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Bronchial epithelial gene expression and interstitial lung abnormalities

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Abstract

Introduction Interstitial lung abnormalities (ILA) often represent early fibrotic changes that can portend a progressive fibrotic phenotype. In particular, the fibrotic subtype of ILA is associated with increased mortality and rapid decline in lung function. Understanding the differential gene expression that occurs in the lungs of participants with fibrotic ILA may provide insight into development of a useful biomarker for early detection and therapeutic targets for progressive pulmonary fibrosis.

Methods Measures of ILA and gene expression data were available in 213 participants in the Detection of Early Lung Cancer Among Military Personnel (DECAMP1 and DECAMP2) cohorts. ILA was defined using Fleischner Society guidelines and determined by sequential reading of computed tomography (CT) scans. Primary analysis focused on comparing gene expression in ILA with usual interstitial pneumonia (UIP) pattern with those with no ILA.

Results ILA was present in 51 (24%) participants, of which 16 (7%) were subtyped as ILA with a UIP pattern. One gene, pro platelet basic protein (PPBP) and seventeen pathways (e.g. TNF-α signalling) were significantly differentially expressed between those with a probable or definite UIP pattern of ILA compared to those without ILA. 16 of these 17 pathways, but no individual gene, met significance when comparing those with ILA to those without ILA.

Conclusion Our study demonstrates that abnormal inflammatory processes are apparent in the bronchial airway gene expression profiles of smokers with and without lung cancer with ILA. Future studies with larger and more diverse populations will be needed to confirm these findings.

Keywords Interstitial lung abnormalities, ILA, Gene expression, Interstitial lung disease, Lung fibrosis

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Introduction

Interstitial lung abnormalities (ILA) are chest computed tomography (CT) findings in persons not known to have interstitial lung disease (ILD) that may represent an early stage of pulmonary fibrosis (PF) [1]. Overlapping associations with genetic polymorphisms [2] and protein biomarkers [3] between those with ILA and idiopathic pulmonary fibrosis (IPF) highlight the fact that these conditions may share pathobiology.

While there are numerous cell types in the lung that likely play important roles in the pathobiology of IPF, growing data suggests that patients with PF have altered patterns of bronchial epithelial cell gene expression [4]. Significant alterations in airway morphology and gene expression signatures have been described in patients with IPF [5–9]. Additionally, several of the single nucleotide polymorphisms that are associated with increased risk for IPF, including MUC5B and DSP are expressed in bronchial airway cells [10, 11]. However, no prior study has characterized bronchial epithelial gene expression among persons with ILA.

To provide an assessment of the genes and pathways associated with ILA, and ILA subtypes we performed bronchial airway epithelial gene expression analyses in patients enrolled in Detection of Early Lung Cancer Among Military Personnel (DECAMP 1 and DECAMP 2) cohorts [5].

Methods

Study population

The DECAMP Study is a multicenter consortium comprised of 15 military treatment facilities, Veterans Affairs hospitals, and academic centers across the United States. Participants were recruited into one of two study protocols, designated as DECAMP-1 and DECAMP-2 [12]. Studies were registered at clinicaltrials.gov as DECAMP 1 (NCT01785342) and DECAMP 2 (NCT02504697) respectively. Briefly, study participants of DECAMP-1 were adults aged 45 and older with indeterminate pulmonary nodules and heavy smoking history whereas, study participants of DECAMP-2 were aged 50–79 with a heavy smoking history and a family history of lung cancer or a personal history of chronic obstructive pulmonary disease (COPD). This study was approved by the Human Research Protection Office (HRPO) for the Department of Defense, and the individual site IRBs for every participating site. All subjects were approached for written informed consent to participate in the study per IRB regulations (Additional file 1: Table S1).

Biospecimen collection in DECAMP

All individuals in the DECAMP study underwent bronchoscopy. Bronchial airway epithelial cells were obtained from brushings of the right mainstem bronchus collected during fiberoptic bronchoscopy with an endoscopic cytobrush (Cellebrity Endoscopic Cytology Brush, Boston Scientific, Boston). The brushes were immediately placed in 1 mL of RNAprotect Cell Reagent (Qiagen, Valencia, CA) and kept at − 80 °C until RNA isolation was performed.

RNA isolation, sequencing and data pre-processing

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Valencia, CA). RNA integrity was assessed by Agilent BioAnalyzer, and RNA purity confirmed using a NanoDrop spectrophotometer. Libraries were generated using the Illumina TruSeq Stranded Total RNA kit and sequenced on the Illumina NextSeq 500 and Illumina HiSeq3000 with 75 base-pair paired-end reads (Illumina, San Diego, CA). For data preprocessing, we developed an automatic pipeline using the Nextflow framework. Quality of FASTQ files was assessed with FastQC. Reads were aligned to the human genome with 2-pass STAR and gene-level and isoform-level expression quantified with RSEM. Splice junction saturation, transcript integrity, and biotype distributions were calculated for each sample with RSeQC. DESeq2 or edgeR was used to identify associations between gene expression profiles and clinical variables while controlling for confounding covariates. Genetic variants were called using the Broad Institute's GATK RNA-seq best-practices workflow. Briefly, duplicates were marked with Picard tools, splitting of intronic reads, realignment around indels, and base quality score recalibration were performed with GATK, and variants were called with Haplotypecaller.

Imaging acquisition in DECAMP

DECAMP-1 utilized CT scans collected as part of routine clinical care while DECAMP-2 utilized a standardized protocol for image acquisition and reconstruction. DECAMP-2 scans were collected using low dose helical computed tomography on a minimum 16-slice scanner. The scans were acquired at 2.5 to 5 mm but reconstructed into 1 mm slice thickness using the soft tissue and lung algorithms. Images from all sites were then de-identified and submitted to the American College of Radiology Imaging Network (ACRIN) Core Laboratory for storage.

Gene expression analysis

The LIMMA package in R (version 3.4.0) was used to assess differential gene expression (DGE). To do this, raw count matrix of gene expression was initially filtered by counts per million (CPM) such that a gene could only be included if its CPM was greater than 1 in 10% of the total number of patients. DGE analysis was then performed using a pairwise comparison between the ILA phenotypes at a false discovery rate (FDR) of 0.05.

The differentially expressed genes (DEG) identified by LIMMA were further analyzed by Enrichr for over-representation analysis. Heatmaps were used to visualize the data and identify unsupervised gene clusters using the "Ward.D2" algorithm. Gene set enrichment analysis (GSEA) [13] was performed on pre-ranked gene lists created by pairwise comparisons between ILA status using Hallmark gene sets [14]. An FDR threshold of 0.01 was applied to select significant hallmark gene sets.

ILA characterization

Measures of ILA were assessed on chest CT scans using a sequential method by readers (including radiologists and pulmonologists) blinded to prior interpretations and participant information and per Fleischner society recommendations [1]. Participants with indeterminate ILA status were excluded from this analysis (Additional file 2: Fig. S1) [15]. ILA with a UIP pattern was specifically characterised in those who had either a probable or definite UIP pattern as previously published [16].

Statistical analysis

Data are presented as means and standard deviations for continuous measurements and number and percentage for categorical features. P values were calculated using Fisher's exact test or t test as applicable. All analyses were adjusted for age, gender, cohort, cancer status, smoking status and sequencing method as confounders. Genes and pathways demonstrating a p-value < 0.05 after adjusting for the false discovery rate (FDR) were considered statistically significant.

Table 1 Baseline characteristics of DECAMP participants comparing with ILA with a UIP pattern to those without ILA and ILA with no UIP variable

	No ILA (N = 162)	ILA with a UIP pattern[a] (N = 16)	ILA with no UIP pattern[a] (N = 35)	*P* value
Age (mean, SD)[a]	64 (8)	70 (6)	67 (6)	**0.004**
Sex				0.21
Male (n, %)	117 (72)	14 (88)	29 (83)	
Female (n, %)	45 (28)	2 (12)	6 (17)	
Race				0.68
White (n, %)	120 (76)	13 (81)	26 (81)	
Black (n, %)	27 (17)	1 (6)	4 (13)	
Asian (n, %)	5 (3)	1 (6)	0 (0)	
Smoking status				0.85
Current (n, %)	74 (46)	7 (44)	14 (40)	
Former (n, %)	81 (50)	9 (56)	20 (57)	
Unknown (n, %)	7 (4)	0 (0)	1 (2.9)	
Spirometry				
FEV1% predicted (mean, SD)[a]	72.0 (20)	93.0 (13)	75.7 (17)	**< 0.001**
FEV1/FVC (mean, SD)	0.6 (0.1)	0.7 (0.1)	0.7 (0.1)	**0.004**
FVC % predicted (mean, SD)[a]	87.6 (18)	99.1 (15)	85.0 (15)	**0.018**
COPD[b]	116 (71.6)	6 (37.5)	19 (54.3)	**0.014**
Radiology[c]				
Emphysema (mean, SD) (% of lung volume < 950HU)[a]	5.4 (7.7)	4.2 (3.4)	3.7 (5.9)	0.57
Cancer[d]				**< 0.001**
Yes (n, %)	13 (8)	8 (50)	7 (20)	
No (n, %)	21 (13)	6 (38)	4 (11)	
Sequencing method				0.42
Total (n, %)	141 (87)	12 (75)	30 (86)	
Exome (n, %)	21 (13)	4 (25)	5 (14)	

[a] *SD* standard deviation, *UIP* usual interstitial pneumonia, *FEV1* forced expiratory volume in 1st second, *FVC* forced vital capacity, *COPD* chronic obstructive pulmonary disease, *HU* Hounsfield units

[b] COPD was defined as FEV1/FVC < 0.7. Spirometry data was not available on 3 (2.5%) participants without ILA

[c] Radiographic data on emphysema was not available for 4 participants (25%) with ILA with a UIP pattern, 21 (60%) participants with ILA with no UIP and and 71 participants (44%) without ILA

[d] Cancer data was available only for DECAMP-1 participants. Additional details about cancer status is provided in Additional file 8: Table S7

Results

Among the 213 participants with both ILA phenotyping and gene expression data, there were 51 (24%) with ILA and 162 (76%) without ILA (Additional file 2: Fig. S1). Of the 51 with ILA, 16 (30%) had a probable or definite UIP pattern (hereafter referred to as 'ILA with a UIP pattern') and 35 (70%) had ILA without a UIP pattern (hereafter referred to as 'ILA with no UIP'). Compared to those without ILA and those with ILA with no UIP pattern, participants with a UIP pattern of ILA were older, and had increased measures of forced expiratory volume in one second (FEV1), forced vital capacity (FVC) and ratios of FEV1 to forced vital capacity (FEV1/FVC) (Table 1). Consistent with these results, participants with a UIP pattern of ILA had lower rates of COPD on spirometry (37.5% vs 71%; Fisher's Exact Test; $p=0.009$; Table 1). Those with ILA both without UIP pattern and with a UIP pattern had more malignant lung nodules compared to those without ILA (Table 1).

After adjusting for multiple testing, no individual gene met statistical significance for differential expression between those with ILA ($n=51$) compared to those without ILA ($n=162$) (Additional file 2: Fig. S1 and Additional file 3: Table S2). Similarly no significant differential expression was observed when comparing ILA with no UIP ($n=35$) and those without ILA ($n=162$) (Additional file 4: Table S3).

Participants with ILA with a UIP pattern compared to those without ILA, demonstrated a > eightfold upregulation of the gene encoding Pro-Platelet basic protein [PPBP] ($FDR<0.05$). At a less stringent threshold, the 122 genes with $p<0.01$ (Additional file 5: Table S4) divided the samples into two clusters including one that was enriched for samples from participants with ILA with a UIP pattern (Fisher's test, $p=0.001$; Fig. 1). We did not detect any other significant differences between the clusters with regard to the characteristics of the participants from whom the samples were obtained. The list of genes with UIP associated gene expression was highly overlapping when COPD was included as covariate (Fisher's Exact Test; $p<1e{-}200$).

Gene set enrichment analysis (GSEA) demonstrated seventeen pathways whose genes were significantly enriched among those with a UIP pattern of ILA when compared to those without ILA ($FDR<0.01$) including tumour necrosis factor-alpha (TNF-α) signalling through nuclear factor kappa-B (NF-κB) (Table 2). 16 out of these 17 pathways were also amongst the 24 pathways

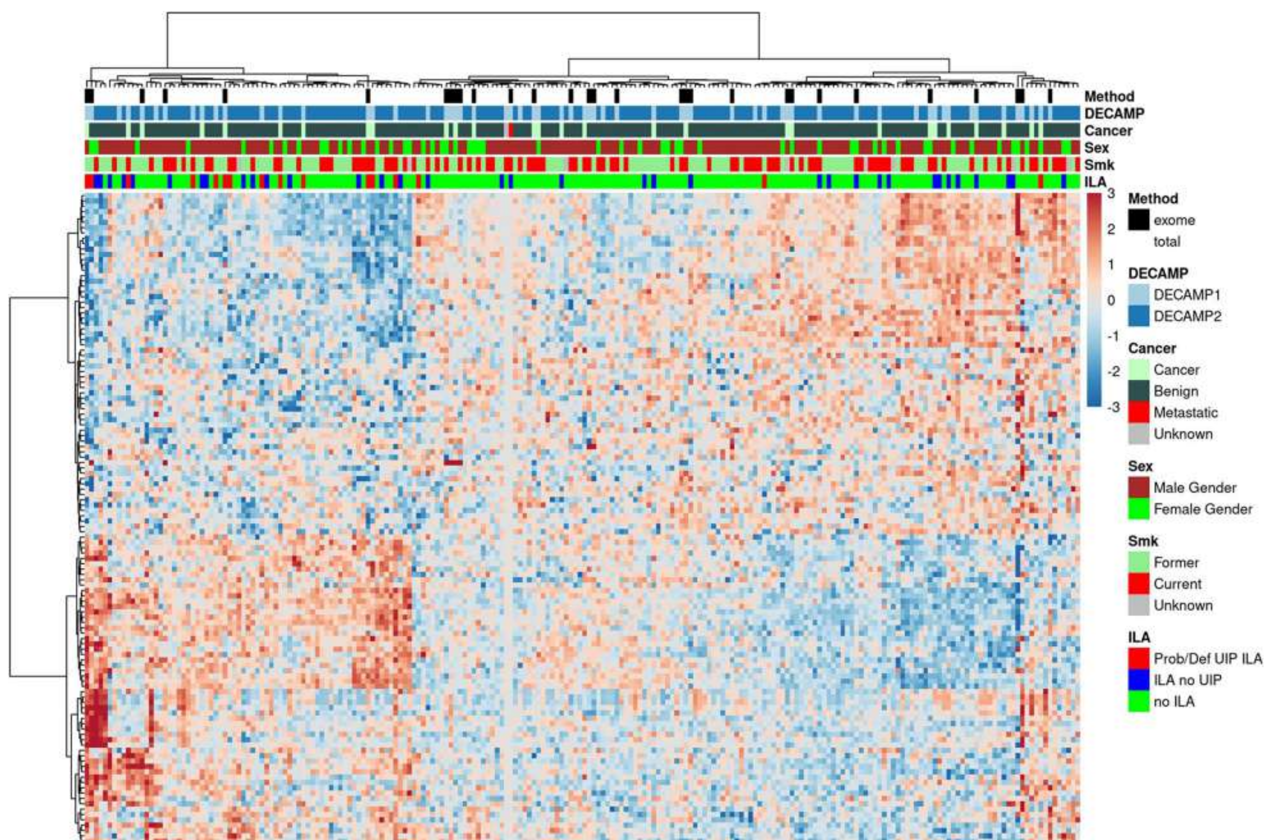

Fig. 1 Heatmap of gene expression differences across study cohort

Table 2 Differentially expressed gene sets in patients with ILA in a probable or definite UIP pattern that met significance

Gene set	ES	P-value	FDR q-value
TNF-α signalling via NFKB	0.658	1.67E−20	8.35E−19
Inflammatory response	0.549	3.90E−10	9.74E−09
Heme metabolism	0.540	1.30E−08	2.16E−07
Interferon gamma response	0.482	1.75E−07	2.19E−06
Oxidative phosphorylation	0.493	2.39E−07	2.39E−06
MYC targets V1	0.471	5.29E−07	4.41E−06
Interferon alpha response	0.562	1.22E−06	8.69E−06
KRAS signalling up	0.468	5.10E−06	3.19E−05
Complement	0.450	1.92E−05	1.07E−04
E2F targets	0.438	2.25E−05	1.13E−04
MTORC1 signalling	0.430	4.22E−05	1.92E−04
IL6 JAK STAT3 signalling	0.550	1.03E−04	4.29E−04
IL2 STAT5 signalling	0.418	2.76E−04	1.06E−03
G2M checkpoint	0.405	3.48E−04	1.24E−03
Protein secretion	0.457	1.94E−03	6.06E−03
Cholesterol homeostasis	0.499	1.90E−03	6.06E−03
Apoptosis	0.391	2.16E−03	6.35E−03

ES enrichment score, *FDR* false discovery rate, *TNF* tumour necrosis factor, *NF-κB* nuclear factor Kappa-B, *MYC* master regulator of cell cycle entry and proliferative metabolism, *KRAS* Kirsten rat sarcoma viral gene oncogene homolog, *E2F* E2F family of transcription factors, *MTORC* mammalian target of rapamycin complex, *IL* interleukin, *JAK* Janus kinase, *STAT* signal transducer and activation of transcription, *G2M* G2-mitotic phase

significantly upregulated (FDR < 0.01) when comparing participants with ILA to that without ILA (Additional file 6: Table S5), a highly significant overlap (hypergeometric test; $p < 7 \times 10^{-7}$). GSEA of the ILA with no UIP group showed 13 pathways that met significance; of which 9 were common to the ones in the ILA with UIP group (hypergeometric test p = 0.003) and 12 in common with the whole ILA group (hypergeometric test; p = 0.0001) (Additional file 7: Table S6).

Discussion

This is the first assessment of bronchial epithelial gene expression among participants with ILA and these findings suggest that both the UIP pattern of ILA and ILA may be associated with similarly altered bronchial airway gene expression at the pathway level.

In this cohort, the most significantly differentially expressed gene was PPBP; a gene within the C-X-C motif cytokine family, specifically ligand 7. While CXCL7 has been associated with non-small cell lung cancer [17], it has also has been implicated in early stages of wound healing and has previously been associated with IPF, both in serum samples as well as bronchoalveolar lavage fluid [18, 19]. It is hypothesized that this cytokine helps to recruit mesenchymal stem cells from bone marrow and promote fibrosis [20].

Genes involved in TNF-α signalling were among the genes most induced in bronchial brushes from individuals with UIP pattern of ILA and in individuals with ILA relative to non-ILA controls. This pathway has similarly been demonstrated to be upregulated in the lungs of patients with pulmonary fibrosis [21], and it's downregulation through the suppression of transforming growth factor-beta (TGF-β) is posited to be one of the therapeutic effects of pirfenidone [22]. These findings suggest that studying persons with ILA, can help to identify biologic pathways that are dysregulated in patients with clinically identified PF.

ILA with no UIP group may represent an intermediate step between the No ILA group and ILA with UIP group based on clinical characteristics and gene expression.

There are limitations to the present study. First, our ability to identify significant pathway enrichment among the genes most differentially expressed despite our overall failure to detect differential expression of individual genes suggests that our study is limited by its small sample size. Second, although analyses were adjusted for the presence of malignancy, it is difficult to exclude the possibility that residual differential background gene expression and the potential covariance of lung cancer and ILA could have affected our results. Third, we cannot rule out that performing this analysis on a subset of DECAMP, due to data availability influenced these results. Specifically, the prevalence of ILA in this cohort is larger than previously described and this may be due to selection bias from using a limited dataset of cohort participants with both ILA phenotyping and RNA sequencing data available. The results of the study are also limited to subjects with a known current or former smoking history and may differ from subjects with ILA without a smoking history. For these reasons, replication of these findings in additional cohorts will be important to validate the observed associations and assess generalizability to other populations.

Conclusion

There are differentially expressed bronchial epithelial cell genes and pathways associated with ILA among patients in DECAMP. Some of the most differentially expressed genes and pathways have been similarly demonstrated to be upregulated among patients with IPF. Additional studies in larger cohorts are warranted to confirm these findings.

Abbreviations

ILA	Interstitial lung abnormalities
DECAMP	Detection of Early Lung Cancer Among Military Personnel
CT	Computed tomography
UIP	Usual interstitial pneumonia
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
PF	Pulmonary fibrosis
RNA	Ribonucleic acid
FDR	False discovery rate
PPBP	Pro-platelet basic protein
GSEA	Gene set enrichment analysis
TNF-α	Tumour necrosis factor-alpha
NF-κB	Nuclear factor kappa-B
CXCL7	C-X-C motif cytokine family, ligand 7
TGF-β	Transforming growth factor-beta

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-023-02536-w.

Additional file 1: Table S1. Institutional Review Board committee names and project approval numbers for each center for the DECAMP Study.

Additional file 2: Figure S1. Study design diagram.

Additional file 3: Table S2. List of differentially expressed genes that met p-value < 0.01 among those with ILA as compared to those without ILA.

Additional file 4: Table S3. List of differentially expressed genes that met p < 0.01 among those without probable or definite UIP pattern of ILA as compared to those without ILA.

Additional file 5: Table S4. List of differentially expressed genes that met p-value < 0.01 among those with Probable or Definite UIP pattern of ILA as compared to those without ILA.

Additional file 6: Table S5. List of differentially expressed pathways comparing ILA with no ILA.

Additional file 7: Table S6. List of differentially expressed pathways comparing ILA no UIP with no ILA.

Additional file 8: Table S7. Cancer breakdown of nodule identified in participants in DECAMP 1 Cohort.

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Author contributions

AAM, ML, XK, MEL, GTO and GMH participated in the experimental design. KX, EB, HH and GMH participated in the data collection. AAM, ML, XK, RKP, TH, JR, FD, SYA, MHC, GTO, JD, HH, MEL, EB and GMH participated in the data analysis and interpretation. AAM prepared the first draft of the manuscript. AAM, ML, XK, RKP, TH, JR, FD, SYA, MHC, GTO, JD, HH, MEL, EB and GMH revised and approved the final version to be published. MHC, MEL, EB, GMH obtained funding for this proposal. All authors are accountable for all aspects of the work.

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Availability of data and materials

The data that support the findings of this study are available from the DECAMP consortium but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of DECAMP consortium.

Declarations

Ethics approval and consent to participate

The DECAMP studies were registered with clinicaltrials.gov and individual IRB approval was obtained from each participating institution. Data provided as Additional files.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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