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Mitochondrial-Related Gene Expression and Macrophage Signatures in Non-Small Cell Lung Cancer, Including Patients with Emphysema as Co-Morbidity

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Abstract

Objective: Our aim is to determine whether mitochondrial dysfunction is a contributing factor to the increased risk of non-small cell lung carcinoma (NSCLC) in COPD patients.

Methods: The clinical relevance of mitochondrial-related gene expression in lung cancer was determined using transcriptomic data from more than 1000 human NSCLC samples. Immunohistochemistry was then used to study cell type specific expression of the relevant mitochondrial-related protein in normal and cancerous lung tissue. Gene set variation analysis (GSVA) was applied in NSCLC datasets to determine the relative expression of specific macrophage transcriptomic signatures.

Results: The expression of 33 mitochondrial-related genes was correlated with NSCLC patient survival. We studied further the expression of PGAM5 and FUNDC1, which are regulators of mitochondrial degradation (mitophagy). In background lung tissue, PGAM5 and FUNDC1 were only expressed in alveolar macrophages, with highest expression in smokers with emphysema compared to healthy smokers and non-smokers. In cancerous tissue, only the malignant epithelial cells and associated macrophages at the periphery of the cancer, expressed PGAM5 and FUNDC1. PGAM5 was also expressed in pre-neoplastic epithelium (squamous dysplasia and carcinoma *in situ*). There was no difference in expression in cancer tissue between the emphysema, healthy smokers and non-smokers group. Macrophages at the edge of the cancer from emphysema patients had a trend towards higher expression of PGAM5 and FUNDC1 compared to those from the other groups. There was a significant correlation between PGAM5 expression in cancer tissue and 9 out of 49 previously defined macrophage transcriptomic signatures with one (module 22) associated with patient survival ($p<0.05$).

Conclusion: PGAM5 is expressed in pre-neoplastic tissue and NSCLC, but not in normal epithelium. The association between PGAM5 expression and lung cancer outcome may be mediated by the induction of specific macrophage phenotypes.

Keywords: Mitochondrion; Lung cancer; PGAM5; FUNDC1; Macrophage; Patient survival

Introduction

Non-small cell lung carcinoma presents with locally advanced or metastatic disease in 80% of cases [1,2], which accounts for its dismal prognosis at 5 years post-diagnosis [3]. Up to 70% of lung cancer smokers have pre-existing COPD prior to cancer diagnosis [4,5]. The high proportion of patients with COPD developing lung cancer is not solely related to the common risk factor of cigarette smoking. COPD patients are at increased risk of developing lung cancer, irrespective of their smoking history [6-8]. Smokers with airflow obstruction have a five-fold increased risk of lung cancer compared to those with normal lung function [4]. As well as airflow obstruction, emphysema diagnosed on computed tomography (CT) is also an independent risk factor for lung cancer [9,10].

Oxidative stress is a well-recognised driver of carcinogenesis and is present in smokers and COPD patients [11]. The airways of patients with COPD demonstrate an additional increase in oxidative stress, compared to healthy smokers and non-smokers [11]. The production of reactive oxygen species from the mitochondrion is the main source of oxidative stress. The above observation of increased oxidative stress in airway epithelial cells in COPD has been at least partly explained by mitochondrial damage [11]. Under increased oxidative stress, metabolically-active cells may undergo an increase in biogenesis and in degradation of damaged mitochondria within autolysosomes (mitophagy) to replace the old mitochondria which have been damaged by oxidative stress. An imbalance between biogenesis and mitophagy may lead to an excess of dysfunctional mitochondria and increased oxidative stress.

The outcome of lung cancer is known to be related to the phenotype of tumour-associated macrophages (M1 *vs.* M2) and their histological location [12]. However, the traditional division of macrophages into the pro-inflammatory M1 subtype versus the anti-inflammatory M2 subtype has now been superseded by a 'spectrum model' of activated macrophages with at least 49 distinct transcriptomic signatures [13]. Metabolic signatures in macrophages are also linked to their polarisation status [14] and changes in mitochondrial-related protein

expression in alveolar macrophages are likely to be accompanied by changes in their transcriptomic signatures.

Our overall hypothesis is that mitochondrial dysfunction is a driving mechanism underlying the increased risk of NSCLC in COPD. We demonstrated enhanced expression of the mitophagy-related proteins PGAM5 and FUNDC1 in malignant epithelial cells, as well as in the alveolar macrophages from normal lung and adjacent to cancer and that their expression was related to the survival of non-small cell lung cancer patients. We then showed that the expression of mitochondrial-related genes was related to specific macrophage phenotypes. Our data suggests that the expression of mitophagy-related proteins in lung cancer is associated with the macrophage phenotype and patient survival.

Methods

Screening for mitochondrial-related candidate genes in lung cancer

The steps involved in this study are outlined in Table 1.

Mitochondrial genes	Selection of mitochondrial related genes using literature mining and molecular signatures database.
Cancer outcome	Selection for genes showing correlation with the survival of cancer patients using a publicly available database.
Differential gene expression in cancer	Selection for genes showing differential gene expression between cancer and normal lung tissue using a publicly available database.
Cell specific protein expression	Demonstrate differential protein expression between cancer and normal tissue *in vivo*. Show cell type specific expression of mitochondrial-related proteins by immunohistochemistry.
Cellular phenotype	Correlate cell type specific expression with phenotype and cancer outcome using publicly available databases.

Table 1: Stepwise approach to screen and assess *in vivo* functions of the most relevant mitochondrial-related genes in lung cancer.

More than 250 mitochondrial-related genes were selected by literature mining and from the Molecular Signatures Database (M8479) [15]. We then determined whether a change in the expression of each tumour-expressed mitochondrial-related gene was associated with a change in patient survival. To this end, we accessed the cancer gene expression and outcome data from the public domain, which have been assembled in the Precog database [16]. The latter contains transcriptomic and survival data of multiple cancer types, and specifically more than 1000 NSCLC (adenocarcinoma and squamous cell carcinoma) cases. In this database, the statistical associations between gene expression and clinical outcomes are assessed by z-scores, which are directly related to p values. Z-scores represent the number of standard deviations from the mean of a normal distribution. $|z|>1.96$ is equivalent to a two-sided $p<0.05$. After selecting for those genes with $|z|>1.96$, we then examined the differential expression of genes between cancer and the normal surrounding tissue, using the publicly available RNA Seq Nexus database [17] that includes transcriptomic data from 151 Stage 1 squamous cell carcinomas, 140 Stage 1 adenocarcinomas and 51 normal lung tissues.

Lung tissue cohort characteristics

Formalin-fixed paraffin-embedded tissue, surplus to diagnostic purposes, was obtained from patients undergoing lung cancer resection, pathologists routinely sampling background lung to detect any co-existing histological abnormalities. Patients' medical records, lung function tests and imaging reports were reviewed. Informed consent was obtained from the donor prior to surgery for use of surgically excised tissues for research purposes. This study was approved by the Health Research Authority, South Central-Hampshire B Research Ethics Committee (REC Reference: 15/SC/0569). The lung cancer patients had not previously undergone radiotherapy or chemotherapy. The patients were divided into non-smokers and smokers' groups which were further subdivided according to chest CT and histological diagnosis of background lung tissue. Healthy smokers were those in which there was no emphysema on CT or histology. The emphysema group included those patients in which emphysema was diagnosed by CT and by histology of background lung.

Immunohistochemistry

Immunohistochemistry for PGAM5 and FUNDC1 was performed on cancer and background ('normal') tissue from lung cancer resections from non-smokers, healthy smokers and COPD patients. For both proteins, heat-mediated antigen retrieval was performed at pH 8.5. The FUNDC1 antibody (Biorbyt) was applied at a 1:50 dilution and the PGAM5 (Abcam) antibody at a 1:300 dilution. To detect the primary antibody, the Optiview DAB detection kit (Ventana Medical Systems) was used and included the secondary antibody. As negative controls, cancer and background lung tissue from the same patients were used, but the primary antibody was not added and was replaced by buffer instead.

Immunostaining was assessed using a semi-quantitative immunohistochemical scoring system (H score) [18]. Briefly, 500 cells of interest (tumour or macrophages) were counted, and an H-score was generated by adding the percentage of strongly stained nuclei multiplied by 3, the percentage of moderately stained nuclei multiplied by 2, and the percentage of weakly stained nuclei multiplied by 1, giving a possible score of range of 0-300 [19].

Gene set variation analysis

Xue et al. have recently defined a set of 49 macrophage transcriptomic signatures which extend the M1/M2 dichotomy of activated macrophages and can be applied *in vivo* [13]. This was done by stimulating human monocytes with multiple activation signals, with identification of central transcriptional regulators associated with macrophage activation.

Gene set variation analysis (GSVA) was used to compare the expression of these macrophage signatures across the cancer datasets GSE31210 and GSE72194. GSVA calculates sample-wise enrichment scores (ES) [20,21]. We compiled 49 gene sets each related to a specific macrophage activation status obtained from Xue et al. [13] and the ES was calculated for each gene set for each subject. Dataset GSE 31210 comprised of transcriptomic and outcome data for 226 primary Stage 1 and 2 lung adenocarcinomas [22]. Dataset GSE72194 [23] combines 5 previous datasets with the clinical and transcriptomic data of 338 adenocarcinomas and 294 squamous cell carcinomas.

Statistical analysis

The immunohistochemical score (H score) was analysed using a Kruskal-Wallis test, with one-way ANOVA tests across the three groups of subjects and Dunn's multiple comparison tests between the groups. ANOVA was used to analyse the ES differences among group means and the Student's t-test was applied to compare the ES differences between the 2 means $p<0.05$ was considered statistically significant.

Results

Screening for mitochondrial-related genes in the pathogenesis of NSCLC

Thirty-three mitochondrial-related genes were differentially expressed between cancer and normal tissue, as well as being associated with a change in survival of the patients with the magnitude of the Precog z score exceeding 1.96 (Table 2). A literature review showed that FADD, NDUFS1 and SIAH2 are known to be involved in the pathogenesis of NSCLC and affected patient survival [24-26]. We therefore focused on factors involved in mitophagy as there is a paucity of data regarding its role in non-small lung cancer pathogenesis despite dysregulation of mitophagy being implicated in the pathogenesis of other cancers [27,28]. Furthermore, changes in the Pink1-parkin mitophagy pathway are known to be involved in the pathogenesis of COPD [30,31].

Immunohistochemistry and patient characteristics

The demographic characteristics of the patients are shown in Table 3. There were a higher proportion of males in the healthy smokers and emphysema groups, compared to the non-smokers. As expected, the FEV1/ FVC ratio was lower in the emphysema group, compared to the other 2 groups. There was no difference in the smoking index between the 2 groups of smokers. Unsurprisingly, there was a predominance of adenocarcinomas in the non-smoking population.

Immunohistochemistry was performed on background lung and cancerous tissue from 3 groups of NSCLC patients: 1. Non-smokers; 2. Healthy smokers and 3. Smokers with emphysema. In background lung tissue, PGAM5 and FUNDC1 were only expressed in alveolar macrophages (Figures 1 and 2).

	Squamous cell carcinoma	Fold change	Adenocarcinoma	Fold change
Oxidative phosphorylation complex	Ndufs1	5	Ndufs1	4
	Ndufv1	2.9	Ndufs6	1.9
	Ndufa10	1.9	Ndufa9	2.4
	Bcs1L	7	Ndufa11	1.8
	ATP5i	4.8	ATP5G1	7.5
	ATP5J	1.8	ATP5J2	3.7
	UQCRC2	1.9	ATP6V1B1	3
Mitophagy	Pink 1	0.5	Pink 1,	0.9
	FUNDC1	2.1	SIAH2	1.4
	PGAM 5	2.5		
Glycolysis	HK3,	0.23	ENO 1	2
	Galm	0.55	ENO2	3.5
			EN03	5
			Aldo A	2.6
Necroptosis	MLKL	0.51	FADD	1.7
	FADD	2.2		
	HSP90AA6P	2.6		
Anti-oxidants			Catalase	0.28
			SOD3	0.5
			PRX	0.18
			GPX 2	74
			sirt 3	1.5
Autophagy			LRPPRC	3

Table 2: List of differentially expressed mitochondrial-related genes between normal and cancer tissue and in which the change in expression in cancer is associated with a change in survival of patients. The differential expression of genes between cancer and the normal surrounding tissue was obtained from the publicly available RNA Seq Nexus database [17]. The fold changes of cancer *vs.* normal tissue expression are provided, with all $p<0.05$. The effect of gene expression on patient outcome was assessed using the Precog database.

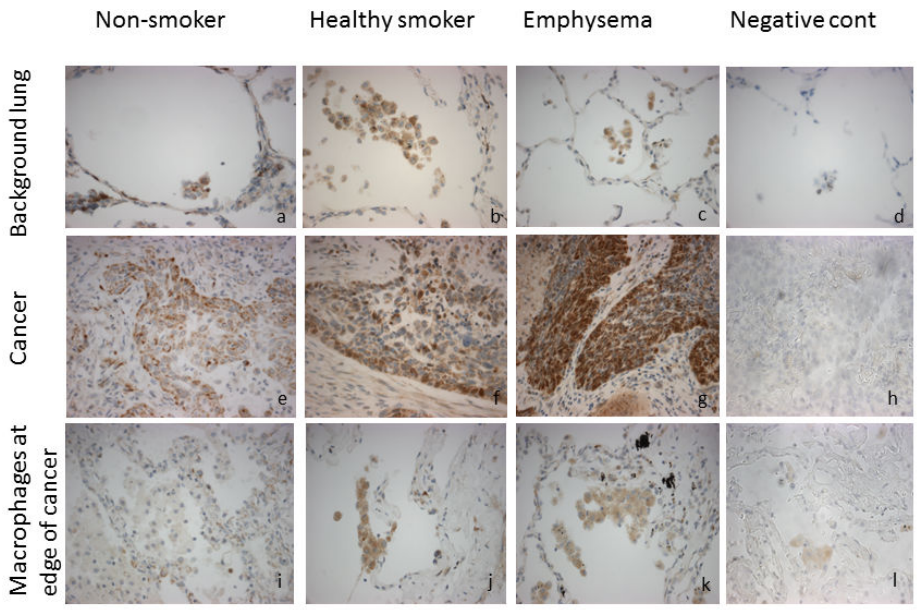

Figure 1: Immunohistochemistry for PGAM 5 expression in background lung (a-d), squamous cell carcinoma (e-h) and alveolar macrophages at the edge of cancer (i-l). Magnification X40. The first row shows background lung tissue with alveolar walls and macrophages in the alveolar space. PGAM 5 is expressed by alveolar macrophages (only cell type showing brown staining in this row), with higher expression in healthy smokers and emphysema groups, compared to non-smokers. The second row shows cancerous tissue with epithelial cells and stromal cells. PGAM 5 is expressed by the malignant epithelial cells (showing brown staining), but not stroma, in all three groups of patients. There was no difference in PGAM5 expression by the epithelial cells across the 3 groups of patients. The third row shows alveolar spaces at the edge of cancerous tissue. PGAM5 is expressed in the alveolar macrophages (only cell type showing brown staining in this row).

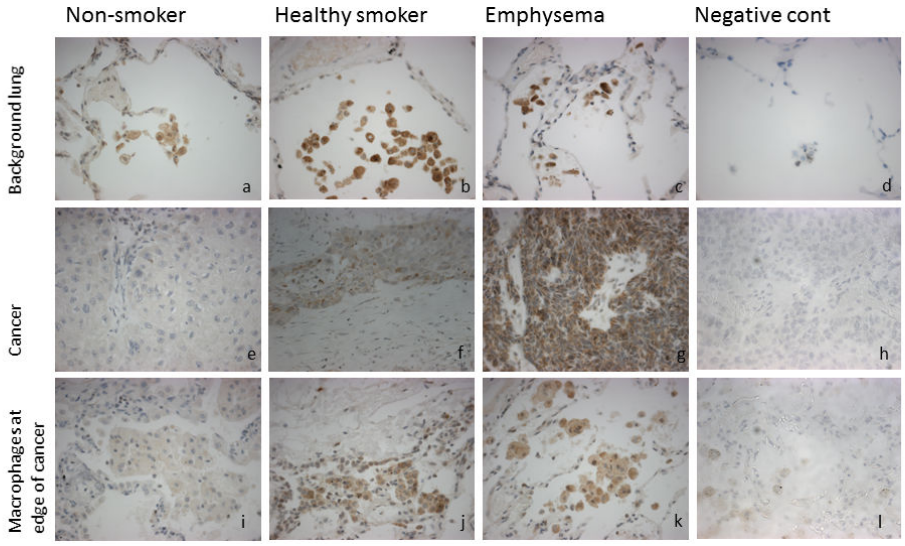

Figure 2: Immunohistochemistry for FUNDC1in background lung (a-d), squamous cell carcinoma (e-h) and alveolar macrophages at the edge of cancer (i-l). Magnification X40. The first row shows background lung tissue with alveolar walls and macrophages in the alveolar space. FUNDC1 is expressed by alveolar macrophages (only cell type showing brown staining in the first row), with higher expression in healthy smokers and emphysema groups, compared to non-smokers. The second row shows cancerous tissue with epithelial cells and stromal cells. FUNDC1 is mildly expressed by the malignant epithelial cells (showing brown staining), but not stroma, in all three groups of patients. There was no statistical difference in FUNDC1 expression in the cancer tissue between the 3 groups of patients. The third row shows alveolar spaces at the edge of cancerous tissue. FUNDC1 is expressed in the alveolar macrophages (only cell type showing brown staining).

Although the number of alveolar macrophages is increased in the healthy smokers (4 fold increase) and emphysema patients (5 fold increase) compared to the non-smokers group, there was a quantitative increase in expression per macrophage of PGAM5 and FUNDC1 (Figures 3 and 4). Expression was detected in alveolar macrophages in lung sections from all subjects studied. There was no expression in alveolar or bronchial epithelial cells in any of the subject groups. The expression of both PGAM5 and FUNDC1 in alveolar macrophages was highest in the emphysema group, compared to healthy smokers and non-smokers (Figures 1-4). However, there was no detectable difference in expression between the healthy smokers and emphysema patients.

In cancerous tissue, only the malignant epithelial cells and alveolar macrophages at the periphery of the cancer expressed PGAM5 and FUNDC1 (Figures 1 and 2). PGAM5 was expressed by the malignant epithelial cells of all tumours (n=30), whether they were adenocarcinomas or squamous cell carcinoma and in pre-neoplastic epithelium (squamous dysplasia and carcinoma *in situ*) (Figure 5). However, there was no expression within atypical adenomatous hyperplasia, a precursor of adenocarcinoma.

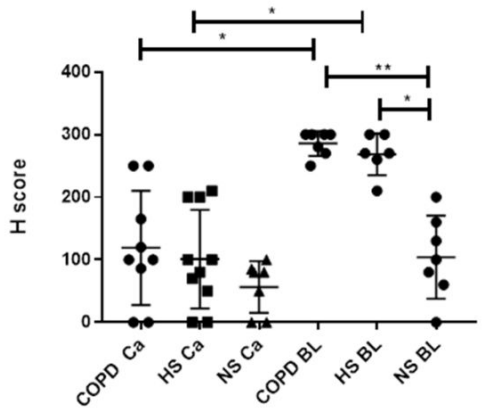

Figure 3: FUNDC1 expression in alveolar macrophages at the edge of cancer (Ca) and from background lung tissue (BL). COPD Ca: Lung Cancer tissue from patients with emphysema; HS Ca: Lung Cancer tissue from Healthy Smokers; NS Ca: Lung Cancer tissue from Non- Smokers. COPD BL: Background Lung tissue from patients with emphysema; HS BL: Background Lung tissue from Healthy Smokers and NS BL: Background Lung tissue from Non-Smokers. The expression of FUNDC1 was higher in alveolar macrophages from the background lung tissue, compared with the alveolar macrophages adjacent to the cancer nest, in the corresponding group of patients. The expression of FUNDC1 in alveolar macrophages (background or cancer tissue) was highest in the emphysema group, compared to healthy smokers and non-smokers. However, there was no detectable difference in expression between the healthy smokers and emphysema patients.

There was no difference in PGAM5 expression by the epithelial cells across the 3 groups of patients, even if an additional comparison of adenocarcinoma or squamous cell carcinoma was carried out across the three groups. There was also no difference in expression between squamous cell carcinoma and adenocarcinoma across the 3 groups of patients.

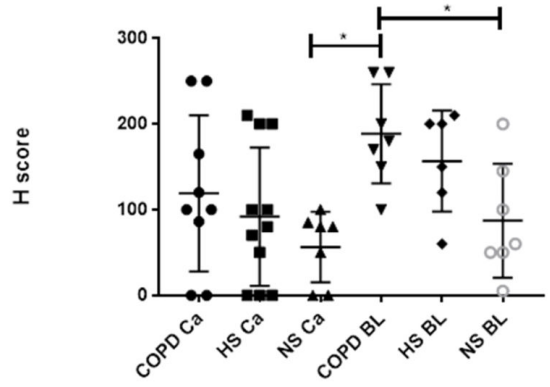

Figure 4: PGAM5 expression in alveolar macrophages at the edge of cancer (Ca) and in background lung tissue (BL). COPD Ca: Lung Cancer tissue from patients with emphysema; HS Ca: Lung Cancer tissue from Healthy Smokers; NS Ca: Lung Cancer tissue from Non-Smokers. COPD BL: Background Lung tissue from patients with emphysema; HS BL: Background Lung tissue from Healthy Smokers and NS BL: Background Lung tissue from Non-Smokers. The expression of PGAM5 in alveolar macrophages was highest in the emphysema group, compared to healthy smokers and non-smokers. However, there was no detectable difference in expression between the healthy smokers and emphysema patients.

With regards to FUNDC1, it was not expressed in tumours from any of the non-smokers studied. The epithelial cells from three tumours (out of 13) from the healthy smokers and 3 (out of 9) from the emphysema group show mild expression for FUNDC1. There was no statistical difference in FUNDC1 expression in the cancer tissue between the 3 groups of patients.

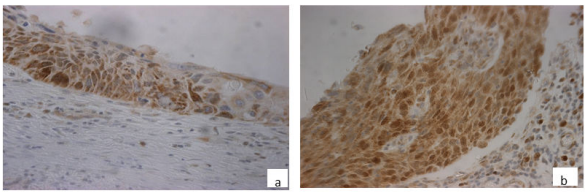

Figure 5: PGAM5 expression in pre-neoplastic epithelium (n=6). Squamous dysplasia (a) and full thickness dysplasia, carcinoma *in situ* (b). There is expression for PGAM5 (indicated by brown staining in cells) in the epithelium of squamous dysplasia and carcinoma *in situ*.

PGAM5 and FUNDC1 were expressed by alveolar macrophages at the edge of the tumour in all sections studied. As previous studies have noted differences in protein expression by alveolar macrophages adjacent to the cancer margin and in lung tissues distant from the

cancer nest [32], we also investigated whether there might be a difference in expression in mitochondrial-related proteins between alveolar macrophages at these 2 histological sites. The expression of FUNDC1 was higher in alveolar macrophages from the background lung tissue, compared with the alveolar macrophages adjacent to the cancer nest, in the corresponding group of patients (Figure 3). With regards to PGAM5, there was a similar trend, but this did not achieve statistical significance (Figure 4). The phenotype of macrophages is dependent on its microenvironment in the lung [33] and this data suggests that the expression of PGAM5 and FUNDC1 may be increased by the proximity of the alveolar macrophages to the cancer.

The role of macrophage phenotype in NSCLC

The immunological phenotype of macrophages has classically been divided into the pro-inflammatory M1 and anti-inflammatory M2 subsets. However, it has been increasingly recognised that this represents an over-simplification of the activated states of the macrophages.

	Non-smoker	Healthy Smoker	Emphysema	p value
Total no of patients	7	13	9	
Age (years)	73.9 ± 10.5	72.0 ± 8.0	67.1 ± 8.7	
Male (% group)	40	54	78	
Smoking index (pack-year)	N/A	53 ± 23.1	61 ± 30.6	
FEV1	1.9 ± 0.4	2.6 ± 0.9	2.2 ± 0.7	
FVC	2.3 ± 0.7	3.3 ± 0.8	3.8 ± 1.1	
FEV1/ FVC (%)	76.3 ± 9.8	75.7 ± 5.1	56.8 ± 9.0	<0.05
Histology	6 ADC, 1 SCC	5 ADC, 8 SCC	6 SCC, 2 ADC, 1 NSCLC	
Cancer Stage	3 Stage1 4 Stage2	9 Stage1 3 Stage 2 1 Stage 3	2 Stage 1 5 Stage 2 2 Stage 3	

Table 3: Characteristics of patients from whom cancer tissue was obtained (ADC: adenocarcinoma, SCC: squamous cell carcinoma).

Initially, we determined whether there was a correlation between the different macrophage signatures and clinical outcome in lung cancer. The dataset GSE 31210 comprised transcriptomic and clinic-pathological data of 226 primary Stage 1 and 2 lung adenocarcinomas and background lung tissue [22]. Using GSVA, two macrophage signatures (modules 22 and 36 which are similar to signatures of monocytes activated by oleic acid and tumour necrosis factor +Pam3CSK4+prostaglandine E2 respectively) were correlated with the overall survival of patients ($p<0.05$), as well as with differential expression between normal lung tissue and Stages 1 and 2 of lung cancer ($p<0.05$) (Figure 6).

Next, we studied whether PGAM5 or FUNDC1 expression was correlated with the macrophage phenotype in tumour tissue in NSCLC dataset GSE72194 [23]. The probe for FUNDC1 was not found in the transcriptomic data. However, there was a positive correlation with one macrophage signature ('module 49') ($r=0.3$) ($p<0.05$), while there was a negative correlation with 8 macrophage signatures ('modules 7, 9, 10, 11, 28, 36, 39 and 42'), including 'module 36' ($r=-0.44$ to -0.24) ($p<0.05$) but not 'module 22'. Therefore, one macrophage signature ('module 36'), which is correlated with PGAM5 expression, is also associated with the outcome of the cancer patients.

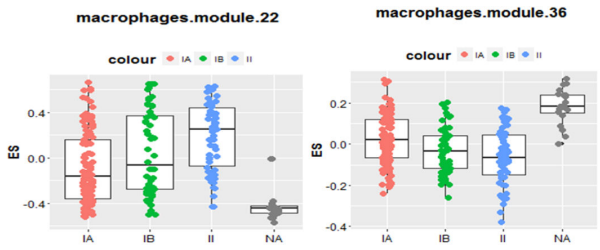

Figure 6: Expression of macrophage signatures in NSCLC, according to stage of cancer. GSVA was used to compare the expression of macrophage signatures across the cancer datasets GSE31210 and GSE72194. GSVA calculates sample-wise enrichment scores (ES). We compiled 49 gene sets each related to a specific macrophage activation status obtained from Xue et al. [13] and the ES was calculated for each gene set for each subject. NA, normal tissue. Two macrophage signatures (modules 22 and 36 which are similar to signatures of monocytes activated by oleic acid and tumour necrosis factor+Pam3CSK4+prostaglandine E2 respectively) showed differential expression between normal lung tissue and Stages 1 and 2 of lung cancer ($p<0.05$).

Discussion

We report for the first time the expression of the mitochondrial-related proteins, PGAM5 and FUNDC1, in lung tissue from COPD and NSCLC patients. PGAM5 and FUNDC1 expression was only detected within alveolar macrophages in non-cancerous lung tissue. The level of expression in alveolar macrophages was higher in smokers than non-smokers, with a trend towards highest expression in emphysematous patients. PGAM5 is consistently expressed in the malignant epithelial cells of lung cancer patients, with no difference in the level of expression across the 3 groups of patients. PGAM5 expression in lung cancer is correlated with specific macrophage phenotypes, some of which are associated with patient mortality. We speculate that products from PGAM5- and FUNDC1-expressing macrophages may affect epithelial cell function and may account, at least in part, for the increased risk of lung cancer in patients with COPD. Further work is needed to elucidate the relevant mechanisms.

Both PGAM5 and FUNDC1 play a role in mitophagy. FUNDC1 is a mitochondrial outer-membrane protein and a receptor for hypoxia-induced mitophagy [34]. It also regulates mitochondrial fission by interacting with DNM1/DRP1 [32]. Mitochondrial protein PGAM5 functions in multiple cell death pathways [35] and regulates mitophagic protection against cell necroptosis [36]. PGAM5 catalyses the dephosphorylation of FUNDC1 which enhances its interaction with microtubule-associated protein 1A/1B-light chain 3, leading to

mitophagy [37]. PGAM5 also promotes inflammasome activation in macrophages [38].

Benign bronchial and alveolar epithelial cells do not express PGAM5 or FUNDC1. Pre-neoplastic epithelium (squamous dysplasia and carcinoma *in situ*) express PGAM5, but not FUNDC1. All malignant epithelial cells, whether from squamous cell carcinomas or adenocarcinomas, express PGAM5. The association of PGAM5 expression with worse prognosis in squamous cell carcinoma and its expression in the sequential transformation of squamous dysplasia into malignant squamous cells suggests its pathological role in lung squamous cell carcinoma. The mechanism by which PGAM5 contributes to the malignant transformation of the epithelium is uncertain. However, this may be related to the action of PGAM5 on cell death pathways, leading to uncontrolled cellular proliferation secondary to its overexpression. PGAM5 may also affect the epigenetic mechanisms controlling the initiation and development of lung cancer. Heterochromatin associated histone methyltransferase G9a is involved in tumour invasion and metastasis *via* depression of cellular adhesion [39]. G9a functions as the main writer of H3K9me2 [40] and plays a role in the maintenance of DNA methylation *via* interaction with DNA methyltransferases [41]. Based on the current study, as PGAM5 might be required for the malignant transformation of the airway epithelial cells, investigation of the patterns of H3K9me2 and DNA methylation at the promoter of PGAM5 might create a novel direction for the study of lung cancer.

There was no difference in the expression of PGAM5 or FUNDC1 between the tumours from non-smokers, healthy smokers and emphysema patients. The level of expression of PGAM5 and FUNDC1 in lung cancer itself cannot therefore explain why patients with COPD have NSCLC with worse outcome again emphasising the importance of macrophage-epithelial cell cross-talk.

PGAM5 and FUNDC1 are also expressed in alveolar macrophages, whether they are from the background lung tissue or adjacent to the cancer margin. Their expression in background lung tissue is increased in healthy smokers and emphysema compared to non-smokers although this did not reach significance for emphysema. However, it is known that the phenotype of alveolar macrophages is altered in COPD [42] and the increased oxidative stress, resulting from excess free radicals in cigarette smoke, leads to mitochondrial damage and increased turnover, including mitophagy. This would be reflected by the increased expression of FUNDC1 or PGAM5 in alveolar macrophages. Increased mitochondrial turnover may also lead to a change in the metabolic phenotype of the alveolar macrophages which is known to be related to their immunomodulatory phenotypes [43]. The activation of PGAM5 in the alveolar macrophages may also lead to the activation of the inflammasome, which is known to be important in triggering the inflammatory reaction in COPD [38]. Overall, our data suggests that factors released from specific subtypes of lung macrophages may affect cancer progression and survival. Future studies are required to examine this functionally.

In cancerous tissue, expression of both FUNDC1 and PGAM5 is observed in alveolar macrophages adjacent to the cancer margin in all three groups of patients. Alveolar macrophages at the edge of the cancer have lower expression of FUNDC1 than in the background lung. A similar phenomenon was observed with PGAM5, but this did not achieve statistical significance. This may be related to the change in phenotype of alveolar macrophages adjacent to the cancer, as has been previously described [32]. These observations therefore suggest that there is an interaction between malignant epithelial cells and the surrounding alveolar macrophages. The mechanism by which this occurs remains uncertain. However, Lisanti and others [44-46] have previously demonstrated that the release of metabolites from cancer cells can affect their immune microenvironment.

In support of this, PGAM5 expression within tumours was correlated with 9 (out of 49) macrophage signatures obtained from Xue et al. [13], one of which (module 22) is associated with the outcome of NSCLC in another dataset. PGAM5 expression is negatively correlated with the macrophage 'module 36' expression in lung cancer, which itself is inversely associated with mortality. This may partly explain our original observation that PGAM5 expression is associated with a worse outcome in NSCLC.

In our study, it was not possible to determine if there are macrophage signatures specific to COPD-associated cancer in contrast to non- COPD associated cancer. We propose that COPD-associated lung cancer has a worse prognosis because of the presence of specific tumour associated macrophage phenotypes associated with worse prognosis.

In summary, the expression of specific mitochondrial-related proteins and macrophage signatures are associated with the outcome of NSCLC. The expression of PGAM5 in lung cancer is correlated with specific macrophage phenotypes, one of which is associated with lung cancer survival. PGAM5 may also play a role in the transformation of malignant airway epithelial cells.

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