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Protein profiling of nasopharyngeal aspirates of hospitalized and outpatients revealed cytokines associated with severe influenza A(H1N1)pdm09 virus infections: A pilot study

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A B S T R A C T

Influenza A viruses (IAV) mutate rapidly and cause seasonal epidemics and occasional pandemics, which result in substantial number of patient visits to the doctors and even hospitalizations. We aimed here to identify inflammatory proteins, which levels correlated to clinical severity of the disease. For this we analysed 102 cytokines and growth factors in human nasopharyngeal aspirate (NPA) samples of 27 hospitalized and 27 outpatients diagnosed with influenza A(H1N1)pdm09 virus infection. We found that the relative levels of monocyte differentiation antigen CD14, lipocalin-2 (LCN2), C-C-motif chemokine 20 (CCL20), CD147, urokinase plasminogen activator surface receptor (uPAR), pro-epidermal growth factor (EGF), trefoil factor 3 (TFF3), and macrophage migration inhibitory factor (MIF) were significantly lower (p < 0.008), whereas levels of retinol-binding protein 4 (RBP4), C-X-C motif chemokine 5 (CXCL5), interleukin-8 (IL-8), complement factor D (CFD), adiponectin, and chitinase-3-like 1 (CHI3L1) were significantly higher (p < 0.008) in NPA samples of hospitalized than non-hospitalized patients. While changes in CD14, LCN2, CCL20, uPAR, EGF, MIF, CXCL5, IL-8, adiponectin and CHI3L1 levels have already been correlated with severity of IAV infection in mice and humans, our study is the first to describe association of CD147, RBP4, TFF3, and CFD with hospitalization of IAV-infected patients. Thus, we identified local innate immune profiles, which were associated with the clinical severity of influenza infections.

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1. Introduction

We are surrounded by an enormous number of microbes, and some of them are pathogenic. Our immune system tries to protect us from pathogenic microbes, yet, from time to time, some of them evade our immune mechanisms and cause symptoms, which may lead to hospitalization and even death [1]. This may happen when pathogens acquire specific mutations in their genomes enabling them to bypass our immune barriers. In addition, our genetics, underlying health conditions, life-style and environmental factors can also influence our immune system and contribute to the development of severe forms of infectious diseases [2].

Human influenza A viruses receive much attention in public because these viruses cause annual epidemics across the globe and occasionally pandemics [3,4]. The most recent influenza pandemic and following seasonal epidemics were associated mainly with viruses of the influenza A(H1N1)pdm09 lineage [5]. Infections with these viruses are manifested as an acute respiratory disease characterized by a sudden onset of high fever, cough, headache, prostration, malaise, and inflammation of the upper respiratory tract. Acute symptoms and fever often persisted for 7–10 days, and in most cases, the infections were self-limited and led to spontaneous recovery. However, in a small proportion of patients, influenza A(H1N1)pdm09 infection resulted in hospitalizations and even death. Often these patients belonged to risk groups, such as the elderly (>65 years old), young children (<5 years old), pregnant women and immunocompromised individuals [3,4]. However, a substantial number of previously healthy young adults were also hospitalized [6–8].

Many different factors could be associated with hospital admission of patients infected with A(H1N1)pdm09 viruses. For example, mutations in the viral genome could enhance virus replication, change virus tropism and modulate human immune and antiviral responses [9,10]. Defects in the host genes could also lower antiviral and general immune responses [11–21]. Furthermore, a variety of underlying conditions (e.g., cardiac and respiratory diseases, immunosuppression, co-infections), abnormal life-style (smoking) and environmental factors (humidity, temperature) increase the risk of hospitalization [22,23].

Here we explored soluble proteins in NPA samples of patients with influenza A(H1N1)pdm09 infection and compared their levels between those admitted to hospital and those treated as outpatients (Finland, 2009–2014). We identified altogether 14 cytokines, which were associated with hospitalizations.

2. Materials and methods

All patients were treated anonymously and samples were encoded. For intensive care unit patients, consent for sample collection was received from relatives or from the patients afterwards. All nasopharyngeal aspirates (NPAs) samples had been sent to HUSLAB for influenza A(H1N1)pdm09 virus diagnosis. Sample collections which demanded direct patient contact and consent procedure were approved by the ethical review committee of the University of Helsinki Central Hospital, Finland (165/13/03/00/2011). All the ethical issues were handled in accordance with the national and EU regulations (Directive 95/46/EC). Other relevant ethical issues, such as good clinical practice and respect of the Finnish legislation and guidelines were applied. No dual use or misuse issues were concerned.

We obtained NPAs from 54 patients. Half of the patients were hospitalized (16 females, mean age = 59 years; 11 males, mean age = 56) and other half were treated as outpatients (11 females, mean age = 40; and 16 males, mean age = 47) during 2009–2014 in Finland. The decision of hospitalization was based on a clinical evaluation of the severity of the disease as judged by an internist in the emergency room. The assessment was carried out according to local practices: if the clinician evaluates that the patient could not manage at home, the patient was hospitalized. As for those attending intensive care unit, the principles of evaluation were consistent with the Infectious Diseases Society of America guidelines on community-acquired pneumonia [24]. Patients for the present study were selected among outpatients, hospitalized patients in the ward or in the intensive care unit. All 54 patients were diagnosed with influenza A(H1N1)pdm09 infections diagnosed by qRT-PCR as described previously [25].

The relative levels of 102 cytokines, chemokines, and growth factors were determined using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems Inc., USA) according to the manufacturer's instructions. Importantly, this immunoassay was chosen because it includes several biomarkers (e.g., CD14, LCN2, CCL20, uPAR, EGF, MIF, CXCL5, IL-8, adiponectin and CHI3L1), which were previously associated with severe IAV infections [26–35]. The results were analysed using ImageJ software. The average signal values of the pair of duplicate spots of each analyte were calculated. The background signal of each film was determined by using the minimum averaged signal value detected on each film. Background signals were subtracted and quantile normalization was used to normalize the data by using Bioconductor's preprocessCore package [Bolstad BM. *preprocessCore: A collection of pre-processing functions. R package version 1.32.0*]. Gene set enrichment analysis was performed using freely available software (http://software.broadinstitute.org/gsea/index.jsp).

Cytokine profiling data was log2 transformed for linear modelling and empirical-Bayes-moderated t-tests using the LIMMA package [36]. To analyse the differences caused by the hospitalization, a linear model was fit to each cytokine/metabolite; the conditions of the volunteers, i.e. hospitalized or non-hospitalized were indicated in the design matrix for this fit. The Benjamini-Hochberg method was used to correct for multiple testing. The significant cytokines were determined at a Benjamini-131 Hochberg false discovery rate (FDR) controlled at 10%. The heatmaps for cytokines and metabolites were generated using the pheatmap package [Raivo Kolde (2015). pheatmap: Pretty Heatmaps. R package version 1.0.8.] based on log transformed profiling data and the clustering of analytes in the heatmap was generated using the complete clustering method on Euclidian distances.

3. Results

To find correlations between patient hospitalizations and NPA cytokine levels, we collected NPA samples from 54 Finnish patients diagnosed by RT-qPCR with A(H1N1)pdm09 influenza virus
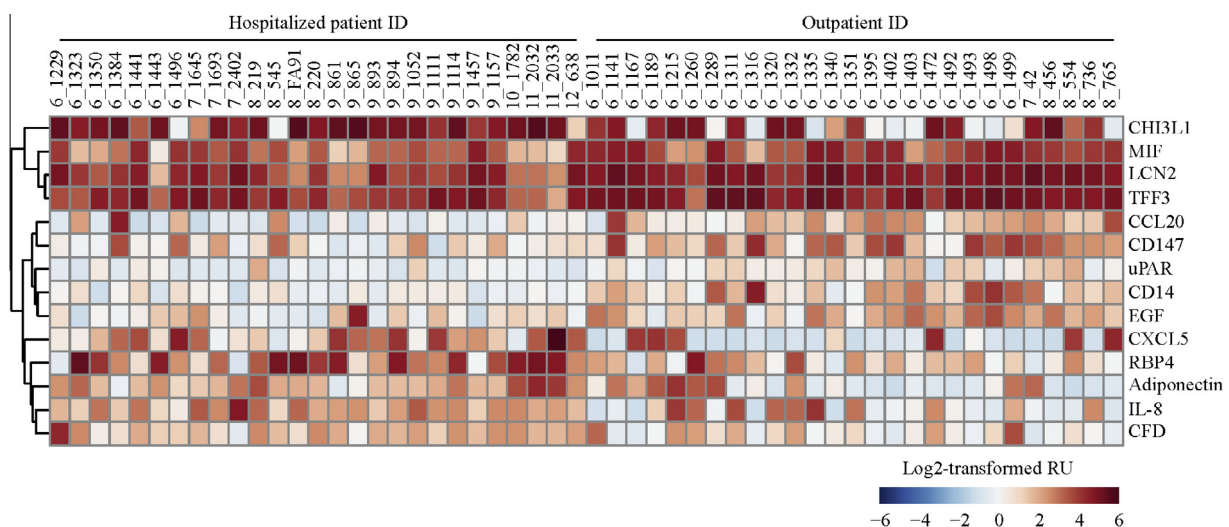
Fig. 1. Profile of 14 cytokines differentially expressed in nasopharynges of 27 hospitalized and 27 outpatients diagnosed with influenza A(H1N1)pdm09 virus infection. A heat map of 14 cytokines is shown. Rows represent cytokines. Columns represent samples. Each cell is coloured according to the average of log2-transformed profiling values. Complete-linkage hierarchical clustering based on Euclidian distances was used for clustering of cytokines.