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Influence of Avian Influenza Virus on Human Inflammatory Gene Expression Profile

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Abstract: Avian influenza A/H5N1 virus considered as a public health risk with systemic inflammatory response syndrome. Outbreaks in poultry usually cause transmission to human by the ora fecal route leading to high rate of morbidity or mortality. Avian influenza A/H5N1 virus stimulates gene expression of immune inflammatory host genes which cause high pathogenic impact on human. From 90 patients suffer from severe respiratory symptoms, only 6 patients were serologically diagnosed infected with avian influenza A/H5N1 virus. Real-Time-Reverse Transcription PCR-array (RT-PCR-array) was used twice (first with acute samples and second with control cycle) to determine the rate of immune-inflammatory gene expression (Gene fold change) in human white blood cells. Our findings reveal that avian influenza virus H5N1 viruses can infect human white blood cells resulting in the induction of 9 proinflammatory cytokines (CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, IFNa, IFNb and TNF). Our observations suggest that avian influenza H5N1 infection can trigger profound white blood cell impact which may play an important role in the relationship between the pathogenesis of avian influenza virus H5N1 and host immune.

Key words: H5N1, gene expression, RT-PCR-array, cytokines, white blood, inflammatory, mortality

INTRODUCTION

Avian influenza A/H5N1 virus, cause a potential mortality rate of more than a hundred million over the world. Many recent studies revealed high impact on human because of interaction between virus and host immune responses which include acute inflammatory response in the form of hypercytokinemia (cytokine storm). The respond of host cells to virus replication resulting in high rate of cellular apoptosis damage (Reemers *et al*., 2010). Oral-fecal route is almost the main route of avian influenza virus transmission from bird-to-human

Bird-to-human transmission of the avian influenza virus is likely by the oral-fecal route (Chakrabarti *et al*., 2010). Immune and genetic factors play a key role in pathogenesis of H5N1 viruses in humans (Peiris *et al*., 2009; Maines *et al*., 2008). The interaction of avian influenza virus and others studies have shown that the high fatality rate of avian influenza virus infections and host immune responses induce overactive inflammatory response in the form of hypercytokinemia which leads to excessive cellular apoptosis and tissue damage (Reemers *et al*., 2010; Lee and Lau, 2007).

Clinical studies have suggested that H5N1 viruses are very strong inducers of various cytokines and chemokines such as Tumor Necrosis Factor (TNF)-alpha Interferon-gamma, IFN-alpha/beta Interleukin (IL)-6, IL-1, Macrophage Inflammatory Protein (MIP-1), Monokine Induced by IFNgamma (MIG) interferon-gamma-Inducible Protein (IP-10), Monocyte Chemoattractant Protein (MCP-1), Regulated on Activation Normal T-cell Expressed and Secreted (RANTES) and IL-8 in both humans and animals (Lee *et al*., 2009; Cameron *et al*., 2008; Matsukura *et al*., 1998).

However, it has also been reported that preventing cytokine response doesn't prevent H5N1 infection and cell death (Salomon *et al*., 2007). Hence, further studies are needed to understand the pathogenesis of H5N1 virus infection, especially at gene expression level. Alveolar epithelial cells and macrophages are the key targets for H5N1 virus in the lungs (Nicholls *et al*., 2007).

Expression microarray analysis is a sensitive method used to study global profiles of gene in cells under different biological conditions (Conesa *et al*., 2016; Clewley, 2004). Recent medical debates were concentrated on: how influenza virus infections affects immune sensitivity and what are the molecular mechanisms that take place at the gene level which genes will be up-regulated or down-regulated, how virus be effective to stimulate specific genes, what is the interpretation for these complications and if there is a possibility to treat these cases at the molecular gene level (Sims *et al*., 2014; Simpson *et al*., 2006). In this study, RT-PCR-array technique was chosen to study the immune cells genomic response to viral infections. To determine whether the immune cell-viral infections will be accompanied by a particular pattern of change in gene expression. To

have data on immune cell's gene regulation (Seyednasrollah *et al.*, 2013; Myskiw *et al.,* 2009). The aim of this study is to detect the effect of H5N1 virus infection on the mRNA expression of inflammatory cytokines genes. This lead to determine the relationship between human inflammatory gene expression and H5N1 virus.

MATERIALS AND METHODS

Patients: About 90 patients were hospitalized at Ibn AL-Haytham Hospital in Amman/Jordan for respiratory tract infection symptoms, the Institutional Review Board (IRB) was obtained to collect blood samples from all patients. A specialist physician has reviewed all data related to patients including: disease symptoms, onset of infection, age, sex and risk factors. Blood samples were collected for serological and molecular viral detection.

Blood samples: Blood samples were collected from patients in the acute phase of the disease (0-2 days) when the patients had high-grade fever (>38.0°C). Specimens were placed on ice and transported lab. Blood samples were divided into two parts: first part (2 mL) is placed in a heparinized tube for viral-type detection using indirect IgM-ELISA test. The second part of blood sample (3 mL) is placed in an EDTA tube to extract RNA from WBCs within 24 h after collection for studying the immune gene expression of leukocytes. Second, samples were taken after recovery (After 1 month as the control samples). Total RNA is purified with blood RNA kits (Invitrogen, USA) according to the manufacturer's protocols.

H5N1 detection: The H5N1 (avian flu) hemagglutinin ELISA was used for the detection of Hemagglutinin (HA) according to the pair set kit instructor.

Real time- PCR array (RT-PCR array): Blood samples with positive H5N1 infections (6 samples) were subjected to study the effect of viral infections on 84 inflammatory genes (SABioscience) to determine the inflammatory gene expression profile of patient's white blood cells after infection with H5N1.

Extraction of RNA from patient's leukocytes: The RNA of the 6 infected patients (acute samples) with the H5N1 viruses were extracted within 24 h of sampling and stored under -87°C. After one month (following recovery) a second sample was obtained from the same patients (control samples) and their RNA was extracted from buffy coat which contains the leukocytes within 24 h and stored at -87°C (Invitrogen, USA).

RNA quantity: Spectrophotometer analysis is carried out to determine the amount of RNA by measuring the Optical Density (OD) at λ_{260}. The $OD_{260/280}$ ratio (Ratio of RNA to protein) is checked and RNA is considered pure if the OD ratio is between 1.7-2.0. Lower ratio (OD<1.7) indicates presence of protein contamination. Using gel electrophoresis good quality RNA appears as a sharp clear two bands (28S and 18S) of ribosomal RNA.

Synthesis of cDNA: The RNA samples were tested for the efficiency of the reverse transcription to produce the cDNA from RNA samples. Random primers were added (1.0 μg) (Promega, USA). The volume is completed to 10 μL by RNase free water. The tube is heated to 70°C for 10 min to melt secondary structure within the template. The tube is cooled immediately on ice to prevent secondary structure from reforming.

Reverse transcriptase mixture preparation: The first cDNA strand was synthesized according to the manufacturer, (SABioscience) and stored at -20°C until next step.

PCR reaction: Add the following components (Promega, USA) to a PCR-tube: 10x PCR buffer (200 mm Tris-HCl, pH 8.4, 500 mm KCl) 5 μL, $MgCl_2$ (50 mm) 1.5 μL, dNTPs Mix (10 mm) 1.0 μL forward primer (10 μM) 1.0 μL, Reward primer (10 μM) 1.0 μL, Taq-DNA polymerase (5 U/μL) 0.4, cDNA first strand synthesis 2 μL, RNase free water complete to 50 μL. The components are mixed and the PCR-tube is placed in the thermo-cycler (Bio-Rad, USA) and run the following program; first cycle (1 cycle, 10 min, 95°C), second cycle (40 cycle, 30 sec, 95°C, 30 sec, 58°C, 60 sec, 72°C).

Gel electrophoresis: Agarose gel 2% (10X Tris Boric Acid EDTA) is prepared (Promega, USA). The gel is supplied with 120 V for 20 min. The DNA was visualized by placing on a UV light source and was photographed directly.

Real time PCR-Array: Pure RNAs were prepared using genomic DNA elimination mixture (SABioscience) and incubated at 42°C for 5 min and chilled on ice immediately for at least 1 min.

cDNA synthesis: The cDNAs were prepared from RNA samples using Reverse Transcriptase (RT) to get reverse transcriptase mixture according to manufacturer (SABioscince). The RT mixture (10 μL) is added to

each 10 μL genomic DNA elimination mixture and the contents are mixed well but gently with a pipette incubated at 42°C for exactly 15 min and then the reaction is stopped immediately by heating at 95°C for 5 min. The ddH_2O (91 μL) is added to each 20 μL of cDNA synthesis reaction then the contents are mixed well. The first strand cDNA synthesis reaction is placed on ice until the next step or stored overnight at -20°C.

Addition of cDNA to RT-qPCR Master Mix: The cDNAs wrer added to RT-qPCR Master Mix (Master mixes contain SYBER Green and reference dye). Then the master mix is used to prepare the experimental mixture according to manufacturer (SABiosciences).

Loading the 96-well PCR-array: The mixture is aliquot across the PCR-arrays, each PCR-array profiles the expression of 84 pathway-specific gene plus 12 wells for controls. The experimental mixture (cDNA and RT-qPCR Master Mix) is added (25 μL) to each well of the PCR-array.

Real-time PCR detection: PCR-arrays Containing experimental mixture were entered into Bio-Rad-iCycler and run using the real-time instrument software using two-step cycling: first cycle (1 cycle, 1 repeat, 10 min, 95°C), second cycle (40 repeat with 15 sec, 95°C, 1 min, 60°C, 1 min, 60°C). SYBR-Green fluorescence is detected and recorded from every well during the annealing step of each cycle.

Dissociation melting curve: Melting curve program is run immediately as: first cycle 1 repeat, 1 min, 95°C: second cycle (1 repeat, 1 min, 55°C: third cycle (80 repeat, 10 sec, 55°C) increase set point temperature after cycle 2 by 0.5°C.

Calculation of fold-change: The resulting threshold cycle (Ct) values for all wells were analyzed using a blank excel spread-sheet SABioscience data analysis template excel file. The ΔCt for each gene is normalized for the house keeping genes (Beta-2-microglobulin, Hypoxanthine phosphoribosyltransferase 1, Ribosomal protein L 13a, Glyceraldehyde-3-phosphate dehydrogenase and actin, beta) in each plate. If the fold-change is greater than 1, then the result may be reported as a fold upregulation. If the fold-change is <1, then the negative inverse of the result may be reported as a fold down-regulation.

Statistical analysis: Since, each gene has 6 values (6 values for acute samples and 6 values for control samples) the difference between the 2 values for each gene is analyzed using the non-parametric Wilcoxon signed ranks-test to calculate the t-value and p-value for each gene.

RESULTS AND DISCUSSION

Our results revealed that H5N1 infections causes high impact on human inflammatory genes represented with increasing of upregulation in the expression of 9 immune-inflammatory genes (CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, IFN, IFN and TNF) (Table 1). Expression rate ranged between 2 Fold Change (FC) for IFN and IFN and 6 FC for CXCL9 and CXCL10 (Fig. 1). CCL2 gene displays chemotactic activity for monocytes and basophils in inflammatory infections, therefore, it is increased with 3 FC after infection with H5N1. This results come in agreement with another study recorded that patients with H5N1 infection have higher serum levels of CCL-2 (Peiris *et al*., 2007, 2009).

mRNA transcript of CCL3 gene increased with 4 FC which plays a key role in viral immune responses through binding to the receptors CCR1, CCR4 and CCR5 and activate the inflammatory response against H5N1 virus. This result is supported with previous studies revealed that H5N1 viruses induce high production of CCL3 gene from macrophages (Chan *et al*., 2005; Guan *et al*., 2004). CCL4 gene encodes Mitogen-Inducible Monokine Protein (MIMP) which has chemokinetic and inflammatory functions after viral infections and expressed with low rate (2 FC) but Chan *et al*. (2005) that infection with H5N1 viruses cause high increasing in the level of CCL4 to stimulate high production of MIMP which in turn creates antiviral inflammatory state.

CCL5 gene is expressed with 3 FC and functions as one of the natural ligands for the chemokine receptor chemokine 5 which acts as a major viral-suppressive factor. Another study recorded lower increasing in CCL5-mRNA with 1.64 FC after 24 h of H5N1 infection (Tatebe *at al*., 2010). Similar result was recorded in another study showed that infection with H5N1 stimulates high expression of CCL5 from macrophages, this increasing reflects its key role as antiviral protein (Guan *et al*., 2004). CXCL9 and CXCL10 genes were upregulated with 6 FC as the highest upregulated genes compared with all other genes in this study (Fig. 2). CXCL9 acts as antimicrobial gene encodes a protein thought to function as chemoattractant for lymphocytes. CXCL10 gene was increased with 6 FC and acts as antimicrobial gene encodes a chemokine which binds to CXCR3 results in pleiotropic effects including activation of monocytes, T-cell migration and natural killer cells. These results coincide with another study revealed that

Table 1: Fold change of upregulated genes after infection with H5N1 virus

Gene position in array	Gene name	Gene symbol	Ct values		Fold change
			Control	Acute	
A10	Chemokine (C-C motif) ligand 2	CCL2	31.3	28	3.0
B04	Chemokine (C-C motif) ligand 3	CCL3	28.7	26.1	4.0
B05	Chemokine (C-C motif) ligand 4	CCL4	25.7	24.4	2.0
B06	Chemokine (C-C motif) ligand 5	CCL5	25.9	22.9	3.0
C11	C-X-C motif chemokine 10	CXCL10	29.6	27.7	6.0
D07	Chemokine (C-X-C motif) ligand 9	CXCL9	24.5	23.0	6.0
D11	Interferon alpha	IFNa	24.4	23.1	2.0
D12	Interferon beta	IFNb	25	23.7	2.0
G05	Tumor necrosis factor	TNF	28.9	27.4	5.0

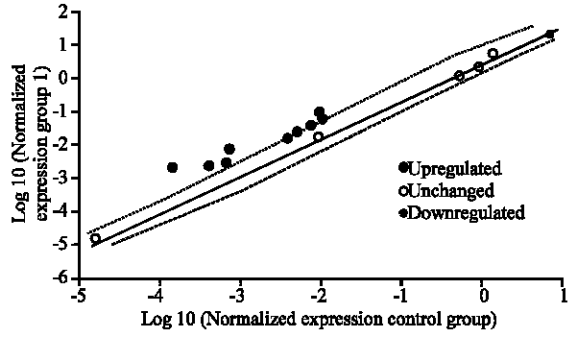

Fig. 1: Scatter plot of 10 log upregulated genes after infection with H5N1

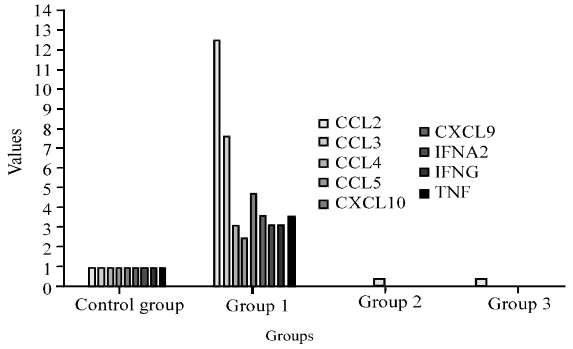

Fig. 2: Rate of gene expression of 10 upregulated genes after H5N1 infection

both CXCL9 and CXCL10 increased (mediated by gamma interferon) after infection with H5N1 (De Jong *et al*., 2006). Another study showed that the level of CXCL10 protein increased after H5N1 infections (Kash *et al*., 2004). Tatebe *et al*. (2010) showed that the expression of CXCL9 and CXCL10 genes were increased more than 3 FC after 24 h of infection with H5N1 virus.

The level of expression of both IFNa and IFNb genes were increased with 2 FC to perform their functions (encode proteins in response to H5N1 infection). Both genes are important for defense against viral infections through activation of transcription of genes such as those

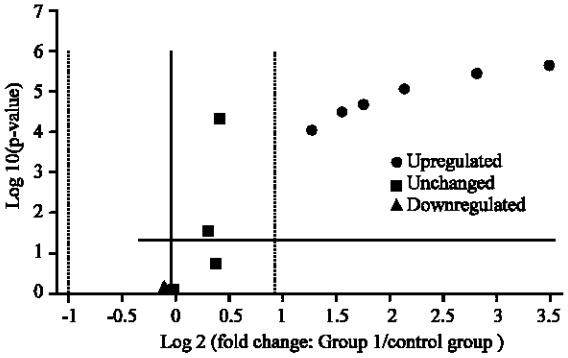

Fig. 3: Volcanic plot of gradual upregulated genes after H5N1 infection

encoding inflammatory cytokines and chemokines. De Jong *et al*. (2006) showed that interferons levels elevated during infection with H5N1.

TNF gene encodes proinflammatory and multifunctional cytokine involved in the regulation of cell proliferation, differentiation and apoptosis after viral infections. The increasing of expression of this gene (5 FC) may reflects its role in cell apoptosis to circumvents the H5N1 infection. Another study recorded that H5N1 viruses induce high upregulation of TNF from macrophages (Chan *et al*., 2005). All upregulated genes showed gradual increasing in their mRNA transcripts during the infection with H5N1 (Fig. 3). Another study revealed that human astrocytic cells infection with H5N1 virus stimulates high expression of TNF mRNA (Ng *et al*., 2010). These results indicate that TNF level is increased after H5N1 infection to trigger apoptosis in infected cells. Gene ontology revealed that human infection with avian influenza virus H5N1 trigger impact effect on expression of 9 inflammatory gene which in turn stimulate 4 key inflammatory pathways: CCL5, CCL2, CCL4, CXCL10, TNF and IFN genes stimulate cytokine-cytokine receptor interaction pathway. CCL5, CCL2 and TNF genes stimulate NOD-like receptor signaling pathway. CCL4,CCL5,CXCL10, IFNA2 and TNF genes stimulate Toll-like receptor signaling pathway. TNF gene stimulates asthma pathway.

CONCLUSION

H5N1 virus impacts human at gene level through upregulation of inflammatory genes which in turn activate very important and complicated immune pathways. Immune pathways which may stimulate cell apoptosis leading to cell die or activate asthma path way that causes severe respiratory dysfunctions. Another pathway triggers high production of inflammatory cytokines which may lead to cytokine storm companioned with severe symptoms usually end up with death. This study may highlights the relationship between H5N1 virus and immune host at gene expression level.

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