Differential proteomic analysis of respiratory samples from patients suffering from influenza

Rahul Chavan^{1,2} · Sandeepan Mukherjee¹ · Ritwik Dahake¹ · Domnic Colvin² · Avinash Kale² · Abhay Chowdhary^{1,3}

Received: 30 March 2016 / Accepted: 20 July 2016 / Published online: 12 August 2016
© Indian Virological Society 2016

Abstract The exact molecular pathways involved in the pathogenesis of influenza are yet unclear. In the present study we investigated the upper respiratory proteome in influenza patients. 200 nasal and throat swab samples were collected from patients suffering from acute respiratory illness. These samples were confirmed for influenza pandemic A/H1N1/2009 and influenza type B using qRT-PCR. 10 similar swabs were collected from healthy individuals and were used as controls. Proteins were extracted from the cell pellets and were subjected to 2-D gel electrophoresis. The differentially expressed proteins were identified using MALDI-TOF. Identified proteins were classified into different functional groups based on functions reported in the databases. 25 % of these proteins were involved in cytoskeletal formation, whereas 14 % were involved in signal transduction. Proteins involved in anti-viral responses, Ca-signaling, transport, and tumor suppression

constituted 10 % each, where as 5 % of proteins each belong to Nicotinic acetylcholine receptor, Protein Synthesis and anti-bacterial proteins. 10 % of the proteins have not been described previously. This is the first report on respiratory proteome profile in Influenza patients. The study emphasizes the role of such profiling studies using multiple platforms for bio-marker discoveries, especially non-invasive diagnostic marker in Influenza and other infectious diseases.

Keywords Pandemic influenza A/H1N1/2009 · Influenza type B · 2-D gel electrophoresis · Mass spectrometry · Differential proteome

Introduction

The US Centers for Disease Control and Prevention (CDC) estimates that seasonal influenza is responsible for an average of more than 20,000 deaths annually. Mortality is highest in infants and the elderly. The 2012–2013 seasons were notable for widespread disease and a higher death rate than was reported in previous years. In addition, the predominant influenza virus subtype was an H3N2, in contrast to dominance by H1N1 subtypes in recent past years. Haffkine Institute is a sentinel surveillance centre under the National Center for Disease Control (NCDC) for the detection of influenza viruses among the population in Mumbai. Previous reports of prevalence studies carried out at Haffkine Institute [33] demonstrated the screening of 100 children for influenza type A and influenza type B and it was noted that 11 were positive for influenza type A and only 5 were positive for influenza type B. The above data indicated the severity of Influenza viruses in India still continues. The need of the hour is to undertake continued

Electronic supplementary material The online version of this article (doi:10.1007/s13337-016-0332-x) contains supplementary material, which is available to authorized users.

✉ Sandeepan Mukherjee
sandeepanm@gmail.com

✉ Avinash Kale
avinash.kale@cbs.ac.in

¹ Department of Virology, Haffkine Institute for Training, Research and Testing, Acharya Donde Marg Parel, Mumbai, Maharashtra 400 012, India

² Department of Chemistry, UM-DAE Center for Excellence in Basic Sciences, Health Centre, University of Mumbai, Vidyanageri Campus, Kalina, Santacruz (East), Mumbai, Maharashtra 400098, India

³ Department of Microbiology, Grant Medical College and Sir J.J. Group of Hospitals, Byculla, Mumbai, Maharashtra 400008, India

surveillance globally, which will help to better define the circulation of influenza viruses as well as to determine optimal periods to implement vaccination programmes among the priority population.

It is also imperative to study the virus-host interactions. In the last years great efforts have been undertaken to reveal the mechanisms of influenza–host cell interactions. Studies focused either on pathogenesis in humans [11], discovery of novel drug targets, antiviral therapies and biomarker research [1], or on analysis of virulence and adaptation strategies of avian influenza virus [5]. Among the Influenza viruses, influenza A has been shown to interact with host cell proteins throughout the virus life cycle [34]. Some interactions elicit alterations in the host proteome, as exemplified by the virus's ability to both induce and evade a host immune response [17], to influence autophagy and apoptosis [12, 24, 36], and to increase viral protein synthesis while shutting down host protein synthesis [14].

Studying virus–host interactions is increasingly reliant on quantitative proteomic techniques such as 2D-DIGE, isotope encoded affinity tag (ICAT), and stable isotope labeling of amino acids in cell culture (SILAC). Several proteomic investigations have been carried out earlier to study the proteomic changes in cell culture based studies with Influenza virus infection [7, 21–23, 40, 42]. Several genomic and proteomic studies have also been carried out in influenza-infected macaques [1, 42]. In a recent study, Andrea L. Kroeker carried out the differential expression of host protein in primary human bronchial airway epithelial (HBAE) cells and she reported that the down regulated proteins generally belong to cell adhesion and lipid metabolism and up regulated proteins were from the host defense responses against influenza infection [44]. Most proteomic studies investigating the effect of influenza infection have been carried out in different cell lines and primary macrophages. Till date, however, only one scientific report [19] has been documented in literature studying nasal secretions from naturally influenza infected patients. Proteins identified in this study are different from those described in influenza-infected cell lines and primary macrophages [7, 22]. Ten proteins were found to be differentially upregulated in the infected children including PLUNC, cystatin S, cystatin SA, S100A9, lipocalin 1 fragments (n52), truncated lactotransferrin, two immunoglobulin (Ig) kappa fragments and one immunoglobulin (Ig) lambda fragment. Luis Teran's findings reveal that the composition of nasal secretions in influenza virus respiratory infections is different from that when children are healthy and may provide further insights into the pathogenesis of respiratory infections caused by seasonal influenza A viruses [37].

With limited data available on virus-host interaction from human patients infected with Influenza, we analyze differential proteomics from the nasal/throat swabs from patients representing Influenza like Illness and Acute Respiratory Infection.

Materials and methods

Sample collection

A total of 200 nasal swabs/throat swabs were collected from July 2011 to July 2012, which comprised of 114 males and 86 females from outpatient department (OPD) and inpatient department (IPD) from hospitals like B. J. Wadia Hospital for Children, L. H. Hiranandani Hospital, King Edward Memorial Hospital, Mumbai. All the samples were referred by medical doctors and were duly archived and were frozen in -80°C for future use. The study was approved by the Institutional Ethics Committee of Haffkine Institute. Identification of viral and bacterial infection from all the 200 samples has been carried out previously [4].

Sample processing

Confirmed positives for pandemic influenza A/H1N1/2009 and influenza type B samples were thawed from -80°C . The samples were centrifuged at $14,000\times g$ for 15 min at 4°C to pellet the cell mass and supernatant of the viral transport medium was discarded. The cell pellet was further washed with phosphate buffer saline (PBS pH 7.4) to remove the remaining contaminants. The cell pellet from 10 different samples from for pandemic influenza A/H1N1/2009 and influenza type B were pooled and cell lysis was performed for 15 cycles of sonication on ice at 30 % amplitude, 5 s pulse with 5 s gap. Centrifugation was performed at $10,000\times g$ for 10 min at 4°C and the cell lysate was used for protein extraction using TRIzol method as discussed below. Thirty samples were used from healthy human volunteers as control. Ten samples were pooled at a time to increase the protein concentration for Iso electric focusing.

Protein extraction

The TRIzol protocol was designed using protocol with minor modifications [32]. In brief, to the cell lysate 1 mL of TRIzol reagent and 200 μL of chloroform were added, mixed vigorously and centrifugation was carried out at $14,000\times g$ for 15 min at 4°C . Upper clear phase was removed carefully. To the remaining phase 300 μL of ethanol was added and incubated at room temperature for

3 min followed by centrifugation at $2000\times g$ for 15 min at 4 °C. To the supernatant, 1:4 volume of ice-cold acetone (kept at -20°C) was added and kept for overnight incubation at -20°C . Protein pellet was thoroughly washed first with 0.3 M guanidium-HCl in 95 % ethanol three times and then two times with ice-cold acetone (kept at -20°C). After air-drying, all protein pellets were dissolved in rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS, 1 % IPG buffer and bromophenol blue dye). Protein quantification was performed using either 2-D quant kit (GE healthcare) or Bradford reagent (Bio Rad) with BSA as a standard.

Two-dimensional gel electrophoresis (2-DE) analysis

Pandemic influenza A/H1N1/2009 and influenza type B protein samples 150 μg were rehydrated on 13 cm IPG strips of pH range 4–7 (GE healthcare) by adding 1 % DTT, 1 % (v/v) respective IPG buffer and De-streak reagent (GE healthcare). First dimension was performed using Ettan IPGphor 3 isoelectric focusing unit (GE Healthcare) with following settings: 50 V for 4 h (Step and hold), 200 V for 4 h (Step and hold), 500 V for 1 h (step and hold), 1000 V for 750 Volt hour (gradient), 8000 V for 13,500 Vh (gradient) and 8000 V for 64,000 Vh (step and hold), 500 V for 4 h (step and hold), resulting in a total of $\sim 80,550$ kVh. Focused IPG strips were treated with equilibration buffer-I and -II containing dithiothreitol (DTT) and iodoacetamide (IAA) for 15 min each. The IPG strips were then placed onto 12.5 % SDS-PAGE gels and the second dimension was carried out. Silver staining was used for all the samples and gels were analyzed using Lab Image software (GE Healthcare). After silver staining, the protein gels were scanned by using GE Image ScannerTMIII and the gel images were subjected to GE 2D Platinum Software and the gels were compare to determine the differential protein spots. Statistical analysis of Anova ($p < 0.05$) and fold difference of 2–8 with match count 2 was used for identification of differential protein spots.

In-gel digestion

In-gel digestion from silver stained gels was carried out using trypsin (Promega). Briefly, the protein spots were washed with water and de-stained with potassium ferric cyanide and sodium thiosulphate. The gel particles were then washed with 50 mM NH_4HCO_3 /acetonitrile 1 + 1 (v/v) for 15 min. The gel particles were dried using vacuum centrifuge, and further reduction and alkylation of gel particles was carried out by 10 mM dithiotretiol and 55 mM iodoacetamide in 50 mM NH_4HCO_3 . The gel particles were allowed to shrink in acetonitrile and again dried using vacuum centrifuge. Freshly prepared

enzyme solution (25 mM NH_4HCO_3 with 5 ng/ μL of trypsin) was added to gel particles and incubated at 37 °C for 30 min. Enough 25 mM NH_4HCO_3 was added to cover the gel particles and incubated overnight at 37 °C. The peptides were extracted from gel pieces by adding series of solvents: 0.1 % TFA in 50 % ACN, 0.1 % TFA in 60 % ACN and 0.1 % TFA in 80 % ACN (100 μL each). All the three fractions were pooled and vacuum dry. The samples were stored at -20°C till Mass Spec analysis. Reconstitute the sample in 5–10 μL of 0.1 % TFA in 50 % acetonitrile before mass spectrometric analysis [35].

Protein identification by MALDI TOF/TOF

The mass spectrometric analysis was carried out on Matrix associated laser desorption ionization—time of flight (MALDI-TOF) instrument (Bruker Daltonik GmbH) at ACTREC, Kharghar, Navi Mumbai. Briefly 2 μL of the extracted protein was mixed with 2 μL (equal volumes) of α -cyano-4 hydroxy-cinnamic acid (HHCA) matrix and was loaded on ground steel MALDI target plate and allowed to dry at room temperature. The MALDI-TOF MS target was subsequently introduced into the MALDI TOF mass spectrometer (Bruker Daltonik GmbH) for automated measurement and data interpretation. Initial manual/visual estimation of the mass spectra was performed using the Flex Analysis 2.4 software (Bruker Daltonik GmbH, Germany). For automated data analysis, raw spectra were processed using the Biotools Version 3.0 software (Bruker Daltonik GmbH, Germany) with default settings. The instrument was calibrated by using standard peptide mix before sample analysis. The instrument was operated in reflection mode using Nd:YAG 335 nm laser, with a mass range of 800–4000 Da. MS peak list was subjected to MASCOT version 2.1 (<http://www.martixscience.com>) search engine for protein identification. The parameters used for mascot search were as follows: trypsin digestion, single missed cleavage, oxidation of methionine and carbamidomethylation of cysteine as variable and fixed modifications respectively, 100 ppm mass tolerance selected for MS and Swiss-Prot database. The taxonomy was set as *Homo sapiens*.

Results and discussion

A total 200 nasal/throat swabs representing respiratory symptoms were collected and diagnosed for seasonal influenza type A (H3N2), pandemic influenza A/H1N1/2009, influenza type B, respiratory syncytial virus (RSV), Human metapneumovirus (hMPV). Out of 200 samples, 37

were found to be positive for pandemic influenza A/H1N1/2009, 24 were positive for influenza type B, 15 for seasonal influenza type A (H3N2), 2 for RSV and none for hMPV [4].

Protein concentration in nasal/throat swabs from a single sample was less as those observed for control samples (1.2 ± 0.30 vs 3.5 ± 0.50 $\mu\text{g}/\mu\text{L}$). The minimum amount of protein load for 13 cm IPG strip has to be more than 100 μg or more for better protein expression for 2-DE analysis. Hence, we had to group the samples, i.e. 10 samples represent one sample with protein concentration more than 100 μg . So as per the confirmed virus positive samples from the group of 200 samples, only three gels could be assessed for pandemic influenza A/H1N1/2009 and two gels for influenza type B. Although a large number of proteins were detected in control sample and pandemic influenza A/H1N1/2009 and influenza type B by the 2-DE technique, only those that were differentially/exclusively expressed/upon infection were studied. Differential analysis of the two-dimensional gels (Fig. 1) using GE 2D Platinum Software revealed five spots differentially expressed in control samples (Supplementary 01), 13 protein spots were exclusively and seen only in pandemic influenza A/H1N1/2009 (Supplementary 02), one protein spot was differentially expressed in influenza type B (Supplementary 03) and 4 protein spots were exclusively expressed. (Supplementary 04).

The proteins which were found to be down-regulated in control healthy samples are reported in Supplementary 01 and those of clinical significance are chondroitin sulfate synthase 3; nutritionally-regulated adipose and cardiac enriched protein (Nrac) and Fibroblast growth factor 3.

Chondroitin sulfate is a major component of extracellular matrix, and is important in maintaining the structural integrity of the tissue. This function is typical of the large aggregating proteoglycans: aggrecan, versican, brevican, and neurocan, collectively known as lecticans. As part of aggrecan, chondroitin sulfate is a major component of cartilage. Loss of chondroitin sulfate from the cartilage is a major cause of osteoarthritis. Chondroitin sulfate readily interacts with proteins in the extracellular matrix due to its negative charges. These interactions are important for regulating a diverse array of cellular activities [2]. Nrac (nutritionally-regulated adipose and cardiac-enriched) is expressed specifically and abundantly in fat and the heart. Both fasting and obesity reduce Nrac expression in white adipose tissue, and fasting reduces its expression in brown fat. Nrac is localized to the plasma membrane, and highly induced during adipocyte differentiation. Nrac is therefore a novel adipocyte marker and has potential functions in metabolism [43]. Fibroblast growth factor 3, the official name of this gene is “fibroblast growth factor receptor 3.” (*FGFR3*). The proteins play a role in several important cellular processes, including regulation of cell growth and division, determination of cell type, formation of blood vessels, wound healing, and embryo development. Fibroblast growth factor receptor (FGFR) 1–4, which belongs to the tyrosine kinase receptor superfamily, also regulates many biological processes including differentiation, proliferation, development, and angiogenesis [27, 29, 39].

The proteins which were found to be exclusively expressed in pandemic influenza A/H1N1/2009 samples are reported in Supplementary 02 and those of clinical

Fig. 1 Representative image of 2DE gel of **a** control samples; **b** pandemic influenza A/H1N1/2009 samples; and **c** influenza type B samples. The protein gels were scanned by using GE Image ScannerTM III, and the images were subjected to GE 2D Platinum

Software and were compared to determine the differential protein spots. Statistical analysis of Anova ($p < 0.05$) and fold difference of 2–8 with match count 2 was used for identification of differential protein spots and are highlighted in red circles (colour figure online)

significance are Mitogen-activated protein kinase kinase kinase 7 (MAPK 7), Zinc finger protein, Pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 2, and Beta-defensin 108B. MAPK 7 and their downstream signaling cascades play crucial roles in immunological and cellular responses to external stimuli. Three well-characterized members of the MAPK superfamily are p38 MAPK, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-jun NH2-terminal kinases (JNK) [10]. These kinases regulate gene expression at both the transcription and post transcription levels by different mechanisms. It has been shown that p38 MAPK and JNK, but not ERK1/2, regulate RANTES expression in influenza virus-infected bronchial epithelial cells. Moreover, MAPK/ERK kinase-specific inhibitors have been reported to inhibit the nuclear export of viral ribonucleoprotein complexes and impair the activity of nuclear-export protein, NEP/NS2, in the virus replication cycle [20]. The zinc finger is a novel motif found in Zinc finger proteins which bind to DNA. Nasser et al. [28] reported that Matrix protein (M1) of influenza virus inhibits its own polymerase; which suggested that a peptide segment of M1 protein with inhibitory properties could serve as an antiviral agent. A peptide synthesized to the Zn²⁺ finger region of the M1 sequence of influenza virus strain A/PR/8/34 centered on amino acids residues 148–166 was shown to be 1000-fold more effective as a polymerase inhibitor than M1. Antiviral activity against influenza type B viruses was reported and also a low level of activity against vesicular stomatitis virus was observed [28]. Pyruvate dehydrogenase complex (PDC), is distributed heterogeneously within the mitochondrial matrix [25] and catalyzes the irreversible oxidation of pyruvate to acetyl CoA. Rapid changes in catalytic activity are achieved primarily by reversible phosphorylation by PDC kinases (PDKs) and phosphatases (PDPs). Humans possess at least four PDK and two PDP isoforms that are expressed differentially among tissues [3]. In one of the studies [41], effects of diisopropylamine dichloroacetate (DADA), a new PDH kinase 4 (PDK4) inhibitor, in mice with severe influenza showed that the infection of mice with influenza A PR/8/34(H1N1) virus resulted in marked down-regulation of PDH activity and ATP level, with selective up-regulation of PDK4 in the skeletal muscles, heart, liver and lungs. These results indicate that through PDK4 inhibition, DADA effectively suppresses the host metabolic disorder-cytokine cycle, which is closely linked to the influenza virus-cytokine-trypsin cycle, resulting in prevention of multi-organ failure in severe influenza. Defensins form a family of antimicrobial and cytotoxic peptides made by neutrophils. Defensins are short, processed peptide molecules that are classified by structure into three groups: alpha-defensins, beta-defensins and theta-defensins. Human β -defensins form an essential component of the

intestinal lumen in innate immunity. The defensive mechanisms of β -defensins include binding to negatively charged microbial membranes that cause cell death and chemoattraction of immune cells. The antimicrobial activity of β -defensin is well reported in vitro against several enteric pathogens and in non-infectious processes such as inflammatory bowel diseases, which alters β -defensin production. However, the role of β -defensin in vivo of interaction with other immune components in host defense against bacteria, viruses and parasites with more complex membranes is still not well known [6].

The protein which is found to be differentially regulated in influenza type B is Interferon-induced protein with tetratricopeptide repeats 2 (IFIT 2) (Supplementary 03), and proteins which are exclusively expressed only in influenza type B and of clinical significance are eukaryotic translation initiation factor 4H (eIF4), Interleukin-1 receptor-associated kinase 4 (IRAK), Elongator complex protein 6 (ELP 6) (Supplementary 04). Interferon-induced protein with tetratricopeptide repeats 2 (IFIT 2), belonging to the IFIT family proteins which are involved in many processes in response to viral infection, mainly by reducing virus replication. The IFIT family proteins contain a TPR (tetratricopeptide repeat) domain, a 34 amino acid motif folding into a helix-turn-helix structure, which mediates protein interactions [9]. IFIT1 and IFIT2 are involved in a nonspecific antiviral program through their direct interactions with eukaryotic initiation factor 3, which subsequently suppresses more than 60 % of translation in cells and viruses during protein synthesis [13, 15, 38]. The IFIT family, especially IFIT1 and IFIT3, restrict DNA and RNA virus replication, such as hepatitis B virus (HBV), human papillomavirus (HPV), hepatitis C virus (HCV), West Nile virus (WNV) and others [26, 30]. Knock down of IFIT1 through RNA interference in human hepatocytes enhanced HCV replication during infection [31]. Eukaryotic translation initiation factor 4H (eIF4) is involved in regulation of translation initiation during protein synthesis. Loss of translation control may lead to cell malignant transformation as consequence of increased rate of protein synthesis and translation activation of mRNA species that are relevant for cell proliferation and survival. Initiation of protein synthesis of most eukaryotic mRNAs is m⁷G-cap-dependent and requires at least 13 eukaryotic initiation factors (eIFs) [16]. Interleukin-1 receptor associated kinases (IRAKs), were originally described as transducers for inducing various inflammatory cytokines and later implicated as critical mediators in regulation of TLR/IL-1R signaling [8]. It is generally believed that IRAK family proteins are pivotal modulators in IL-1/TLR signaling transduction. As a member of the IRAK family, more focus has been on the critical function of IRAK2 in inflammation and immune responses [18]. Thus, it may be worthwhile to

Fig. 2 Pie-chart representation of the distribution of the differentially expressed protein obtained by comparing pandemic influenza A/H1N1/2009 and influenza type B with the control samples

investigate whether there are additional IRAK variants possessing different activities in mediating TLR/IL-1R-induced NF- κ B activation. Elongator complex protein 6 (ELP 6) is also alternatively termed as Elongator Acetyltransferase Complex Subunit 6 which is a Protein Coding gene. It acts as subunit of the RNA polymerase II elongator complex, which is a histone acetyltransferase component of the RNA polymerase II (Pol II) holoenzyme and is involved in transcriptional elongation. Elongator may play a role in chromatin remodeling and is also involved in acetylation of histones H3 and probably H4. It is believed that ELP 6 is also involved in cell migration.

The proteins from the Control group like Chondroitin sulphate synthase, Nutritionally-regulated adipose and cardiac enriched protein (Nrac) and FGF3 which form the extracellular matrix in various cell types are down-regulated when compared with the infectious Influenza group. These results are consistent with Andrea L. Kroeker's study [19] who reported that the down regulated proteins generally belong to cell adhesion and lipid metabolism. The proteins from pandemic influenza A/H1N1/2009 are MAPK7, Zinc finger proteins, Pyruvate dehydrogenase kinases and Beta-defensin 108B are mostly involved in anti-viral mechanism [10, 20, 28] and also help in host defense system. The proteins from influenza type B majorly IFIT2 along with Interleukin-1 receptor-associated kinase (IRAK) plays a central role in cell signaling processes as cited earlier [9, 18]. Our comparison of the pandemic influenza A/H1N1/2009 and influenza type B with the control samples leads to the observation that a majority of the differentially expressed proteins belong to the cytoskeletal group (25 %) followed by those

responsible for signal transduction (14 %). Second set (10 % each) contains anti-viral; calcium signaling and metabolism; tumor suppression proteins; and transport proteins. One group of uncharacterized proteins also constitute 10 % of the population. The third set (5 %) contains anti-bacterial, nicotine acetylcholine and protein synthesis proteins (Fig. 2).

It could be elucidated that the proteins from the control group could be used as diagnostic markers since these proteins are down-regulated by a significant fold change in the influenza group. The proteins from the pandemic influenza group can be taken up for further studies to specify their roles as anti-viral agents and hence could be used as novel therapeutics. The proteins from the influenza type B are mostly involved in cell signaling processes, so these proteins could be used to understand the mechanism of viral infection in host cellular responses after tagging them with either fluoro-chromes or radioisotopes.

This study in our knowledge is one of the first reports of the upper respiratory proteome study in Influenza infection. Further studies, involving multiple omics platforms are required to understand the molecular pathways involved in the pathology of influenza. Such studies will help us identify biomarkers which may be used as diagnostic or therapeutic targets.

Acknowledgments The authors would like to thank technical staff of Mass-spectrometry facility at ACTREC, Navi Mumbai for their assistance in protein sequencing experiments. The study was funded by Intra-mural fund of Haffkine Institute for Training, Research and Testing, Mumbai, India.

References

1. Baas T, Baskin CR, Diamond DL, Garcia-Sastre A, et al. Integrated molecular signature of disease: analysis of influenza virus-infected macaques through functional genomics and proteomics. *J Virol*. 2006;21:10813–28.
2. Baeurle SA, Kiselev MG, Makarova ES, Nogovitsin EA. Effect of the counterion behavior on the frictional-compressive properties of chondroitin sulfate solutions. *Polymer*. 2009;50(7):1805–13.
3. Bowker-Kinley MM, Davis WI, Wu P, Harris RA, Popov KM. Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. *Biochem J*. 1998;329:191–6.
4. Chavan RD, Kothari ST, Zunjarrao K, Chowdhary AS. Surveillance of acute respiratory infections in Mumbai during 2011–12. *Indian J Med Microbiol*. 2015;33:43–50.
5. Chen LM, Davis CT, Zhou H, Cox NJ, Donis RO. Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. *PLoS Pathog*. 2008;5:e1000072.
6. Cobo ER, Chadee K. Antimicrobial human β -defensins in the colon and their role in infectious and non-infectious diseases. *Pathogens*. 2013;2:177–92.
7. Coombs KM, Berard A, Xu W, Krokhin O, Meng X, Cortens JP, et al. Quantitative proteomic analyses of influenza virus-infected cultured human lung cells. *J Virol*. 2010;84(20):10888–906.
8. Croston GE, Cao Z, Goeddel DV. NF- κ B activation by interleukin-1 (IL-1) requires an IL-1 receptor-associated protein kinase activity. *J Biol Chem*. 1995;270:16514–7.
9. D'Andrea LD, Regan L. TPR proteins: the versatile helix. *Trends Biochem Sci*. 2003;28(12):655–62.
10. Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. *Annu Rev Immunol*. 2002;20:55–72.
11. Formek JL, Korth MJ, Katze MG. Use of functional genomics to understand influenza-host interactions. *Adv Virus Res*. 2007;70:81–100.
12. Gannage M, Dormann D, Albrecht R, Dengjel J, Torossi T, Ramer PC, et al. Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. *Cell Host Microbe*. 2009;6(4):367–80.
13. Guo J, Hui DJ, Merrick WC, Sen GC. A new pathway of translational regulation mediated by eukaryotic initiation factor 3. *EMBO J*. 2000;19(24):6891–9.
14. Hale BG, Randall RE, Ortin J, Jackson D. The multifunctional NS1 protein of influenza A viruses. *J Gen Virol*. 2008;89(Pt 10):2359–76.
15. Hui DJ, Terenzi F, Merrick WC, Sen GC. Mouse p56 blocks a distinct function of eukaryotic initiation factor 3 in translation initiation. *J Biol Chem*. 2005;280:3433–40.
16. Jackson RJ, Hellen CU, Pestova TV. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol*. 2010;11:113–27.
17. Kash JC, Goodman AG, Korth MJ, Katze MG. Hijacking of the host-cell response and translational control during influenza virus infection. *Virus Res*. 2006;119(1):111–20.
18. Keating SE, Maloney GM, Moran EM, Bowie AG. IRAK-2 participates in multiple Toll-like receptor signaling pathways to NF κ B via activation of TRAF6 ubiquitination. *J Biol Chem*. 2007;282:33435–43.
19. Kroeker AL, Andrew PE, Halayko J, Coombs KM. Response of primary human airway epithelial cells to influenza infection: a quantitative proteomic study. *J Proteome Res*. 2012;11:4132–46.
20. Kujime K, Hashimoto S, Gon Y, Shimizu K, Horie T. p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulates RANTES production by influenza virus-infected human bronchial epithelial cells. *J Immunol*. 2000;164:3222–8.
21. Li C, Bankhead A, Eisfeld AJ, Hatta Y, Jeng S, Chang JH, et al. Host regulatory network response to infection with highly pathogenic H5N1 avian influenza virus. *J Virol*. 2011;85:10955–67.
22. Lietzen N, Ohman T, Rintahaka J, Julkunen I, Aittokallio T, Matikainen S, et al. A Quantitative subcellular proteome and secretome profiling of influenza A virus-infected human primary macrophages. *PLoS Pathog*. 2011;7(5):e1001340.
23. Liu HC, Hicks J, Yoo D. Proteomic dissection of viral pathogenesis. *Dev Biol (Basel)*. 2008;132:43–53.
24. Ludwig S, Pleschka S, Planz O, Wolff T. Ringing the alarm bells: signalling and apoptosis in influenza virus infected cells. *Cell Microbiol*. 2006;8(3):375–86.
25. Margineantu DH, Brown RM, Brown GK, Marcus AH, Capaldi RA. Heterogeneous distribution of pyruvate dehydrogenase in the matrix of mitochondria. *Mitochondrion*. 2002;1:327–38.
26. Matthys V, Gorbunova EE, Gavrillovskaia IN, Pepini T, Mackow ER. The C-terminal 42 residues of the Tula virus Gn protein regulate interferon induction. *J Virol*. 2011;85:4752–60.
27. Mima T, Ueno H, Fischman DA, Williams LT, Mikawa T. Fibroblast growth factor receptor is required for in vivo cardiac myocyte proliferation at early embryonic stages of heart development. *Proc Natl Acad Sci USA*. 1995;92:467–71.
28. Nasser EH, Judd AK, Sanchez A, Anastasiou D, Bucher DJ. Antiviral activity of influenza virus M1 zinc finger peptides. *J Virol*. 1996;70:8639–44.
29. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev*. 2005;16:159–78.
30. Rath AV, Cantalupo PG, Sarkar SN, Pipas JM. Induction of interferon-stimulated genes by Simian virus 40 T antigens. *Virology*. 2010;406:202–11.
31. Raychoudhuri A, Shrivastava S, Steele R, Kim H, Ray R, Ray RB. ISG56 and IFITM1 proteins inhibit hepatitis C virus replication. *J Virol*. 2011;85:12881–9.
32. Reddy PJ, Rao AA, Malhotra D, Sharma S, Kumar R, Jain R, et al. A simple protein extraction method for proteomic analysis of diverse biological specimens. *Curr Proteom*. 2013;10:298–311.
33. Roy S, Patil D, Dahake R, Mukherjee S, Athlekar SV, Deshmukh RA, Chowdhary A. Prevalence of influenza virus among the paediatric population in Mumbai during 2007–2009. *Indian J Med Microbiol*. 2012;30(2):155–8.
34. Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, et al. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. *Cell*. 2009;139(7):1255–67.
35. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal Chem*. 1996;68:850–8.
36. Sumbayev VV, Yasinska IM. Role of MAP kinase-dependent apoptotic pathway in innate immune responses and viral infection. *Scand J Immunol*. 2006;63(6):391–400.
37. Teran LM, Ruggeberg S, Santiago J, et al. Immune response to seasonal influenza A virus infection: a proteomic approach. *Arch Med Res*. 2012;43:464–9.
38. Terenzi F, Hui DJ, Merrick WC, Sen GC. Distinct induction patterns and functions of two closely related interferon-inducible human genes, ISG54 and ISG56. *J Biol Chem*. 2006;281:34064–71.
39. Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. *Nat Rev Cancer*. 2010;10:116–29.
40. Vester D, Rapp E, Gade D, Genzel Y, Reichl U. Quantitative analysis of cellular proteome alterations in human influenza A

- virus infected mammalian cell lines. *Proteomics*. 2009;9(12):3316–27.
41. Yamane K, Indalao IL, Chida J, Yamamoto Y, Hanawa M et al. Diisopropylamine dichloroacetate, a novel pyruvate dehydrogenase kinase 4 inhibitor, as a potential therapeutic agent for metabolic disorders and multiorgan failure in severe influenza. *PLoS One*. 9(5). doi:[10.1371/journal.pone.0098032](https://doi.org/10.1371/journal.pone.0098032).
42. Zhang L, Zhang X, Ma Q, Ma F, Zhou H. Transcriptomics and proteomics in the study of H1N1 2009. *Genom Proteom Bioinform*. 2010;8(3):139–44.
43. Zhang R, Yao F, Gao F, Abou-Samra AB. Nrac, a novel nutritionally-regulated adipose and cardiac-enriched gene. *PLoS One*. 2012;7(9):e46254.
44. Zinman G, Brower-Sinning R, Emeche CH, Ernst J, Huang GT, Mahony S, et al. Large scale comparison of innate responses to viral and bacterial pathogens in mouse and macaque. *PLoS One*. 2011;6(7):e22401.