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Urokinase plasminogen activator receptor polymorphisms and airway remodelling in asthma



To the Editor:

In the past decade, several asthma genes have been identified [1]; however, the key challenge is to determine how these genetic changes contribute to the underlying lung biology. We identified the urokinase plasminogen activator receptor (*PLAUR*) as an asthma susceptibility gene by positional cloning [2]. We showed that the same single nucleotide polymorphisms (SNPs) were associated with soluble *PLAUR* levels in blood, airway hyperresponsiveness (AHR) and accelerated lung function decline in asthma; a clinical feature linked to airway wall remodelling [2]. Therefore, we hypothesised that *PLAUR* may contribute to structural changes in asthma *via* increased levels of the membrane bound or soluble receptor. We subsequently showed that *PLAUR* levels were elevated in the airway epithelium of asthma patients and that *PLAUR* has a role in epithelial repair responses [3]. The aim of the current study was to 1) test for association between *PLAUR* SNPs and markers of airway remodelling using bronchial biopsies from asthma patients; and 2) test for association between SNPs and staining for *PLAUR* in airway tissue.

This study utilised bronchial biopsy samples from 137 asthma patients. These subjects were previously recruited in the northern Netherlands and re-examined during 2002–2006 [4]. Lung function testing and AMP provocation tests were performed and subjects were considered to have AHR when an AMP concentration of $<320 \text{ mg}\cdot\text{mL}^{-1}$ caused a 20% fall in forced expiratory volume in 1 s (FEV₁). DNA samples were available in all 137 subjects. Bronchoscopy, collection and processing of the bronchial biopsies were performed as previously described, including quantification of remodelling markers [4]. Quantification was performed on the largest of three biopsy sections taken. Clinical characteristics of the asthma patients investigated and details on genotyping and immunohistochemistry are available on request. The study was approved by the University Medical Center Groningen ethics committee and participants signed informed consent forms.

Bronchial biopsies were characterised for: 1) basement membrane thickness (μm); 2) sub-epithelial vasculature (number of CD31⁺ vessels per 0.1mm² of submucosa); 3) percentage intact epithelium; 4) basal



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epithelial proliferation (percentage of Ki67⁺ cells in basal epithelium); 5) collagen III deposition (% positive per 200 μm -area under basement membrane); 6) lung function (FEV₁ % predicted and FEV₁/vital capacity pre- and post-bronchodilator); 7) AHR (provocative concentration causing a 20% fall in FEV₁ AMP <320 mg·mL⁻¹); and 8) PLAUR (immunohistochemistry) epithelial expression (low/high expression).

Basement membrane thickness was calculated by dividing surface area by length (μm), based on computer-assisted measurements. The number of CD31⁺ (DAKO, Cambridgeshire, UK) vessels in the submucosal area was counted in the whole section (excluding areas of epithelium, muscle and mucus glands), therefore measuring the number of vessels per area (0.1mm²). Epithelial layer integrity was assessed on haematoxylin and eosin stained biopsy sections and expressed as the percentage of basement membrane covered with normal, intact epithelium. Reproducibility of the measurements was confirmed by a single observer performing repeated measurements in 10% of all cases.

22 SNPs spanning *PLAUR* were genotyped [2] (data available on request from the authors) and allele frequencies did not deviate from Hardy-Weinberg equilibrium ($p < 0.05$). There was some redundancy in the genotyping with r^2 values > 0.8 for: rs4803648/rs4802189/rs4251938, rs2356338/rs4251831 and rs344780/rs344781.

Normalisation of variable distribution was performed by natural logarithm transformation. Multiple linear regression models were used to test for associations of *PLAUR* SNPs with all continuous outcomes and logistic regression for AHR. As the number of subjects with the homozygote mutant genotype was low in the two subgroups ($n < 10$), only dominant (heterozygotes and homozygotes mutants pooled *versus* wild types) models were used, adjusted for age, sex, smoking and inhaled corticosteroid (ICS) use. A simple linear and logistic regression model was used to assess associations between epithelial *PLAUR* expression and clinical and remodelling parameters, smoking and ICS use. Associations of epithelial *PLAUR* expression with $p < 0.05$ in univariate analyses were also tested in multivariate models. Genetic analysis was performed separately in never- and ever- (current and ex-) smokers. p -values of < 0.05 were considered statistically significant (2-sided tests). As there was *a priori* evidence of an association between the SNPs chosen for study and asthma and lung function related phenotypes [2, 5], we have not corrected for the number of SNPs and tests completed. Importantly, the outcome variables in our analyses are not independent (*e.g.* for basement membrane thickness and percentage intact epithelium, the correlation coefficient is -0.294 ; $p < 0.001$) and several SNPs are in linkage disequilibrium making, for example, Bonferroni correction over conservative. This approach is in line with other similar studies [6]. We therefore report associations for p -values of < 0.001 – 0.05 .

11 of the 22 SNPs analysed showed association with one or more outcomes including: AHR, basal epithelial proliferation, basement membrane thickness and collagen III deposition (figure 1a). Seven of these 11 SNPs had previously been associated with asthma and four with lung function decline in asthma (figure 1a). Of interest, eight of these 11 SNPs showed an association with basal epithelial proliferation (Ki67), identifying a potential role for *PLAUR* in epithelial homeostasis as suggested previously [3]. This observation is in line with findings showing that epithelial cells overexpressing *PLAUR* have altered proliferation *in vitro* [7]. In addition, soluble cleaved *PLAUR* is elevated in induced sputum [8] and lavage fluid following allergen challenge in asthma patients [9].

PLAUR protein expression in biopsies was prominent in the epithelium as anticipated [3]. Staining was apical suggesting increased soluble *PLAUR*; however, we cannot exclude antibody or sample driven differences when we compare with previous staining [3] using a different, now unavailable antibody (figure 1b–e). We identified individuals with high ($n=50$) and low ($n=41$) epithelial staining. Of interest, ICS use was associated with lower *PLAUR* levels in the epithelium (adjusted OR=0.301, 95% CI 0.122–0.745; $p=0.009$), showing ICS responsiveness as suggested previously [9]. We tested for association between *PLAUR* staining intensity and remodelling outcomes and showed that strong epithelial *PLAUR* staining was associated with reduced basal epithelial proliferation (37% decrease, adjusted $p=0.010$, figure 1f). These data identify that epithelial *PLAUR* levels relate to basal epithelial proliferation *in vivo*. However, we did not identify an association between *PLAUR* SNPs and the epithelial *PLAUR* staining, potentially due to insufficient power (data not shown). While a strength of our study is measurement of *PLAUR* expression in lung tissue, this was limited by it being a categorical rather than a quantitative measurement as samples for Western blotting were unavailable. Previously, we demonstrated that several of these 5' region SNPs (rs2356338, rs4493171, rs1994417, rs346043) were associated with serum/plasma *PLAUR* levels [2].

In the ENCODE dataset [10], SNPs identified in figure 1a as associated with airway remodelling measures, were enriched for DNase sites in tracheal epithelial cells (11.1 fold enrichment; $p=0.013$) and enhancer sites (*e.g.* in K562 cells, 17.7 fold enrichment; $p=0.0006$). However, analysis of a lung expression quantitative trait loci dataset did identify a role for *PLAUR* SNPs as determinants of *PLAUR* mRNA levels at the 10% false discovery rate level in total lung [11]. This however does not necessarily imply that the SNPs are unimportant in specific cells, *e.g.* epithelial cells.

a) SNP	Outcome	This study		Previous study [2]	
		β	p-value	Asthma	Decline
rs4803648-A 3'UTR	AHR (odds ratio estimates)	0.448	0.044	Risk	Accelerated
	Basal epithelial proliferation Ki67+	0.482	0.001		
	Collagen III deposition	5.223	0.037		
rs4802189-A 3'UTR	AHR (odds ratio estimates)	0.406	0.034	Risk	Accelerated
	Basement membrane	-0.743	0.010		
	Basal epithelial proliferation Ki67+	0.449	0.005		
rs4251938-G 3'UTR/intron	Basement membrane thickness	-0.679	0.020	Risk	Accelerated
	Basal epithelial proliferation Ki67+	0.592	$\leftarrow 0.001$		
rs2302524-C Lys220/175Arg	Basal epithelial proliferation Ki67+	0.300	0.049	Risk	Accelerated
rs4251864-C Intron 3	AHR (odds ratio estimates)	3.034	0.019	No	No
rs4251831-C Intron 1	Basal epithelial proliferation Ki67+	-0.365	0.009	No	ND
rs2356338-T 5'-649	Basal epithelial proliferation Ki67+	-0.443	0.001	Risk	Accelerated
rs344779-T 5'-3665	Basal epithelial proliferation Ki67+	0.383	0.006	No	ND
rs4493171-T 5'10533	Collagen III deposition	4.595	0.048	Protect	ND
rs1994417-T 5'-13183	% intact epithelium	0.248	0.026	No	ND
	Collagen III deposition	5.895	0.011		
rs346043-C 5'-20458	Basal epithelial proliferation Ki67+	-0.337	0.019	Risk	ND

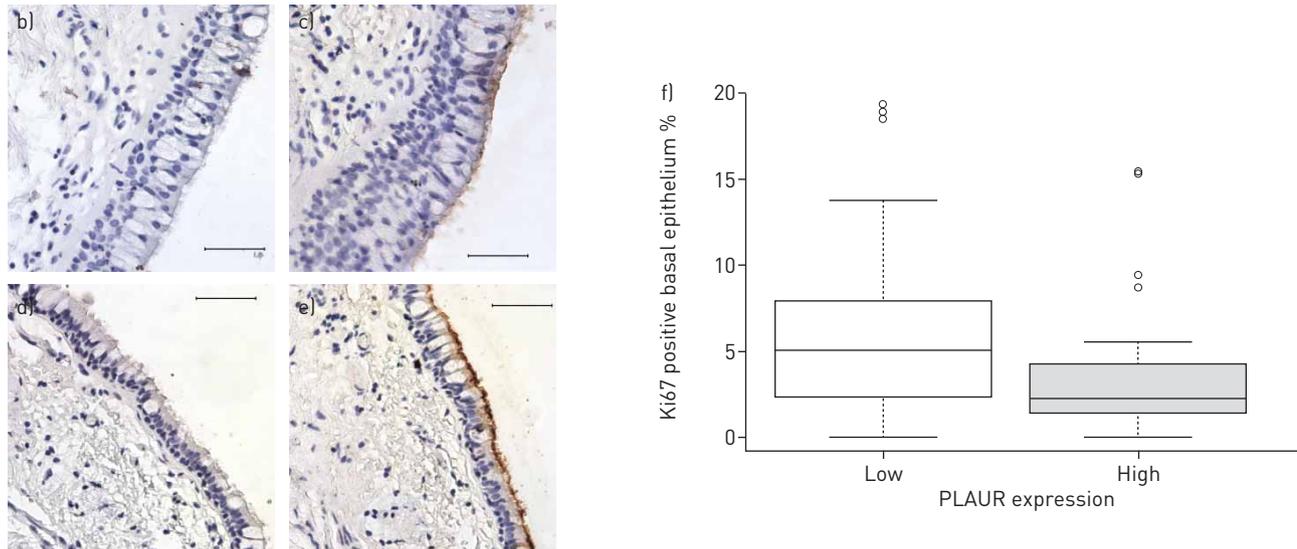


FIGURE 1 Main associations between *PLAUR* (urokinase plasminogen activator receptor) single nucleotide polymorphisms (SNPs) and markers of airway remodelling, and immunohistochemical analyses of *PLAUR* protein expression in asthma patient biopsies. a) Genetic associations. All SNP associations are adjusted for age, sex, inhaled corticosteroid use and ever-smoking. Smoking was an identified confounder for this dataset (data not shown). SNP associations with various remodelling outcomes are presented in comparison with previous associations with asthma and/or decline in lung function in asthmatic subjects [2]. Examples of b) isotype control and c) low level *PLAUR* expression. Examples of d) isotype control and e) high level *PLAUR*. b-e) Scale bars=50 μ m. f) Basal epithelial cell proliferation (Ki67) is significantly higher in low (n=41) versus high (n=50) *PLAUR* categorised biopsies (p=0.005). Boxes represent the median and 25–75th percentiles and whiskers the minimum and maximum values (excluding outliers, shown as dots). UTR: untranslated region; AHR: airway hyper-responsiveness; ND: not determined.

Cigarette smoke can induce *PLAUR* expression in the airways and may contribute to the bronchial epithelial phenotype [12, 13]. Although investigating a possible SNP interaction with smoke exposure would be of interest to this study, we were constrained by limited numbers of smokers (ever-smokers n=75; never-smokers n=61).

PLAUR is a serine protease receptor with many downstream effects including ECM degradation as well as non-plasmin functions, e.g. cell migration and proliferation [14]. The current study provides further indications that PLAUR may be involved in airway remodelling in asthma through alterations in epithelial function, which is, at least in part, driven by genetic polymorphisms. Further investigation into the role of PLAUR in asthma may provide therapeutic opportunities to target airway remodelling in asthma.



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PLAUR polymorphisms and levels are associated with markers of airway remodelling in lung biopsies of asthma patients <http://ow.ly/X5Srl>

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