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BRIEF REPORT

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# Epigenome-wide effects of vitamin D on asthma bronchial epithelial cells

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**ABSTRACT**

Vitamin D is a nutrient and a hormone with multiple effects on immune regulation and respiratory viral infections, which can worsen asthma and lead to severe asthma exacerbations. We set up a complete experimental and analytical pipeline for ATAC-Seq and RNA-Seq to study genome-wide epigenetic changes in human bronchial epithelial cells of asthmatic subjects, following treatment of these cells with calcitriol (vitamin D3) and Poly (I:C)(a viral analogue). This approach led to the identification of biologically plausible candidate genes for viral infections and asthma, such as DUSP10 and SLC44A1.

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## To the editor

Vitamin D may prevent or attenuate severe asthma exacerbations and viral respiratory infections through epigenetic regulation of innate and adaptive immune mechanisms [1] through processes such as chromatin folding. Genome-wide chromatin accessibility assays such as ATAC-Seq (Assay for Transposase Accessible Chromatin and high-throughput sequencing) [2] can identify transcriptionally active sections of chromosomes, thus helping us define the binding status of transcription factors, determine nucleosome occupancy, and construct gene regulatory networks. We report the use of ATAC-Seq and total RNA-Seq to evaluate the genome-wide effects of vitamin D, with and without a viral analogue (Poly (I:C)) on asthmatic bronchial epithelial cells.

Asthmatic (AHBEC) bronchial epithelial cells were cultured and stimulated with sham (culture media), calcitriol, poly (I:C) (to simulate viral infection), or both. ATAC-Seq was performed using one sample per treatment to detect chromatin sections open for transcription; the resultant DNA fragments were purified, amplified, and sequenced. Sequencing data was aligned to the reference genome with Bowtie2 [3], and peaks were called with MACS2 [4] and annotated with HOMER. Chromatin accessibility was quantified as read coverage within the merged ATAC-Seq peak regions for each sample, and the count matrix was normalized with library sizes and transformed to counts per million (CPM) using edgeR [5]. Pairwise fold-change of the chromatin accessibility was then calculated based on CPM. Pathway enrichment analysis was performed on genes annotated to the differential peaks, using Fisher's exact test.

RNA-Seq was performed using the same conditions as for ATAC-Seq. RNA-Seq was performed in three biological replicates where cell line AHBEC obtained from ATCC was cultured from different passages and on different dates, and in one set of three technical replicates to confirm that the open chromatin determined by ATAC-Seq corresponds to changes in gene expression. The RNA-Seq data was further validated using real-time PCR of detected genes using three biological and three technical replicates. RNA-Seq reads were aligned to the reference genome with STAR [6], and TPM (Transcripts Per Kilobase Million) value was quantified for each gene

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in each sample using RSEM [7]. Bigwig files containing the read coverage information of every sequencing sample were generated for the visualization in the UCSC Genome Browser [8].

The quality of the ATAC-Seq data is shown in Figures S1 and S2. The mapping rates were high (>95%), and the average number of ATAC-Seq peaks (accessible chromatin regions) was 17,125 (detected by MACS2 *callpeak*), but with wide variation depending on treatment protocol and library size (Table S1).

Clustering analysis on the chromatin accessibility indicated clustering of: Sham and Calcitriol treated cells, Poly I:C and Calcitriol +Poly I:C treated samples (Figure S3). Differential peaks were identified between six pairwise comparisons among the four treatments for Asthma AHBEC (n = 616) (Figure 1) with more than a three-fold absolute change on the log2 transformation of CPM in any of the pairwise comparisons. Among these differential peaks, 52 were highly correlated (absolute correlation >0.9) to the expression of corresponding annotated genes (Table S2). The top 4 correlated ATAC-Seq differential peaks and RNA-Seq genes are shown in Figure 2. We used QRT-PCR to validate the top 4 genes from RNA-Seq (Figure S5). We also performed analyses for differential gene expression between each treatment (Calcitriol, Poly I:C, and Calcitriol +Poly I:C) versus Sham (Table S3) using the R package DESeq2. We summarized the log fold-changes for the differentially expressed genes from RNA-Seq using adjusted p-value <0.05 and the corresponding differential peaks from ATAC-Seq that share the same gene symbol annotations. For Calcitriol vs Sham,

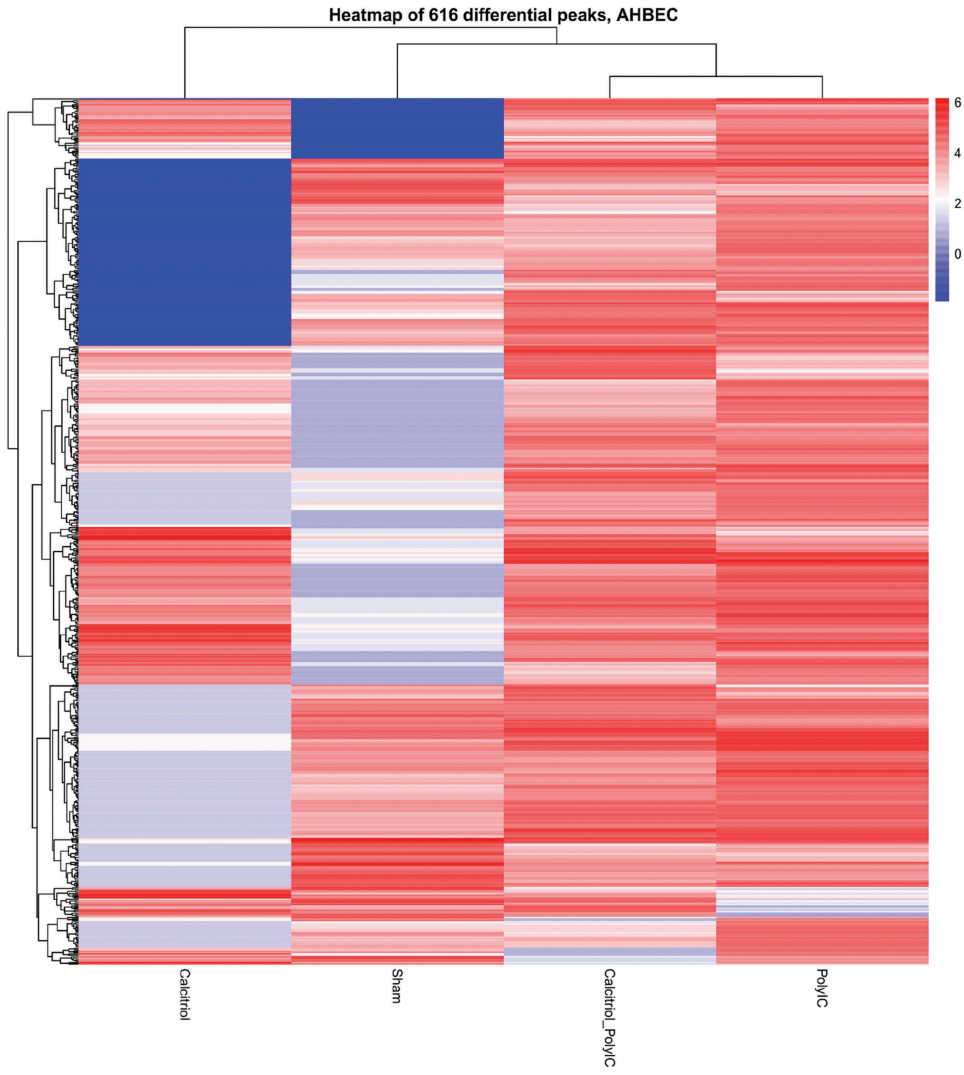


**Figure 1.** Heatmap of chromatin accessibility in the differential peaks for AHBEC.

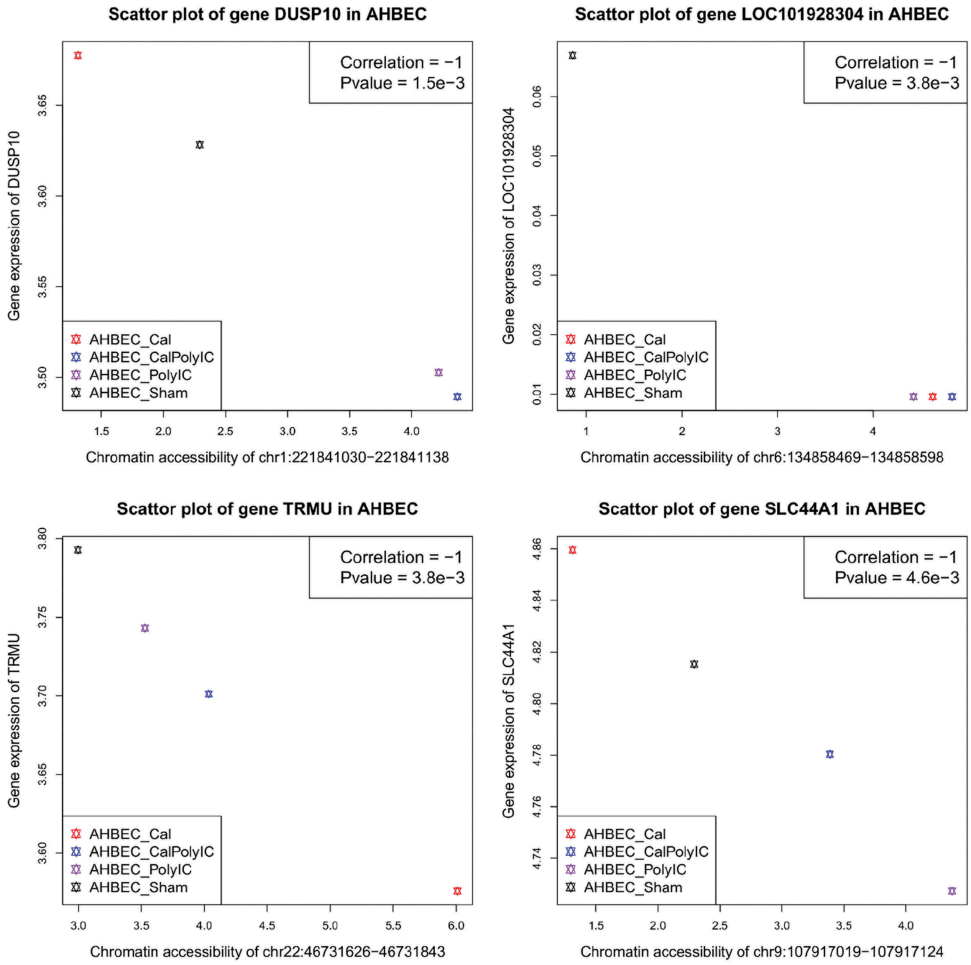


**Figure 2.** Scatter plots of top correlated ATAC-Seq differential peaks and RNA-Seq genes.

there were no differentially expressed genes. We have 92 differentially expressed genes between Poly I:C vs. Sham and 364 differentially expressed genes between Calcitriol+PolyI:C vs. Sham. The corresponding peaks from ATAC-Seq that share same gene annotations (table S3) include 4 and 11 overlapped peaks with corresponding genes between PolyIC vs. Sham and Calcitriol+PolyIC vs. Sham, respectively. TBX3 gene has a log fold-change of 3.56 between PolyIC vs. Sham. In addition, the peak annotated with SLC8A1 gene also has a relatively high log fold-change of 2.72. For Calcitriol+PolyI:C vs. Sham, we can also notice that the peaks annotated to TBX3 and SLC8A1 are differential based on more than three-fold absolute changes. Besides, the significant differential peak also includes the one annotated to the ZCCHC2 gene. In a genome-wide study of a gene by environment interaction (GWIS) of depressive symptoms and stressful life events, a single nucleotide polymorphism (SNP) intronic to ZCCHC2 (rs17070072) was significant ($p = 1.46 \times 10^{-8}$) [9].

Large absolute log fold changes (near 3 or >3) for differential peaks between PolyI:C vs. Sham (genes SLC8A1 and TBX3) and Calcitriol+PolyI:C vs. Sham (genes SLC8A1, TBX3 and ZCCHC2) and those for the corresponding differentially expressed genes annotated to them are shown in Figure S6.

Through pathway enrichment analysis of genes annotated to the differential peaks, we identified 101 pathways significantly enriched (FDR < 0.05) by the differentially accessible genes. The pathway analysis in the asthma cell line 'AHBEC' showed enrichment in cancer-related and metabolic pathways (Table S4). Genes with the highest fold-change between treatments included *DUSP10, LOC101928304, TRMU*, and *SLC44A1* (Table S2). Dual specificity phosphatase 10 (DUSP10), also known as MAP kinase phosphatase 5 (MKP5) is a key negative regulator of IL-33-induced

cytokine production in Th2 cells [10]. Mkp5-deficient cells produced greatly enhanced levels of pro-inflammatory cytokines during innate immune responses and exhibited greater T-cell activation than their wild-type counterparts [11]. MKP5 was shown to protect against sepsis-induced acute lung injury [12]. Furthermore, deficiency of MKP5 results in reduced influenza virus replication in the lung, which is associated with increased type I IFN production [13] while overexpression of MKP2 and MKP5 inhibited IFNβ promoter activity in response to LPS or poly (I:C) stimulation [14]. DUSP10 negatively regulates the inflammatory response to rhinovirus through IL-1β signalling [15].

DUSP10 is a primary vitamin D target is expressed in human peripheral blood mononuclear cells (PBMCs) [16]. showed that changes in the expression of DUSP10 and 11 other VitD target genes in human PBMCs at the start and the end of the 5 months vitamin D-intervention (VitDmet study) were systematically correlated with the alteration in the circulating form of vitamin D3, 25-hydroxyvitamin D3 (25(OH)D3). Furthermore, VDR target gene DUSP10 gene expression appears to be the most comprehensive biomarker for vitamin $D_3$ responsiveness of human individual [17].

*LOC101928304* is an uncharacterized non-coding RNA, and TRMU (tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase) is a nuclear gene that encodes a mitochondrial tRNA-modifying enzyme. *SLC44A1* (solute carrier family 44 members 1) also called Choline like transporter family is proposed to supply choline for the synthesis of cell membrane phospholipids in an NA(+)-independent manner. The physiological relevance of SLC44A1 is indicated by its likely involvement in membrane synthesis for cell growth or repair, and also by its role in phospholipid production for the generation of lung surfactant. In transfusion-related acute lung injury, antibodies to SLC44A2 cause a deleterious aggregation of granulocytes [18].

In the analysis of differential gene expression between each treatment (Calcitriol, Poly I:C, and Calcitriol +Poly I:C) versus Sham, there were no differentially expressed genes, based on the adjusted p-value, for Calcitriol vs. Sham. We extracted the log fold-change in ATAC-Seq for the top 5 differentially expressed genes from the results of all the peaks (not the 616 differential peaks we reported) between PolyIC vs Sham and Calcitriol + PolyIC vs Sham. For the genes with multiple peaks, we took the peak with the largest absolute log fold-change (Table S5).

In comparing Poly IC vs Sham we identified dual specificity phosphatase 4 (DUSP4), Activin A receptor type 2B (ACVR2B) and T-Box3 (TBX3). DUSP4 belongs to the same family as DUSP10, the top 1 gene we report in our correlation analysis. It regulates corticosteroid sensitivity via dephosphorylation of JNK1 and GR-$Ser^{226}$ . DUSP4 activation by formoterol restores impaired corticosteroid sensitivity, indicating that DUSP4 is crucial in regulating corticosteroid sensitivity, and therefore might be a novel therapeutic target in severe asthma [19]. ACVR2B, a member of the transforming growth factor (TGF)-β superfamily of secreted factors, is a potent negative regulator of muscle growth. Systemic Blockade of ACVR2B Ligands Protects Myocardium from Acute Ischaemia-Reperfusion Injury [20]. T-box genes encode transcription factors involved in the regulation of developmental processes. TBX3 is the earliest expressed member of the T-box transcription factor family and is involved in the maintenance and induction of pluripotency [21].

In comparing Calcitriol + PolyIC vs. Sham we identified DUSP4, TBX3 and Macrophage Migration Inhibitory Factor (MIF). MIF plays a role in the regulation of macrophage function in host defense through the suppression of anti-inflammatory effects of glucocorticoids. MIF also antagonizes glucocorticoid inhibition of T-cell proliferation in vitro by restoring IL-2 and IFN-γ production [22]. Furthermore, MIF overexpression promotes the proliferation and migration of Airway Smooth Muscle Cells (ASMCs) by upregulating the activity of the ERK1/2 and FAK signalling and the expression of matrix metalloproteinase (MMP)-2 [23]. In mouse models of allergic asthma, the lack of MIF causes an almost complete abrogation of the cardinal signs of the disease including mucus secretion, eosinophilic inflammation, and airway hyperresponsiveness. Additionally, blocking the expression of MIF in animal models leads to a significant reduction of pathological signs of eosinophilic inflammation [24].

We uploaded the bam and bigwig files to the NSF-funded site CyVerse Discovery Environment

(https://de.cyverse.org/de/) that supports free data hosting and created custom tracks on UCSC Genome Browser to visualize read coverage in these matched ATAC-Seq and RNA-Seq samples (http://genome.ucsc.edu/s/zhangr100/AHBEC_VitD). Our chromatin accessibility patterns were similar to the reference DNase I hypersensitivity clusters in the ENCODE database. For instance, integration of ATAC-Seq and RNA-Seq data indicated the effect of chromatin accessibility on the expression of *DUSP10* (Figure S4).

Vitamin D is a hormone with multiple effects on immune regulation and respiratory viral infections, which may affect asthma morbidity. We set up an experimental and analytical approach to ATAC-Seq and RNA-Seq to study genome-wide epigenetic changes in human bronchial epithelial cells of asthmatic subjects, following treatment of these cells with vitamin-D and Poly I:C. This approach led to the identification of biologically plausible candidate genes for viral infections and asthma *(DUSP10)* a Vitamin D Receptor 'VDR' target gene is a key negative regulator of IL-33-induced cytokine production in Th2 cells [10]. For instance, deficiency of MKP5 results in reduced influenza virus replication in the lung, which is associated with increased type I IFN production [13]. Type I IFN signalling promotes the maintenance of lung epithelial tight junctions during *S. pneumoniae* infection thereby reducing the passage of the pathogen from alveoli into the lung parenchyma [25,26].

*SLC44A1* gene is involved in phospholipid production for the generation of lung surfactants. Surfactant Proteins play a role in: (1) lowering surface pressure of the alveolus [27], (2) enhancing bacterial and viral clearance by opsonizing pathogens and facilitate their phagocytosis immune cells such as macrophages and monocytes [28] and (3) immunomodulation: studies have shown that surfactants proteins SP-A and SP-D bind directly to allergens and particles such as pollen grains [29], house dust mite allergen [30], and *Aspergillus fumigatus* allergen [31], inhibiting specific IgE binding to allergens and subsequently decreasing allergen-induced histamine release.

Additional experiments are needed to validate our findings, ideally on a larger set of cells from subjects with asthma and matching control subjects.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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