

Consecutive Rhinovirus Infection of Epithelial Cells Alters Chrono-Inflammatory Expression Network

Weckmann M^{1,2*}, Becker T³, Pech M^{1,2}, Koch CE⁴, Oster H⁴ and Kopp MV^{1,2}

¹Department of Pediatric Allergy and Pulmonology, Children's Hospital at the University of Lübeck, Germany

²Airway Research Center North (ARCN), German Center for Lung Research (DZL), Germany

³Imaging Platform at the Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA

⁴Department of Medical Chronophysiology, University of Lübeck, Lübeck, Germany

Abstract

Introduction: Asthma exacerbations are associated with viral upper respiratory tract infections, and rhinovirus (RV) is the major cause of these virus-associated exacerbations. Circadian clocks regulate physiological rhythms via transcriptional networks. These networks contain several feedback mechanisms inducing complex tissue specific patterns of gene activation and repression that help to maintain and regulate important biological processes. For example, the neutrophil inflammation of the lung is gated by epithelial circadian oscillators. We hypothesized that recurrent, human rhinovirus infection (HRVI) of *in vitro* cultured cells alter the circadian-gated inflammatory networks and imprint a methylation pattern similar to the network found in asthmatic patients.

Results: We measured the methylation of clock and inflammatory genes in the BEAS2-B cell line after 1, 3 or 5 consecutive rhinovirus infections. Hierarchical clustering of methylation levels identified distinct clusters of numbers of infection (NOI: 1,3 and 5) for clock and inflammatory genes in BEAS2-B cells. Furthermore, a linear regression model of the methylation level was used to identify significantly increased or decreased loci ($p < 0.01$). In non-infected cells, clear clusters of RNA expression of circadian clock genes *CRY1*, *PER2*, *PER3*, *CLOCK* were found to be negatively associated with interleukin 8 (IL-8) expression. After HRVI a partial reversal is observed and the expression of the *CLOCK* gene is positively correlated to IL-8 expression. Similarly, in asthmatic patients, a strong positive correlation between *CLOCK* and *IL-8* was found.

Conclusion: This study provides first insights in how repeated viral infections (e.g. HRVI exacerbations in asthma) may introduce persistent changes to circadian-gated inflammatory networks involved in governing neutrophil recruitment. This could offer novel diagnostic strategies to identify and define certain asthmatic endotypes and lead to novel therapeutic approaches based on circadian rhythm stabilization.

Keywords: Asthma; Gene expression; RNA; Epigenetic

Introduction

Despite considerable efforts in research and clinical management the incidence rate of asthma in early childhood has not changed for many years. Recurrent lower respiratory tract viral infections with Rhinovirus (RV) or Respiratory Syncytial Virus (RSV) are indicative for the development of asthma later in life [1-3]. However, the underlying mechanisms driving this association, remain largely unknown. Up to 80% of asthma exacerbations are associated with viral upper respiratory tract viral infections with rhinovirus (RV) as their major cause of these virus-associated exacerbation [4,5].

Circadian clocks regulate physiological rhythms via transcriptional networks. These networks contain several feedback mechanisms inducing complex tissue specific patterns of activation and repression. Gibbs et al. elegantly described in their recent work, how neutrophil inflammation of the lung is gated by the epithelial circadian oscillators. Selective depletion of the clock gene *BMAL1* in club cells resulted in overexpression of *CXCL5*, a chemoattractant for neutrophils, after *in vivo* stimulation with LPS [6]. Bozek and colleagues predicted, based on the presence of circadian transcription factor binding sites, an extensive network of clock-controlled genes (CCG). Therefore, small shifts in clock gene expression, or reduced sensitivity to circadian signals (e.g. by methylated promoter regions of target genes) may already induce substantial cascading amplification and dysregulation further downstream [7]. Recent data on epigenetic modifications of rhinovirus infections indicate profound effects on methylation patterns in epithelial cells *ex vivo* [8].

Patients with asthma show marked circadian variations in their

airway hyperreactivity, symptoms and treatment response. It has been shown that neutrophil lung infiltrates are critically elevated in nocturnal asthma, which is correlated with increased symptoms and even mortality [9,10]. These neutrophilic asthma phenotypes are considered as refractory to therapy such as high-glucocorticosteroid treatment [11-14]. We hypothesized a perturbation of circadian-gated inflammatory networks after recurrent, human rhinovirus infection (HRVI) *in vitro* and similarly changed correlations in asthmatics nasal epithelial cells.

Methods

Tissue cell culture

BEAS2-B and human primary nasal epithelial cells were cultivated within BEGM (Lonza, CC3170) precoated (1:50 PureCol, Advances Bio Matrix, 5005-B) 6 well plates (Sarstedt, 833920300). The HRVI was performed in passage 2 (primary cells) with 3×10^5 cells, at multiplicities

***Corresponding author:** Dr. Markus Weckmann, Department of Pediatric Allergy and Pulmonology, Children's Hospital at the University of Lübeck, Germany, Tel: 0049-451-500-5838, Fax: 0049-451-500-4193; E-mail: markus.weckmann@uksh.de

Received September 25, 2017; **Accepted** September 29, 2017; **Published** September 30, 2017

Citation: Weckmann M, Becker T, Pech M, Koch CE, Oster H, et al. (2017) Consecutive Rhinovirus Infection of Epithelial Cells Alters Chrono-Inflammatory Expression Network. J Mol Genet Med 11: 282 doi:10.4172/1747-0862.1000282

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of infection (MOI) of 10 (RV-16) and harvested 48 h post infection. As infection control another 3×10^5 cells of each donor were treated like the infected cells without a HRVI. For BEAS2-B, consecutive infections were repeated as described above with a 24 h infection period and giving cells time to reach 70% confluency after termination of infection (no virus present). Each consecutive infection was paired with a non-infected control.

Genome wide analysis of DNA methylation and RNA expression

HumanMethylation450 BeadChip Kit was used to analyse DNA methylation and RNA sequencing. Measurements to determine RNA expression were carried out at the Institute of Clinical Molecular Biology, Christian-Albrechts-University of Kiel, following the manufacturer's protocols. The genome wide analysis of loci specific DNA methylation was performed using 500 ng bisulfite-converted genomic DNA (EZ DNA methylation Kit, Zymo Research, D5001) and the HumanMethylation450 BeadChip Kit (Illumina, WG-314-1003). β -values were Illumina normalized (Partek Genomic Suite, Partek). For RNA sequencing 1000 ng of total RNA were applied with the HiSeq PE Cluster Kit v4, cBot (Illumina, PE-401-4001), the HiSeq SBS Kit v4 (Illumina, FC-401-4003) and the HiSeq2500 System (Illumina).

Study population

Our study cohort is a subpopulation of the KIRA cohort (part of The ALLIANCE cohort) and was recruited from January 2014 to February 2015 in Luebeck (Germany). We included 10 children with doctors diagnosed asthma based on the criteria of the GINA guidelines and 10 children without asthma (controls). Primary epithelial cell scrapings for epithelial cell culture were done in all children. Individuals with fever ($\geq 38.5^\circ\text{C}$) or signs of upper or lower respiratory tract infection during the last two weeks prior to the planned visit were excluded.

Modelling and statistical analysis

Correlation maps were computed using JMP13 (SAS, USA) based on multivariate Pearson coefficient analysis. In BEAS2-B cells, only changes in methylation with linear regression coefficients of greater than 0.5 and $p < 0.01$ for number of infections (NOI) were selected for clock genes (*CRY1*, *CRY2*, *PER2*, *PER3*, *CLOCK*, *IL8*, *IL6*, *IL4*, *IL5*, *IL32*, *IL13*, *CXCL10*, *CXCL1*, *TLR10*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR6*, *TLR8*, *NR1D1*, *INF2*, *CCL5*, *CXCL5*, *MUC5AC*), asthma key genes and known genes involved in neutrophil recruitment.

Results

We infected BEAS2-B cells with rhinovirus 16 for 24 h for five consecutive times. During the virus free periods, we allowed the cells to recover and reach 70% confluency. Methylation of clock and asthma related genes (Supplement Table 1) in bronchial epithelial cells showed strong correlation with increasing numbers of infections (Figure 1A). Ward hierarchical clustering separated distinct clusters for every infection number [1,3,5]. Methylation showed not only positive (cg02010763, $\Delta\beta$ -value: 0.11, $p < 0.001$) increases but also reductions (cg15616511: $\Delta\beta$ -value -0.13, $p < 0.001$) when a linear regression model across all numbers of infections was applied. These changes coincided with significant alterations in chrono-inflammatory network. RNA expression patterns (RNASeq, Figure 1B) of infected and non-infected BEAS2-B cells were plotted as correlation maps (Pearson coefficient, blue=-1, red=1). In non-infected cells, clear correlation clusters exist (circadian clock genes *CRY1*, *PER2*, *PER3*, *CLOCK*) and these were negatively correlated with, e.g. interleukin (IL)

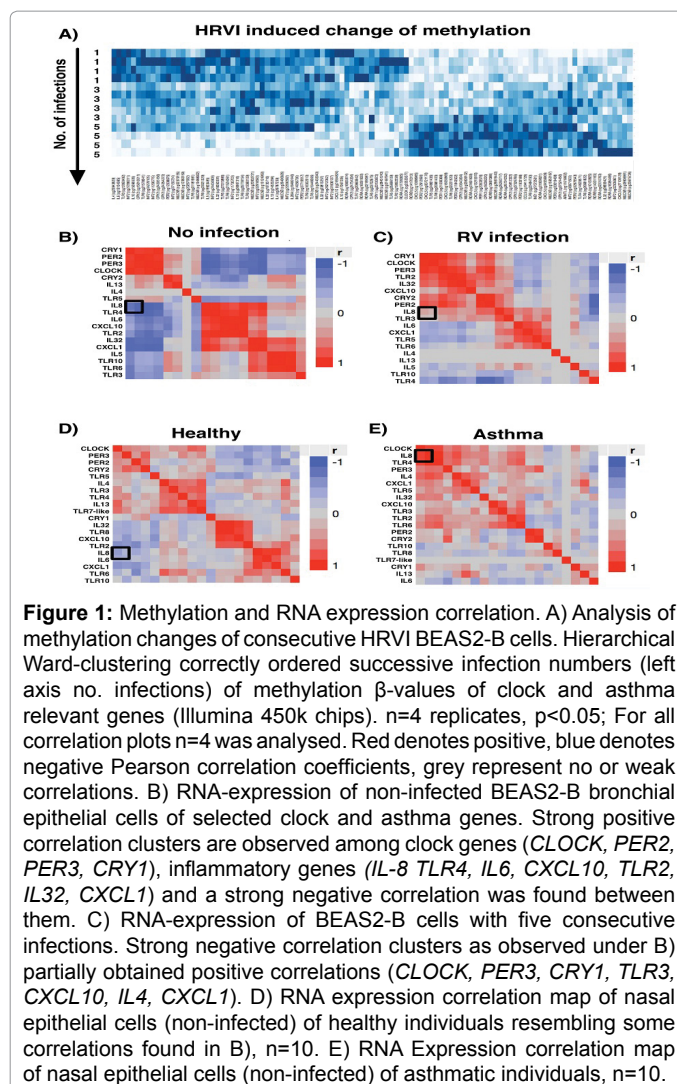


Figure 1: Methylation and RNA expression correlation. A) Analysis of methylation changes of consecutive HRVI BEAS2-B cells. Hierarchical Ward-clustering correctly ordered successive infection numbers (left axis no. infections) of methylation β -values of clock and asthma relevant genes (Illumina 450k chips). $n=4$ replicates, $p < 0.05$; For all correlation plots $n=4$ was analysed. Red denotes positive, blue denotes negative Pearson correlation coefficients, grey represent no or weak correlations. B) RNA-expression of non-infected BEAS2-B bronchial epithelial cells of selected clock and asthma genes. Strong positive correlation clusters are observed among clock genes (*CLOCK*, *PER2*, *PER3*, *CRY1*), inflammatory genes (*IL-8* *TLR4*, *IL6*, *CXCL10*, *TLR2*, *IL32*, *CXCL1*) and a strong negative correlation was found between them. C) RNA-expression of BEAS2-B cells with five consecutive infections. Strong negative correlation clusters as observed under B) partially obtained positive correlations (*CLOCK*, *PER3*, *CRY1*, *TLR3*, *CXCL10*, *IL4*, *CXCL1*). D) RNA expression correlation map of nasal epithelial cells (non-infected) of healthy individuals resembling some correlations found in B), $n=10$. E) RNA Expression correlation map of nasal epithelial cells (non-infected) of asthmatic individuals, $n=10$.

-8 a neutrophil chemoattractant. In infected cells, this pattern changed substantially (Figure 1C), where asthma relevant genes such as *IL-32* and neutrophil chemoattractants like *IL-8* favored a rather positive correlation. We next assessed, if a similar pattern would be observed in healthy and asthmatic children, when nasal epithelial cells were cultured but not infected. Healthy, RNA expression (Figure 1D) shows patterns reminiscent of those seen in non-infected BEAS2-B cells, but with an overall weaker correlation. In asthma, a strong correlation was found between *CLOCK* and *IL-8*. *IL-32* also showed a shift towards positive correlation, albeit weaker than that observed in tissue culture (Figure 1E, RNA expression). This suggests that HRVI not only alters methylation in the cell's clock genes but also leads to a disruption of chrono-inflammatory expression networks.

Discussion

Here we present first evidence that consecutive infections with human rhinovirus induce changes in the DNA methylation of several clock genes in *in vitro* in a cultured bronchial epithelial cell line. Furthermore, these changes decouple clock genes expression from genes involved in neutrophil inflammation and asthma, respectively. While expression patterns in RV-infected BEAS-2B cells resembled those in epithelial cells of asthmatic children, healthy controls showed

patterns more similar to those from uninfected BEAS-2Bs. This supports the notion of an altered circadian-inflammatory network present in asthma.

Recent evidence is emerging from clock gene knock-out studies that circadian control is crucial for controlling inflammation, e.g. neutrophil inflammation. Gibbs et al. showed in an elegant study, that local depletion of *BMAL1* increases the production of CXCL5 by club cells in the lung. As a result, neutrophil recruitment augmented after lipopolysaccharide stimulation and clearance of *Streptococcus pneumoniae* infection was hampered [6]. In line with the critical importance of the systemic secretion of cortisol as the circadian synchronizer, the authors identified the oscillating occupancy of the CXCL5 promoter corticosteroid response element (CRE) in club cells imperative for the control of normal neutrophil recruitment, which clearly indicates regulatory potential for epigenetic modifications such as methylations [6]. Similarly, Ehlers and colleagues found mice with postnatal *BMAL1* knockout to show exaggerated Sendai virus and influenza susceptibility. In addition, these animals developed an asthma-like phenotype and expression analysis of bronchial brushings (SARP cohort) showed clear evidence of an altered clock activity in asthmatics [15]. Specific virus infection of the lungs apparently seems to modify clock and inflammatory gene expression networks. In a mouse model of tobacco smoke exposure and influenza infection, inflammatory genes (e.g. interleukin 6) showed phase-shifts very similar to what we observed in our correlation maps [16]. Whereas under control (wildtype and “air”) conditions interleukin 6 is off-phase with the *CLOCK* gene, it becomes synchronized under viral infections.

Conclusion

Our data may offer a first glimpse on how consecutive viral infections may lead to persistent changes in chrono-inflammatory networks involved in governing neutrophil inflammation in asthmatic individuals, especially after HRV1 exacerbations. *IL-8* was negatively correlated with *CLOCK* expression in healthy individuals but correlated positively in asthmatics. This effect was introduced after up to five infections with HRV of BEAS2-B cells which also strongly altered methylation in several cell clock genes. However, further research including larger asthma cohorts and clinical studies are essential to better understand the impact of viral infection on persisting changes of methylation and their effects on asthma pathogenesis. This may offer novel strategies to identify certain asthma-endotypes. On the other hand, elucidating changes in the chrono-inflammatory network in asthmatic patients might lead to new therapeutic strategies, e.g. anti-viral medication, corticosteroid dose timing, or rhythm stabilization.

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