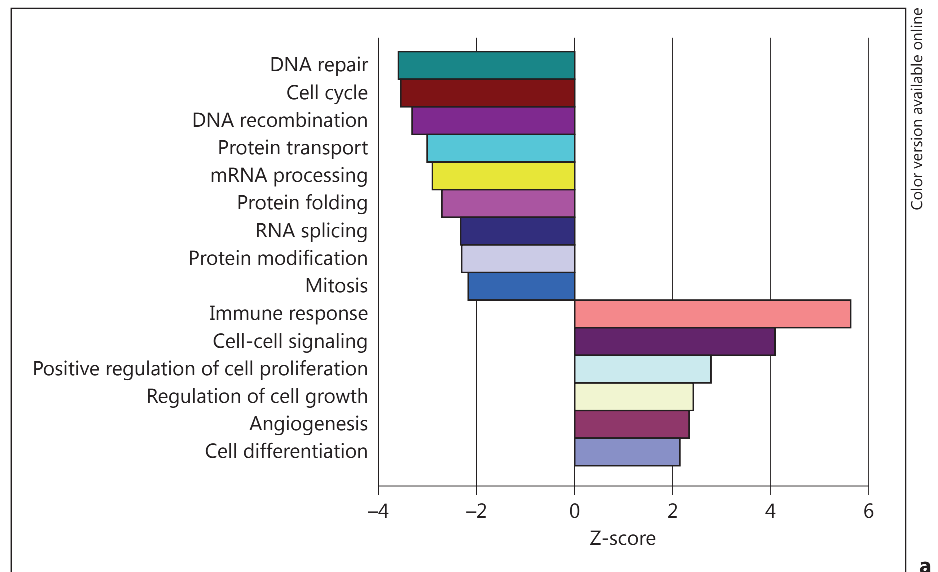

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Name	Z-score	p
Immune response	5.62313	0.00000002
Cell-cell signaling	4.08641	0.00004381
Positive regulation of cell proliferation	2.78731	0.00501482
Regulation of cell growth	2.42919	0.01513276
Angiogenesis	2.33298	0.01964921
Cell differentiation	2.13658	0.03263253
DNA repair	–3.61924	0.00029547
Cell cycle	–3.32591	0.00034175
DNA recombination	–3.32591	0.00088129
Protein transport	–3.02015	0.00252648
mRNA processing	–2.92245	0.00347289
Protein folding	–2.72142	0.00650017
RNA splicing	–2.36233	0.0181603
Protein modification	–2.32602	0.02001723
Mitosis	–2.19131	0.0284293

b

Fig. 1. Dysregulated biological pathways relative to uninfected birds by GAzer. (Z-score <0, downregulated; Z-score >0, upregulated). **a** Map of GAzer to identify the most dysregulated biological pathways. **b** Z-score and p value of dysregulated pathway.

GO analysis through use of the Gazer [15] (http://expressome.kobic.re.kr/GAzer/index.faces). GAzer is a Web-based integrated tool. The basic idea of gene set analysis is to compare two groups by looking at changes in sets of genes, rather than looking at the individual gene level. Therefore, this type of analysis compensates for the fact that small changes not seen at the gene level are often detected when the gene set as a whole is examined. The predicted gene interaction networks among differentially expressed genes found in this study were analyzed by STRING (Search Tool for the Retrieval of Interacting Genes/Proteins; http://string-db.org/) [16].

Real-Time PCR Verification

The expression of selected genes was verified by real-time PCR (7500 Real-Time PCR System, ABI). Primers were designed and synthesized by Shenergy Biocolor Bioscience and Technology Company (Shanghai, China; primers listed in table 1, 18S referenced to [17]). Total RNA was prepared from thymuses using the AxyPrep™ Multisource Total RNA Miniprep Kit (AXYGEN, USA). 1 μg of total RNA was reverse-transcribed into first-strand cDNA using PrimeScript RT Master Mix (TaKaRa, USA) following the manufacturer's instructions, and synthesized cDNA was diluted 1:10 with nuclease-free water. 1 μl of diluted cDNA, 400 nM primers, and 10 μl of SYBR Green Master Mix were used for real-time PCR in a final volume of 20 μl. The amplification conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and then 60 °C for 34 s. Dissociation curves were generated to analyze individual PCR products after 40 cycles. Gene expression was normalized against the expression of chicken 18S mRNA. The analyses of relative gene expression data were performed by the $2^{-\Delta\Delta CT}$ method [18]. Data were compared with Student's t test by the statistical package for social sciences (version 16.0). Genes showing FC values above 2 or below –2 and an unpaired t test $p < 0.05$ were defined as significantly changed.

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Table 4. Validation of microarray results by real-time PCR

Gene symbol	Probe set ID	Gene expression fold change after MDV infection at different time points							
		7 dpi		14 dpi		21 dpi		28 dpi	
		microarray	real-time PCR	microarray	real-time PCR	microarray	real-time PCR	microarray	real-time PCR
ANXA1	Gga.4884.1.S1_at	1.03	2.29	1.61	1.01	3.32	1.86	4.75	4.82
B-LA	Gga.4037.2.A1_s_at	4.59	4.21	–1.69	–1.42	–12.50	–3.92	8.85	1.57
BLB2	Gga.5147.1.S1_x_at	2.80	1.48	–10.18	–10.41	–3.03	–3.10	–1.54	–3.57
CASP6	Gga.2960.1.S1_at	1.51	1.78	–1.18	–1.85	–2.38	–12.08	–2.50	–2.36
CD74	Gga.4414.1.S1_s_at	–1.27	3.01	1.36	–1.37	–2.08	–5.69	2.09	1.24
CD8A	Gga.12943.1.S1_s_at	–1.11	6.91	–1.41	–2.09	–8.33	–6.80	–3.57	–3.31
CD8B	Gga.574.1.S1_at	–1.61	1.75	–1.19	–1.98	–9.09	–9.07	–4.00	–12.96
GADD45A	Gga.1927.1.S1_at	–1.92	–1.08	–1.20	–1.47	–2.08	–2.73	–1.39	1.27
HIF1A	Gga.4933.1.S1_at	1.50	3.58	–1.04	–1.10	1.80	–1.41	2.04	1.63
HSP90AA1	GgaAffx.12967.1.S1_s_at	–1.67	1.92	–1.30	–1.92	–5.56	–19.60	–4.00	–11.83
NPM1	Gga.12636.1.A1_at	–1.30	1.46	–2.08	–1.43	–2.63	–4.44	2.72	–2.13
p21	Gga.1114.1.S1_at	1.50	2.85	–1.10	–1.45	2.78	4.13	2.57	5.96
p53	Gga.706.1.S1_at	3.69	3.53	–1.23	–1.07	1.71	–2.37	1.85	1.31
RB1	Gga.2986.3.S1_a_at	1.02	2.13	1.11	–1.56	–1.41	–6.15	–2.27	–2.15
TLR2	Gga.98.2.S1_a_at	2.45	6.42	–1.10	1.02	–1.79	–6.99	–1.92	1.22
TLR4	GgaAffx.4379.1.S1_at	2.05	6.35	6.86	–1.23	–7.69	–3.91	2.48	1.25
VAV3	Gga.2876.1.S2_a_at	1.20	2.38	–1.41	–2.59	–2.78	–26.71	–1.47	–7.72
VIM	Gga.9346.3.S1_a_at	1.11	2.69	1.21	1.18	1.07	1.69	2.09	4.32

Results

Chicken Thymus Responses to MDV Infection

The number of differentially expressed genes with 2-fold or higher changes (see online suppl. file 1; see www.karger.com/doi/10.1159/000370069 for all online suppl. material) are as follows: 1,250 genes (7 dpi), 834 genes (14 dpi), 1,958 (21 dpi), and 2,306 genes (28 dpi). We found that genes involved in the immune response upregulated at 7 dpi included MHC classes I and II as well as type II interferons, some interleukins and interleukin receptors (IL1-β, IL-6, IL-8, IL-15, IL-18, IL18R1, IL21R, and IL22RA1). We also found interferon response [e.g. the signaling molecule interferon regulatory factor 1 (IRF1), IRF7, and IRF10]. In addition, several genes associated with innate immune were upregulated, including the Toll-like receptors TLR2, TLR3, TLR4, TLR6, TLR7, and TLR15, and the chemokines CCL1, CCL4, and CCL19. In contrast, the genes involved in tumor development and metastasis were upregulated at 21 and 28 dpi in infected thymuses. These genes include jun oncogene (JUN), v-src sarcoma viral oncogene, vascular endothelial growth factor A, IRF4, signal transducer and activator of transcription 3 (STAT3), and wingless-type MMTV integration site family, member 5A. These findings suggest that MDV-activated T cells in late infection in thymuses induce the activated transformed T cells to develop cancer.

Analysis of Differentially Expression Genes

In order to determine which biological process was altered during MDV infection, the microarray data (online suppl. file 1) were further analyzed using GOEAST to determine GO term enrichment. Tables 2 and 3 show the biological process which was significantly ($p < 0.05$) affected during the host responses to MDV infection. We found the upregulated expression genes involved in immune and inflammatory response at 7 dpi. Angiogenesis, cytoskeleton organization, cell adhesion, and signal transduction was shown at 21 and 28 dpi. The overall expression level of host genes at four stages was analyzed by GAzer. Upregulated biological processes in infected birds included cell proliferation, angiogenesis, and immune response (fig. 1). Other biological processes including cell cycle, mitosis, mRNA processing, protein folding, modification, and transport were downregulated.

Real-Time PCR Validation of Array Data

To confirm the microarray data, real-time PCR was performed on 18 selected genes. Fold change was calcu-

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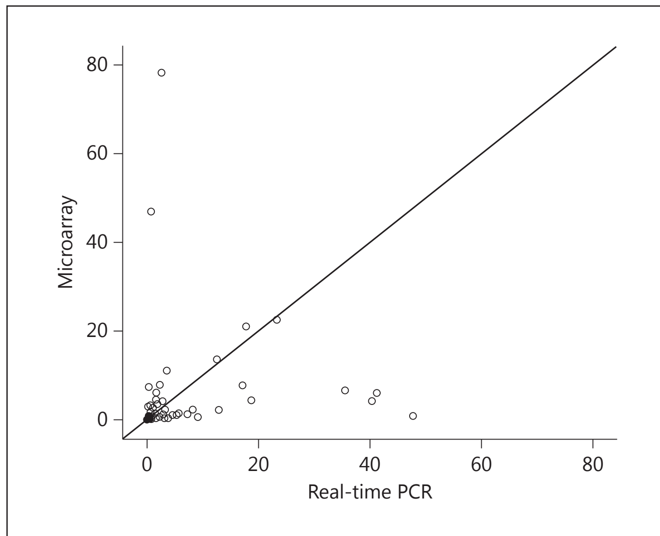

Fig. 2. Scatter plots of microarray and real-time PCR data; the x-axis and y-axis represent the values of 2-fold changes.

lated using the $2^{-\Delta\Delta CT}$ method and expression values which were normalized against the expression of 18S. In table 4, the negative values suggest the fold change is <1; the raw data of fold changes were transformed based on a 2-fold change and then made into a scatter plot (fig. 2). We found that the real-time PCR data were similar to the observations using the microarray (table 4; fig. 2). A correlation coefficient between real-time PRC data and microarray observations is 0.831 ($p = 0.00061$). Thus, the PCR data supported the microarray results.

Discussion

Antiviral Innate and Adaptive Immune Response

We found that an induced innate response has been established at 7 dpi, in which chemokines, proinflammatory cytokines and Toll-like receptors were activated in infected thymuses. The expression of some chemokines (e.g. CCL1 and CCL4), interleukins (e.g. IL1-β, IL-6, IL-8, and IL-18) and interleukin receptors (e.g. IL18R1) as well as genes involved in the interferon response (such as IRF1, IRF7, and IRF10) was upregulated. It has been found that MDV infection leads to the upregulation of TLR3 and TLR15 in chicken spleen tissues at 5 dpi and downregulation of TLR5 in liver tissues [4, 12]. TLRs play a key role in the innate immune system and they recognize molecules that are broadly shared by pathogens [19, 20]. In this study, TLR2, TLR3, TLR4, TLR6, TLR7, and TLR15 were all significantly upregulated during the early infection with MDV. Some of them then obviously downregulated at 21 dpi (online suppl. file 1). One, at expression level of TLR2, was further verified by real-time PCR (table 3). Downregulation of TLR will interfere with interactions between the receptor molecule and the viruses like other herpesviruses [21, 22], perhaps because the host cannot clear MDV-infected cells.

Likewise, adaptive immune responses were also altered by MDV. Several genes involved in antigen process and presentation, such as CD8, CD4, and MHC II, were decreased in infected chicken thymus after 7 dpi. These could be caused by downregulation of MHC-mediated antigen presentation. These results were consistent with other reports [12, 23, 24]. Furthermore, we found that CD28, a costimulatory molecule that is essential for the activation of T cells, was severely decreased. This may be a potential mechanism of the immune suppressive/evasive activities of MDV. However, MHC class I molecules were upregulated in the chicken thymuses infected with MDV at different stages. This phenomenon needs to be investigated further in the future.

Cell Cycle and Apoptosis

GO enrichment analysis of different gene expressions indicated that apoptosis and cell cycle were downregulated during the host response to infection with MDV (fig. 2). Apoptosis has been shown to be a common host cell response to viral infection [25], which is advantageous for the host as it eliminates virus-infected cells. Current evidence indicates that MDV has evolved multiple mechanisms to thwart this cellular response to protect cells against apoptosis [26, 27]. One of the latest discoveries is that the Meq oncoprotein of MDV inhibited p53-dependent transcription and apoptosis by interacting directly with p53 [26]. In our study, we further found that several genes of p53-dependent apoptosis pathway including CHEK2, CYCS, APAF1, and PERP were downregulated in infected thymuses at 21 and 28 dpi. Earlier studies showed that Bcl-2 blocks a caspase-dependent pathway of apoptosis in HSV-1 infected cells for virus replication [28]. The downregulated expression of Bcl-2 was found in our study. Ohashi et al. [29] also found that the expression of Bcl-2 was downregulated in both CD4+ and CD8+ T cells prepared from MDV-infected chickens at 3 weeks after infection. No Bcl-2 transcript was detected in MD tumor-derived MSB1 and MTB1 cell lines. These results suggest that MDV-inhibited apoptosis makes itself grow in the cells and then transforms them.

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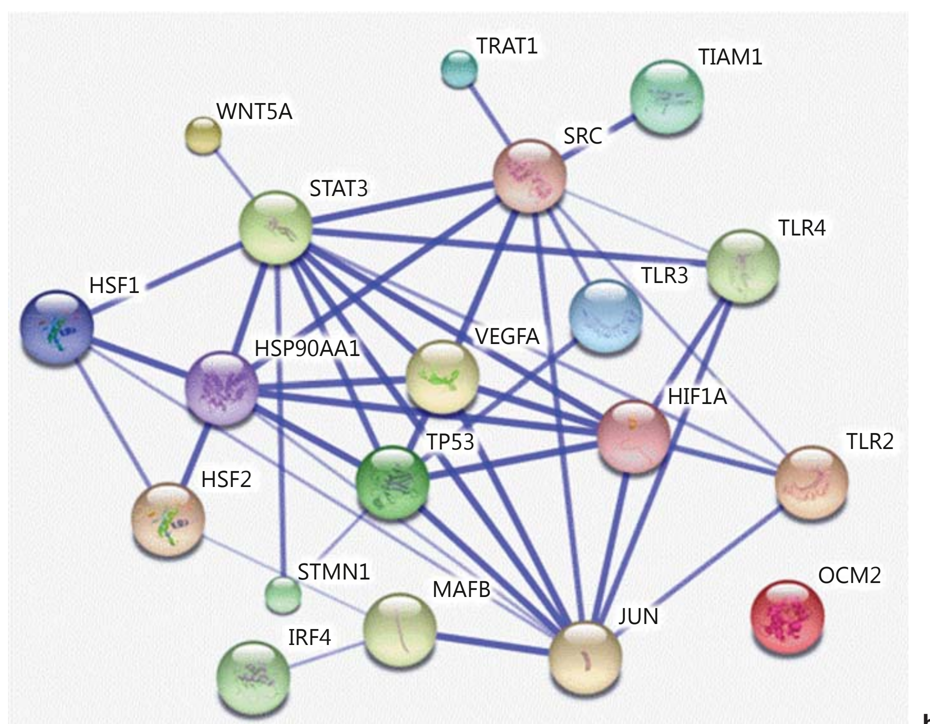
Gene	Description
HIF1A	hypoxia-inducible factor 1, alpha-subunit (basic helix-loop-helix transcription factor)
HSP90AA1	heat shock protein 90 kDa alpha (cytosolic), class A member 1
IRF4	interferon regulatory factor 4
JUN	jun oncogene
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B
OCM2	oncomodulin 2
SRC	v-src sarcoma viral oncogene
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
STMN1	stathmin 1
TCBA1	T-cell lymphoma breakpoint-associated target 1
TIAM1	T-cell lymphoma invasion and metastasis 1
TP53	tumor protein 53
TRAT1	T-cell receptor-associated transmembrane adaptor 1
VEGFA	vascular endothelial growth factor A
WNT5A	wingless-type MMTV integration site family, member 5A

a

Color version available online



b

Fig. 3. The main differently expressed host genes involved in MDV transformation and their interaction network based on the STRING database. **a** Genes involved in angiogenesis, cell proliferation, apoptosis, cell immune-related signaling pathways, and MDV transformation. **b** Network of gene-gene interactions based on the STRING database.

Herpesviruses elicit a cell cycle arrest by virus-encoded proteins to block cellular DNA replication and support efficient viral replication [30]. For MDV, it is known that Meq can bind to several factors that are involved in cell cycle control, including CDK2, p53, and RB. However, the mechanism of cell cycle arrest induced by MDV remains unclear. Our microarray analysis results show that thymuses infected with MDV induced upregulated expression of p21 and GADD45, which are known downstream targets of p53. Corresponding to these changes, CDK6, cyclin D, cyclin E, CDC2, and cyclin B, which are critical regulators of G1 and G2 cell cycle arrest, were mainly downregulated at 21 and 28 dpi. Upregulation expression of p21, an important cell cycle arrest factor, was further verified by real-time PCR analysis. The increased expression of p21 in infected cells was also found in other herpesviruses [31, 32].

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Potential Host Genes Involved in MDV Transformation

There is little literature on the potential host genes involved in MDV transformation in chicken thymuses. In this study, we found the expression of genes involved in angiogenesis, cell proliferation, apoptosis, and cell immune-related signaling pathways (e.g. JAK-STAT, MAPK, JUN, and Wnt pathways) was upregulated at 21 and 28 dpi. We listed some tumor-related genes involved in MDV transformation (fig. 3a). According to the network of these genes interactions (fig. 3b), some genes such as STAT3 and JUN can broadly interact with other genes.

The JAK-STAT signaling pathway is involved in regulating cytokine-dependent gene expressions. Dysregulation of this pathway is associated with cellular transformation [33, 34]. Chen et al. [35] and Vaysberg et al. [36] verified that JAK-STAT signaling plays a role in EBV-associated malignancies. In this study, we observed upregulation expressions of the STAT3 gene in infected thymuses with MDV. Activation of STAT3 occurs in many tumors and is correlated with transformation and anti-apoptotic effects in malignancies [33]. The increased expression of STAT3 induced by MDV might be contributed to MDV transformation. JUN pathways play a critical role in malignant transformation. It could be activated in infected cells by herpesviruses such as EBV and KSHV [37–39]. The expression of JUN was also found to be upregulated in infected thymuses. Levy et al. [40] confirmed that Meq can transform chicken cells via the Jun pathway.

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