

## CHRONIC OBSTRUCTIVE PULMONARY DISEASE

# Transforming growth factor- $\beta_1$ genotype and susceptibility to chronic obstructive pulmonary disease

L Wu, J Chau, R P Young, V Pokorny, G D Mills, R Hopkins, L McLean, P N Black

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See end of article for authors' affiliations

Correspondence to:  
Dr P N Black, Department  
of Medicine, Auckland  
Hospital, Private Bag  
92024, Auckland, New  
Zealand; pn.black@  
auckland.ac.nz

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**Background:** Only a few long term smokers develop symptomatic chronic obstructive pulmonary disease (COPD) and this may be due, at least in part, to genetic susceptibility to the disease. Transforming growth factor  $\beta_1$  (TGF- $\beta_1$ ) has a number of actions that make it a candidate for a role in the pathogenesis of COPD. We have investigated a single nucleotide polymorphism at exon 1 nucleotide position 29 (T→C) of the TGF- $\beta_1$  gene that produces a substitution at codon 10 (Leu→Pro).

**Methods:** The frequency of this polymorphism was determined in 165 subjects with COPD, 140 healthy blood donors, and 76 smokers with normal lung function (resistant smokers) using the polymerase chain reaction and restriction enzyme fragment length polymorphism.

**Results:** The distribution of genotypes was Leu-Leu (41.8%), Leu-Pro (50.3%), and Pro-Pro (7.9%) for subjects with COPD, which was significantly different from the control subjects (blood donors: Leu-Leu (29.3%), Leu-Pro (52.1%) and Pro-Pro (18.6%),  $p=0.006$ ; resistant smokers: Leu-Leu (28.9%), Leu-Pro (51.3%) and Pro-Pro (19.7%),  $p=0.02$ ). The Pro<sup>10</sup> allele was less common in subjects with COPD (33%) than in blood donors (45%; OR=0.62, 95% CI 0.45 to 0.86,  $p=0.005$ ) and resistant smokers (45%; OR=0.59, 95% CI 0.40 to 0.88,  $p=0.01$ ).

**Conclusions:** The proline allele at codon 10 of the TGF- $\beta_1$  gene occurs more commonly in control subjects than in individuals with COPD. This allele is associated with increased production of TGF- $\beta_1$  which raises the possibility that TGF- $\beta_1$  has a protective role in COPD.

**A**lthough chronic obstructive pulmonary disease (COPD) is a consequence of smoking, only 15–20% of long term smokers develop symptomatic airflow obstruction.<sup>1</sup> This suggests that there is a genetic predisposition to COPD and this is supported by family studies,<sup>2</sup> but the genetic polymorphisms that have been identified—such as  $\alpha_1$ -antitrypsin deficiency—only account for a small proportion of the individuals who develop COPD.<sup>3</sup>

Transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) is a cytokine with many different effects on cell proliferation and differentiation and on inflammation.<sup>4,5</sup> Some of these actions could protect against the development of COPD. TGF- $\beta_1$  can inhibit matrix metalloproteinases that may contribute to the development of emphysema through the digestion of elastic fibres.<sup>6,7</sup> It also promotes the formation of elastin<sup>8,9</sup> and this could help repair damage to the lungs of individuals who are smokers and who are at risk of developing COPD. With these considerations in mind, we have looked for an association between COPD and one of the polymorphisms in the TGF- $\beta_1$  gene.

Previous studies have identified a number of polymorphisms in the TGF- $\beta_1$  gene.<sup>10–13</sup> One of these is a polymorphism at nucleotide +869 (T869C) that produces a Leu→Pro substitution in codon 10. This occurs in the signal peptide sequence of the exon 1 and replaces one non-polar amino acid (leucine) with another (proline). The Pro<sup>10</sup> allele is associated with increased formation of TGF- $\beta_1$ .<sup>14,15</sup> An association of this polymorphism has been reported with scleroderma.<sup>16</sup> The Pro<sup>10</sup> allele has also been linked to bone mineral density<sup>17</sup> and the regulation of blood pressure.<sup>14,17</sup> In our study we have looked at the frequency of the polymorphism at codon 10 in patients with COPD and in two groups of control subjects (healthy blood donors and smokers with normal lung function (resistant smokers)). Polymerase chain reaction (PCR) and restriction enzyme fragment length polymorphism (RFLP) were used to analyse the genotype of the polymorphism.

## METHODS

Blood samples were collected from two groups of patients with COPD. The first group comprised 61 patients admitted to Auckland or Waikato hospitals with an exacerbation of COPD. Lung function in these subjects was measured immediately before discharge. The second group comprised 104 outpatients with stable COPD. In both groups of patients the lung function was measured after short acting bronchodilators had been withheld for 6 hours or more. Spirometric tests were performed according to ATS criteria.<sup>18</sup> As well as having a doctor's diagnosis of COPD, all of the patients were  $\geq 40$  years old, had a smoking history of  $\geq 20$  pack years, forced expiratory volume in 1 second (FEV<sub>1</sub>) of  $\leq 60\%$  predicted, and ratio of FEV<sub>1</sub> to forced vital capacity (FVC) of  $\leq 70\%$ . Subjects who were known to have bronchiectasis or a primary diagnosis of asthma were not included in the study. Blood samples were collected into EDTA and/or CPT tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) before extraction of DNA.

Blood samples were also collected from two groups of control subjects. The first were 140 healthy blood donors and samples were collected at the time they donated blood. All of the blood donors completed a brief questionnaire that included a question on ethnicity. Lung function was not measured in this group but none had symptomatic COPD. The second control group comprised individuals who had not developed COPD despite smoking. They were members of the Returned Services Association and were only included in the study if they were  $\geq 40$  years old, had a smoking history of  $\geq 20$  pack years, FEV<sub>1</sub>  $\geq 85\%$  predicted, and FEV<sub>1</sub>/FVC  $\geq 70\%$ . All of the patients and control subjects were of white ethnic origin.

The study was approved by the North Health ethics committee and all of the subjects provided written informed consent.

### DNA extraction

CPT tubes were centrifuged at 1500 g for 20 minutes at room temperature, the peripheral blood mononuclear layer was harvested according to the manufacturer's instructions, and the cell pellet stored at -80°C until DNA extraction. Blood samples collected into EDTA tubes were treated with sucrose lysis buffer and the nuclear cell pellet stored at -80°C until DNA extraction. Genomic DNA was extracted using DNAzol (Invitrogen, Carlsbad, CA, USA) following standard protocols given by the manufacturer. Stock DNA was stored at a concentration of 500 ng/ $\mu$ l at -20°C and PCR reactions were prepared using working DNA stocks of 100 ng/ $\mu$ l.

### PCR amplification

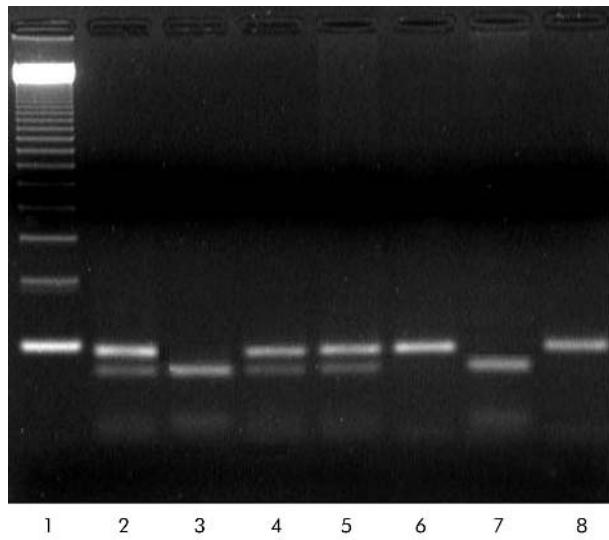
Reaction mixtures of 25  $\mu$ l were prepared containing 200 ng DNA, 1 U Taq polymerase (Invitrogen), 0.2 mM dNTPs, 0.5  $\mu$ M of each primer (Invitrogen), PCR buffer (containing 10 mM Tris HCl, pH 8.3, 50 mM KCl) and 1.0 mM MgCl<sub>2</sub>. The primer sequence was based on the study by Syrris *et al.*<sup>10</sup> The forward primer sequence was ACCACACCAGCCCTGTTGC and the reverse primer sequence was AGTAGCCACAGCAGCGGTAGCAGCTGC. Using a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA, USA), PCR was performed with an initial denaturation at 94°C for 3 minutes. This was followed by 33 cycles, with denaturation at 94°C for 50 seconds, annealing at 66°C for 1 minute, then elongation at 72°C for 1 minute. A final elongation of 10 minutes at 72°C then followed. An amplification check was carried out using electrophoresis with a 2.5% agarose gel (Invitrogen) in 0.5% TAE buffer (40 mM Tris acetate, 5.7% glacial acetic acid, 2 mM Na<sub>2</sub>EDTA.2H<sub>2</sub>O) containing 0.25  $\mu$ l ethidium bromide. 4  $\mu$ l of amplification product and 3  $\mu$ l of loading buffer (consisting of 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were added to each well. Electrophoresis was performed in 200 ml 0.5% AE buffer with 2.5  $\mu$ l ethidium bromide with a voltage of 90 mV for approximately 80% of the length of the gel.

### Restriction enzyme digestion

3.0  $\mu$ l of the amplification product was digested with 4 U PstI restriction endonuclease enzyme (Roche Diagnostics, Hague Road, IN, USA) in a 12  $\mu$ l volume mixture containing 0.9  $\mu$ l SuRE/Cut Buffer H (Roche Diagnostics). The reaction mixture was incubated at 37°C for 1 hour. Restriction enzyme digested PCR products were subjected to electrophoresis in a 2.5% agarose gel (Seakem; FMC, Rockland, ME, USA) at -70 mV for 2 hours in 200 ml TBE buffer. Using ultraviolet transillumination after ethidium bromide staining, the products were visualised and the size of the product was determined using 123 bp ladder (Invitrogen). In each digestion, control samples were included from subjects who had been confirmed by DNA sequencing as Leu/Leu, Leu/Pro and Pro/Pro, respectively. A gel of the RFLP digestion products is shown in fig 1.

### Sequencing

DNA sequencing was performed on 10 random samples to confirm the match between the product sequence and the established TGF- $\beta$ 1 sequence. PCR reactions were performed as described above but scaled up to 100  $\mu$ l. The PCR product was purified using High Pure PCR Purification Kit (Roche Diagnostics) following the manufacturer's instructions. 500  $\mu$ l of absolute ethanol and 15  $\mu$ l 3 M NaOAc were used to precipitate DNA from the 100  $\mu$ l buffer (from the kit) that was used to elute the DNA from the column matrix. The mixture was left at -80°C for 1 hour, centrifuged at 13000 rpm for 2 minutes, then the pellet was washed twice with 500  $\mu$ l 75% ethanol and redissolved in sterile double



**Figure 1** Agarose gel showing RFLP for polymorphism in the TGF- $\beta$ 1 gene at nucleotide +869 (T869C) producing a Leu→Pro substitution at codon 10. Lane 1 shows 123 bp DNA ladder. Lanes 2, 4 and 5 show the Pro-Leu genotype, lanes 3 and 7 show the Leu-Leu genotype, and lanes 6 and 8 the Pro-Pro genotype.

distilled deionised water. Sequencing reactions were performed using dye primer reactions with the above primers and Big Dye (Applied Biosystems, Foster City, CA, USA) chemistry and analysed on an ABI 377A apparatus (Applied Biosystems).

### Statistical analysis

The distribution of genotypes in the subjects with COPD, blood donors, and resistant smokers was compared using 2 × 2 contingency tables with Fisher's exact test or 2 × 3 contingency tables with the  $\chi^2$  test. These tests were all two sided. In addition, the influence of smoking (pack years), age, and sex on the risk of being a resistant smoker or a patient with COPD for the different TGF- $\beta$ 1 genotypes was modelled using logistic regression (SAS version 8, SAS Institute, Cary, NC, USA). The baseline characteristics of the different groups were compared using Fisher's exact test for sex and the Wilcoxon test for independent groups for smoking history and age.

### RESULTS

The characteristics of the 165 subjects with COPD are shown in table 1. Information on duration of symptoms was available in 145 patients. The first control group comprised 140 healthy blood donors. In a second control group of 76 smokers who had not developed COPD (resistant smokers) the mean FEV<sub>1</sub> was 95.8% predicted. The resistant smokers

**Table 1** Characteristics of subjects with COPD and resistant smokers

	COPD (n = 165)	Resistant smokers (n = 76)
Mean (SD) smoking history (pack years)	44.9 (22.5)	45.8 (31.3)
Mean (SD) age (years)	66.8 (10.3)	53.7 (10.8)
Male (%)	55.2	56.0
Mean (SD) FEV <sub>1</sub> (% predicted)	32.6 (13.3)	95.8 (10.9)

did not differ from the patients with COPD in smoking history or sex but were younger ( $p = 0.06$ , table 1).

Sequencing of the PCR product confirmed that the observed sequence for the Leu<sup>10</sup> and Pro<sup>10</sup> alleles at codon 10 was the same as the expected sequences.

The distribution of genotypes was the same for the blood donors (29.3% Leu-Leu, 52.1% Leu-Pro, and 18.6% Pro-Pro) and the resistant smokers (28.9% Leu-Leu, 51.3% Leu-Pro, and 19.7% Pro-Pro;  $p = 0.98$ ). The distribution of genotypes for the COPD subjects was 41.6% Leu-Leu, 50.0% Leu-Pro, and 8.4% Pro-Pro, which was significantly different from the blood donors ( $p = 0.006$ ) and from the resistant smokers ( $p = 0.02$ , table 2). There was no significant deviation in the genotype frequency from the Hardy-Weinberg equilibrium. The Pro<sup>10</sup> allele occurred less frequently in the subjects with COPD (33%) than in the blood donors (45%; odds ratio 0.62 (95% CI 0.45 to 0.86),  $p = 0.005$ ) and the resistant smokers (45%; odds ratio 0.59 (95% CI 0.40 to 0.88),  $p = 0.01$ ).

In the logistic regression the odds of being a resistant smoker as opposed to a COPD patient were modelled for the Pro-Pro genotype versus the Leu-Leu genotype and for the Pro-Pro genotype versus the Leu-Pro genotype. The unadjusted odds ratio was 3.2 (95% CI 1.5 to 9.6,  $p = 0.003$ ) for Pro-Pro versus Leu-Leu. Following adjustment for age, sex, and smoking history, the odds ratio did not change (3.2 (95% CI 1.1 to 9.2),  $p = 0.03$ ). For Pro-Pro versus Leu-Pro the unadjusted odds ratio was 2.7 (95% CI 1.1 to 6.2),  $p = 0.02$ , and the adjusted odds ratio was 2.2 (95% CI 0.8 to 6.0),  $p = 0.13$ . Information on smoking history was not available for most of the blood donors and they could not be included in the logistic regression.

The three different genotypes within the patients with COPD were compared to determine whether there were differences in age, smoking, or lung function. Patients with COPD with the Pro-Pro genotype had a higher mean smoking history (53.9 pack years) than patients with the other genotypes (Leu-Pro 44.4 pack years, Leu-Leu 43.7 pack years) while having a similar mean age (Pro-Pro 65.0 years, Leu-Pro 67.7 years, Leu-Leu 66.1 years) and mean FEV<sub>1</sub> % predicted (Pro-Pro 34.3%, Leu-Pro 33.9%, Leu-Leu 30.6%), but none of these variables differed significantly between the groups.

## DISCUSSION

We have shown that the proline allele at codon 10 of the TGF- $\beta_1$  gene is less common in subjects with COPD than in control subjects. The Pro<sup>10</sup> allele has been shown to be associated with higher levels of TGF- $\beta_1$  in serum<sup>14,15</sup> and increased TGF- $\beta_1$  mRNA in peripheral blood mononuclear cells.<sup>14</sup> Furthermore, the Pro-Pro genotype is associated with higher serum concentrations of TGF- $\beta_1$  than the Pro-Leu genotype which, in turn, is associated with higher concentrations than the Leu-Leu genotype. This suggests that the high producer genotype for TGF- $\beta_1$  may protect against the development of COPD. Our findings are consistent with those reported by Arkwright *et al* in cystic fibrosis.<sup>19</sup> They did not find any difference in the frequency of the Pro<sup>10</sup> allele between controls and subjects with cystic fibrosis, but the Pro<sup>10</sup> allele was less common in subjects with cystic fibrosis who had a rapid decline in lung function. In other words, the

Pro<sup>10</sup> allele had a protective effect on lung function in their study as it did in ours.

A recent study using an animal model of emphysema adds plausibility to the suggestion that increased production of TGF- $\beta_1$  may protect against the development of COPD. The integrin  $\alpha_v\beta_6$  activates latent TGF- $\beta$ .<sup>20</sup> Mice that lack this integrin (Itgb6 null mice) develop age related emphysema. When transgenes for TGF- $\beta_1$  were inserted into these mice so that they constitutively expressed active TGF- $\beta_1$ , the changes in the lung associated with the deletion of the integrin gene no longer occurred. In these mice the protective effects of TGF- $\beta_1$  appear to be related to inhibition of macrophage metalloelastase (MMP 12) which degrades elastin.

In any genetic association study there is always the concern that there may be confounding because the cases and controls are not well matched. The background frequency of an allele can vary in different ethnic groups and problems can arise if the ethnic background of the cases and controls differs.<sup>21</sup> We only included subjects of white ethnic origin in this study because of evidence that there are ethnic differences in the frequency of the Pro<sup>10</sup> allele. Suthanthiran *et al* reported that the Pro<sup>10</sup> allele was significantly more common in African-Americans than in white subjects.<sup>14</sup>

Ideally, we would have reduced the chance of confounding by identifying controls and cases in the same way—for example, both groups would have been identified from a cross sectional, community based cohort study. This would be a much more difficult study to undertake because of the large number of individuals that would have to be screened to identify a sufficient number of patients with symptomatic COPD. We were not in a position to undertake such a study. Nonetheless, there are a number of features of our study that make it less likely that our findings are due to confounding by non-genetic factors. We undertook a logistic regression controlling for age, sex, and smoking history and this did not change the findings. The adjusted and non-adjusted odds ratios were the same and the results remained statistically significant. In addition, other studies of white subjects have reported a distribution of genotypes similar to that in our control group.<sup>14,17</sup> The ECTIM study was one of the largest studies of TGF- $\beta_1$  polymorphisms. This study compared subjects with myocardial infarction and control subjects from three centres in France and one in Northern Ireland and found no difference in the distribution of the codon 10 genotypes between cases and controls.<sup>13</sup> For the 1192 subjects the distribution of genotypes was Leu/Leu 34%, Leu/Pro 48%, and Pro/Pro 17.8%. This distribution of genotypes is similar to that seen in our control group. We also found the same distribution of genotypes in the two different control groups (blood donors and resistant smokers) that were identified independently of each other. Although we think it is unlikely, we cannot completely exclude the possibility that our results are due to unexplained confounding so our findings need to be confirmed by other studies.

In this study we did not include patients with a diagnosis of bronchiectasis but some of the patients may still have had a degree of bronchiectasis. O'Brien *et al*<sup>22</sup> obtained high resolution computed tomographic (CT) scans of 110 patients presenting to their general practitioner with an acute

**Table 2** Distribution of genotypes in COPD and control subjects

	Leu-Leu	Leu-Pro	Pro-Pro
COPD patients (n=165)	69 (41.6%)	83 (50.0%)	13 (8.4%)
Blood donors (n=140)	41 (29.3%)	73 (52.1%)	26 (18.6%)
Resistant smokers (n=76)	22 (28.9%)	39 (51.3%)	15 (19.7%)

exacerbation of COPD; in 29% there was evidence of bronchiectasis on the CT scan. We also excluded individuals with a primary diagnosis of asthma, but some of the subjects may have had co-existent asthma even though their predominant problem was COPD. Further studies are necessary to determine if subjects with COPD and co-existent asthma or bronchiectasis differ from other subjects with COPD in their distribution of codon 10 genotypes.

If TGF- $\beta_1$  does indeed protect against the development of COPD, our study does not establish a mechanism and any speculation about this is tentative. In emphysema there is disruption and fragmentation of elastic fibres in the alveolar walls.<sup>23</sup> The resulting loss of elastic recoil leads to premature collapse of the small airways during expiration and this is thought to explain the airflow obstruction that occurs in COPD. TGF- $\beta_1$  could conceivably act to prevent the degradation of elastin by inhibiting the expression of matrix metalloproteases as it does in *Itgb6* mice. It is also possible that TGF- $\beta_1$  may be acting to promote the synthesis of elastin<sup>7,8</sup> and, as a result, it could have a role in repairing the loss of elastic fibres that occurs as a result of smoking. TGF- $\beta_1$  also has anti-inflammatory actions<sup>3,4</sup> and these could be relevant because COPD is characterised by inflammation with increased numbers of CD8+ lymphocytes and macrophages in the airways,<sup>24,25</sup> but additional research will be necessary to elucidate the role of TGF- $\beta_1$  in COPD.

#### Authors' affiliations

- L Wu, J Chau, V Pokorny, Department of Molecular Medicine, University of Auckland, Auckland, New Zealand  
 L Wu, R P Young, P N Black, Department of Medicine, University of Auckland, Auckland, New Zealand  
 G D Mills, Respiratory Medicine, Waikato Hospital, Hamilton, New Zealand  
 R Hopkins, Respiratory Services, Green Lane Hospital, Auckland, New Zealand

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