Circulating RNA differences between patients with stable and progressive idiopathic pulmonary fibrosis

To the Editor:

Idiopathic pulmonary fibrosis (IPF) is a chronic disease characterised by progressive decline in pulmonary function. The rate of decline can vary, with some patients remaining stable over longer periods of time and others rapidly progressing [1]. The variable progression of this disease makes it difficult to elucidate pathogenic pathways involved in the initiation and progression of IPF. Advances in high-throughput gene-expression analyses have led to improvements in our understanding of disease biology and prognostic gene signatures. We hypothesise that IPF has a unique circulatory transcriptional profile compared to healthy controls, with additional differences between stable and progressive disease likely related to disease pathogenesis.

The study cohort consisted of consenting patients from the Australian IPF Registry with clinical/physiological/radiographical findings consistent with the diagnosis of IPF. All work was approved by the Royal Perth Hospital Ethics Committee (HREC/2011-138), and the Sydney Local Health Network (HREC/15/RPAH/28). Baseline forced vital capacity (FVC) and diffusing capacity for cabon monoxide (D_{LCO}) were assessed ±6 months from the time of blood collection, and the longitudinal FVC and D_{LCO} trajectories were determined ±6–12 months from the baseline lung function using a linear regression model. A decline in FVC ⩾10% and/or D_{LCO} ⩾15% within 6–12 months of baseline was used to define progressive IPF. No patients were on anti-fibrotic medications at blood collection.

An initial 10 patients from each group had plasma isolated and RNA extracted. Expression of over 135 000 transcripts were analysed by microarray (Human Clariom D; ThermoFisher Scientific), and expression profiles were compared between stable and progressive IPF samples. The top targets with a minimum two-fold difference between the two IPF groups were identified and droplet digital PCR (ddPCR; BioRad) was used to validate expression differences and compare absolute expression measurements between an independent cohort of stable (n=33) and progressive (n=24) IPF patients and disease-free healthy controls (n=15). In contrast to other methods, ddPCR provides an absolute, objective quantification of the number of mRNA transcripts with high precision. This is based on partitioning samples into thousands of uniformly nanolitre-sized droplets, undergoing end-point PCR, and template concentration was determined using Poisson's statistical analysis of the ratio of positive (containing amplified target) to negative (no detectable amplified target) droplets detectable.

Five independent formalin-fixed paraffin-embedded (FFPE) IPF, four healthy lung control FFPE specimens, IPF and normal fibroblast cells lines as well as five COPD plasma samples used as a disease control group, were all analysed to confirm expression of genes detected in IPF patient circulation. A549 respiratory adenocarcinoma cell line was included as a positive control for gene expression analyses and for assay quality control. p-values for relative gene expression levels of each transcript detected by the microarray and ddPCR were generated using one-way ANOVA adjusted for multiple comparisons (Kruskal–Wallis with Dunn's multiple comparisons test) or Mann–Whitney test. The predictive performance of gene expression levels was examined using Cox proportional hazards regression analysis adjusting for age, FVC baseline, gender and GAP stage (gender, age and FVC/D_{LCO}). Global inter- and intra-group variability of

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Gene expression differences were found in the circulation of IPF patients *versus* healthy controls, of which five were expressed in higher concentrations in progressive *versus* stable IPF, potentially providing insight into disease pathogenesis https://bit.ly/3bakHCL

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the data was carried out by performing a principal component analysis (PCA). Statistical analyses were performed on SPSS version 24.

The mean±SD age was 71±7 years in the IPF stable group (n=33; 21 males); 65±10 years in the IPF progressive group (n=24; 15 males); and 62±10 years in the healthy control group (n=15; eight males). Lung function at baseline in the stable group was FVC 79±26% predicted and D_{LCO} 49±15% predicted, *versus* FVC 78±18% predicted and D_{LCO} 43±13% predicted in the progressive group. There were 15 never smokers (stable 12; progressive three), 38 ex-smokers (stable 19; progressive 19), three current smokers (stable two; progressive one) and one unknown.

From a total of 135 750 transcripts analysed in the microarray, 127 genes were differentially expressed between stable and progressive IPF patients. The microarray data was further filtered, and the most abundant eight transcripts with more than two-fold gene expression difference between IPF groups were selected for validation.

Validation by ddPCR confirmed seven of the eight transcripts (*TAF2*, *NT5C2*, *JAK1*, *TAOK1*, *TRAM1*, *RP11-726G23.6* and *MIR6841*) were differentially expressed between IPF and healthy controls, of which five of the transcripts (*TAF2*, *NT5C2*, *JAK1*, *TRAM1* and *RP11-726G23.6*) were observed at higher concentrations in progressive *versus* stable IPF samples (figure 1a), with strongest evidence for *TAF2* (p=0.0413). ddPCR verification also confirmed higher expression of the seven transcripts in IPF lung tissue and IPF fibroblasts relative to healthy lung tissue and fibroblasts derived from normal controls. Immunolocalisation staining by immunohistochemistry was carried out on the five IPF lung FFPE samples to characterise significantly expressed *TAF2*. Stronger TAF2 expression was observed in the cytoplasm of bronchial epithelial cells, alveolar epithelial cells, smooth muscle cells and fibroblasts in IPF tissue (figure 1b) relative to healthy lung (figure 1c). *TAF2* expression was predictive of increased mortality (p<0.05) on multivariate Cox regression. PCA revealed that *TAF2* and *RP11-726G23.6* expression had a positive predictive relationship with IPF progression status (p=0.036).

a)

Gene	Count	IPF (n=57)	Healthy (n=15)	p-value	Progressive (n=24)	Stable (n=33)	p-value
NT5C2	Min	0	0		0	4.0	
	Median	18.9	6.0	<0.0001	24.6	15.0	0.0889
	Max	137.3	13		122.5	137.3	
JAK1	Min	0	0		0	3.9	
	Median	36.0	2.0	<0.0001	37.6	36.0	0.7698
	Max	149.2	10		131.0	149.2	
TAOK1	Min	0	0		0	0	
	Median	17.3	3.0	<0.0001	17.2	17.3	0.6163
	Max	136.9	11.0		136.9	76.9	
TRAM1	Min	0	0		0	0	
	Median	57.9	4.0	<0.0001	61.2	55.7	0.4750
	Max	726.9	14.0		726.9	315.0	
RP11-726G23.6	Min	0	0		0	0	
	Median	10.7	0	<0.0001	12.8	9.3	0.2974
	Max	80.2	28.0		80.2	23.5	
TAF2	Min	0	0		0	0	
	Median	7.5	0	0.0118	12.0	5.5	0.0413
	Max	106.2	27.0		106.0	35.5	
MIR6841	Min	0	0		0	0	
	Median	12.2	7.7	0.0354	10.3	12.3	0.5908
	Max	77.3	36.5		77.3	63.6	

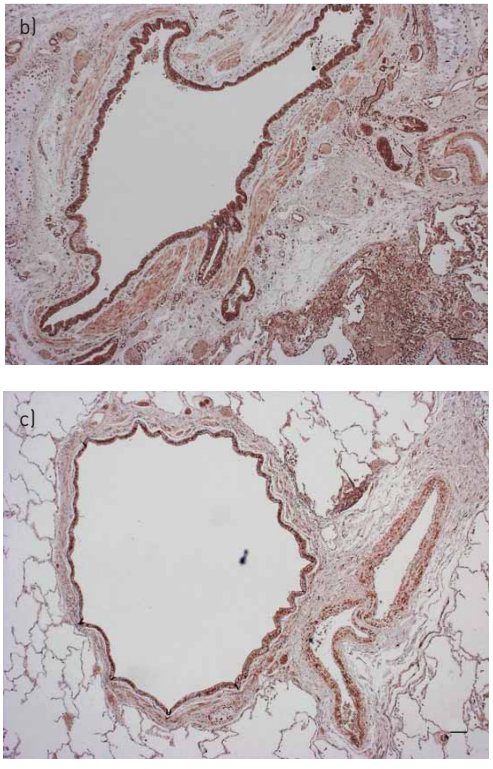

FIGURE 1 Differentially expressed genes that distinguish idiopathic pulmonary fibrosis (IPF) from healthy controls. a) Comparative concentrations of each gene between IPF *versus* healthy and progressive *versus* stable IPF. Level of *TAF2* transcript was higher in progressive IPF compared with stable. p-values were generated using Kruskal–Wallis test or Mann–Whitney test, adjusted for multiple comparisons with Dunn's multiple comparisons test. Representative examples of 4X TAF2 protein staining in b) formalin-fixed paraffin embedded IPF lung tissue section and c) healthy normal lung control tissue. Scale bars 100 μm. Stronger TAF2 expression was observed in the cytoplasm of bronchial epithelial cells, alveolar epithelial cells, smooth muscle cells and fibroblasts in IPF tissue relative to healthy lung.

This study aimed to investigate the circulating transcriptome in stable *versus* progressive IPF. Analysis of gene expression identified seven transcripts in the plasma (confirmed in IPF lung tissue) that were differentially expressed in IPF compared to healthy controls, with a trend of increased circulating levels of these transcripts in progressive *versus* stable IPF. Specifically, expression of *TAF2* was significantly higher in progressive *versus* stable IPF, which may represent a marker indicative of disease progression. Interestingly, gene expression analysis in circulation of a cohort of COPD patients showed significantly increased expression relative to healthy controls in all but *MIR6841,* where the strength of evidence was more moderate (p=0.055), further indicating the likely relevance of these genes in a chronic fibrosing and remodelling lung disease setting.

TAF2 (TATA-box binding protein associated factor-2) encodes an integral component of the core transcriptional machinery for RNA polymerase II. TAF proteins regulate differentiation and proliferation, important factors in the pathogenesis of IPF [2]. Interestingly, lung data from the Human Protein Atlas Tissue Gene Expression Profiles dataset has reported the expression of *TAF2* predominantly in pneumocytes and endothelial cells, making up 50–75% of the expression relative to all cell types in the lung [3].

NT5C2 (5′-Nucleotidase, Cytosolic-II) encodes a hydrolase that serves an important role in cellular purine metabolism and cell survival [4]. The RNAseq study by Nance *et al.* [5] reported higher expression of the *NT5C2* gene in IPF lung tissue relative to healthy controls. Its function in the maintenance of intracellular nucleotide pool homeostasis has been described in neurological disorders and leukaemias, and requires further investigation in IPF [6].

JAK1 (Janus kinase 1), is a tyrosine kinase protein involved in the activation of several signal transduction pathways involved in differentiation, proliferation, survival and migration. STAT3 acting downstream of JAK1 is a key regulator of fibroblast phenotype [7].

TAOK1 (thousand and one amino acid protein kinase-1) encodes a protein kinase involved in the stress-activated MAPK pathway, regulating a DNA damage response and apoptosis [8, 9]. The MAPK signalling cascade is known to regulate cellular process involved in fibrogenesis, such as epithelial–mesenchymal transition. Although *TAOK1* has never been described in IPF, it has been reported to exacerbate liver fibrosis *via* the overexpression of α-smooth muscle actin [10].

TRAM1 (translocation associated membrane protein-1), encodes a protein which forms part of the mammalian endoplasmic reticulum (ER), facilitating the translocation of proteins across its membrane. TRAM1 is upregulated under conditions of ER stress, which might be relevant in IPF [11, 12].

RP11-726G23.6 and *MIR6841* are non-coding genes that have lost their ability to code for proteins. *MIR6841* specifically is a non-coding microRNA (miRNA) likely involved in post-transcriptional regulation of gene expression [13]. Although not described in IPF, *MIR6841* is associated with *RICTOR* (RPTOR independent companion of MTOR complex-2), a protein coding gene that forms a subunit of mTORC2 (mammalian target of rapamycin complex-2) which is known to be associated with pulmonary fibrosis [14].

Taken together, the above-mentioned circulatory genes were upregulated in IPF relative to healthy controls, with significantly higher expression of *TAF2* in progressive *versus* stable IPF. Notably, this was supported in the RNAseq study by Nance *et al.* [5], reporting higher expression of *TAF2*, *NT5C2* and *TRAM1* genes in IPF *versus* healthy lung tissue in a smaller cohort (n=8 IPF patients *versus* n=7 healthy controls) with undefined disease progression status.

Another relevant study explored the predictive outcome of circulating RNA in IPF using a 52-gene expression microarray. Herazo-Maya *et al.* [15] revealed the significant improvement in outcome predictive accuracy when their 52-gene risk profiles were added to a patient's GAP index. Unlike our study, their measured outcomes included transplant-free survival and mortality, using pooled data relating to age, gender, FVC % pred and immunosuppressive therapy, and not absolute percentage decline in lung function test, as per our study. Furthermore, their study based the gene risk profiles on a 52-gene signature in RNA isolated from peripheral blood mononuclear cells, whereas our study only explored genes free in the circulation. This could be a possible explanation as to why neither studies identified overlapping genes. Another strength of our study was the inclusion of a second chronic lung disease, COPD, which interestingly displayed similarly increased levels of these circulatory genes. The ability to characterise circulatory biomarkers could increase our understanding not only of the pathogenesis and progression of IPF, but potentially other chronic fibrosing/remodelling lung diseases, such as COPD. Validation of our findings in an independent cohort will need to be carried out, and although one statistically significant difference was observed in gene expression between progressive and stable IPF, trends were observed, and larger cohorts will be needed to confirm true significance. Most of the implicated proteins have potential to be involved in pathogenic mechanisms of IPF, so future functional studies focused on these biomarkers are warranted.

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