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Accelerated decline in lung function in cigarette smokers is associated with *TP53/HDM2* polymorphisms

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Abstract

In vitro studies have shown that p53 mediates a protective response against DNA damage by causing either cell-cycle arrest and DNA repair, or apoptosis. These responses have not yet been demonstrated in humans. A common source of DNA damage in humans is cigarette smoke, which should activate p53 repair mechanisms. The level of p53 is regulated by HDM2, which targets p53 for degradation. The G-allele of a polymorphism in intron 1 of *HDM2* (*rs2279744:G/T*) results in higher HDM2 levels, and should be associated with a reduced p53 response and hence more DNA damage and corresponding tissue destruction. Similarly, the alleles of a polymorphism (*rs1042522*) in *TP53* that encode arginine (G-allele) or proline (C-allele) at codon 72 cause increased pro-apoptotic (G-allele) or cell-cycle arrest activities (C-allele), respectively, and may moderate p53's ability to prevent DNA damage. To test these hypotheses we examined lung function in relation to cumulative history of smoking in a population-based cohort. The G-alleles in *HDM2* and *TP53* were found to be associated with accelerated smoking-related decline in lung function. These data support the hypothesis that p53 protects from DNA damage in humans and provides a potential explanation for variation in lung function impairment amongst smokers.

Introduction

The product of the p53 gene (*TP53* in humans) is important for preventing cancers. We know this because defects in *TP53* are responsible for Li-Fraumeni Syndrome, which results in multiple cancer phenotypes (1), and mutations in *TP53* that either inactivate or alter p53 function are commonplace in many human malignancies (www.p53.iarc.fr). Furthermore,

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mice deleted for, or harbouring mutations within, the mouse p53 gene (*Trp53*) are highly cancer prone (2, 3).

How p53 functions to prevent cancers is not entirely clear. However, p53 is a transcription factor that responds to many different cellular stresses, including DNA damage (4, 5). p53 protein is present at very low concentrations in normal cells, but upon stress, p53 concentration increases several fold due to phosphorylation, which prevents it from being targeted for degradation by the E3 ligase HDM2 (6, 7). Once stabilised, p53 can transactivate several downstream genes which leads to (a) the induction of apoptosis to eliminate DNA damaged cells, (b) transient cell cycle arrest allowing DNA repair to occur, or (c) permanent cell cycle arrest (senescence) (4, 5). Thus, in these ways, p53 protects the organism from exposure to environmental insults, including DNA damage. These protective responses of p53 are all likely to contribute to tumour suppression to a greater or lesser extent, depending on tissue type and the nature of the stress.

Functional polymorphisms in *HDM2* and *TP53* have been identified in humans (8). The G-allele of a polymorphism in intron 1 of *HDM2* (*rs2279744:G/T*) is known to result in increased levels of HDM2 due to the creation of a binding site for the common transcription factor Sp1 (9). This leads to reduced p53 protein levels and an attenuated p53 response. In some cases, individuals homozygous for this allele are more tumour prone (7). In *TP53*, the alleles of *rs1042522* that encode arginine (G-allele) or proline (C-allele) at codon 72 are thought to influence the nature of the biological response of p53 to stress (10-15). p53 protein harboring the arginine residue (p53^{R72}) has been reported to strongly interact with HDM2, resulting in enhanced nuclear export and association with the mitochondria. This appears to promote the apoptotic activity of p53. In contrast, there are data suggesting that p53^{P72} is more likely to induce cell cycle arrest in response to DNA damage. Consistent with the evidence that these polymorphisms confer different biological activities on p53, their allele frequencies vary with both ethnicity and latitude (16).

Although the ability of p53 to respond to environmental stresses in cell culture and in animal models is well-documented (4, 5), there are few data demonstrating that this occurs in humans. A common environmental source of DNA damage in humans is cigarette smoke which contains many mutagenic compounds. If p53 is protecting cells from DNA damage caused by exposure to mutagens, such as those in cigarette smoke, the degree of protection should vary according to the strength and nature of the p53 response. Thus individuals with the G-allele of the *HDM2* polymorphism (*rs2279744:G/T*) with a weaker p53 response may be less able to deal with exposure to cigarette smoke than individuals with the C-allele. Similarly, the degree of protection from exposure to cigarette smoke will vary with the biological nature of the p53 response and, therefore, individuals differing in the *rs1042522* polymorphism should exhibit different degrees of protection. The hallmark of smoking-induced airway damage is a reduction in lung function--as measured by Forced Expiratory Volume in 1 second (FEV₁) and by the ratio of FEV₁ to Forced Vital Capacity (FEV₁/FVC)--which persists despite inhalation of a bronchodilator, indicating "irreversible" impairment (17). To test these hypotheses, these measurements were made in a population based cohort between ages 18 and 32 years in relation to cumulative history of smoking. We report that the extent of lung function decline after exposure to cigarette smoke exposure does indeed vary with the strength and nature of the p53 response.

Results

***HDM2* G allele is associated with accelerated decline in lung function after cigarette smoke exposure**

Post-bronchodilator measurements of FEV₁ and FVC were performed in a population-based cohort study spanning young adulthood, from ages 18 to 32 years. Tobacco smoking was assessed on 4 occasions between ages 18 to 32. As expected, there was a significant main effect of cigarette smoke exposure on lung function change between these ages. The number of pack-years smoked was significantly associated with lower post-bronchodilator FEV₁ values and lower FEV₁/FVC ratios at age 32 after adjustment for these measures at age 18 ($p=0.018$ and $p=0.001$ respectively). However, there was no relationship between the *HDM2* or *TP53* polymorphisms and measures of lung function at either age 18 or 32 years (all p values >0.2).

Next, we examined whether the relationship between smoking and lung function was moderated by genetic variation in *HDM2* (*rs2279744:G/T*). Among individuals homozygous for the G-allele, cumulative smoking history was associated with lower post-bronchodilator FEV₁ values, whereas this association was not significant among individuals with the T-allele (Table 1), yielding a statistically significant interaction between the number of *HDM2* G-alleles and smoking ($p_{\text{int}}=0.004$). Thus, a weaker p53 response in cigarette smokers is associated with an accelerated decline in lung function. The association between smoking history and post-bronchodilator FEV₁/FVC ratios also appeared to be stronger among individuals homozygous for the G-allele than among T carriers, but the interaction between smoking and the number of *HDM2* G-alleles did not reach statistical significance ($p_{\text{int}}=0.15$).

The *TP53* G allele is associated with lung function decline in smokers

The above results suggest that p53 protects lung tissue from cigarette smoke exposure, as hypothesized. We therefore asked whether smoking-related impairment of lung function varied according to the nature of the p53 response. Lung function measurements in relation to smoking history were analysed with respect to the *TP53 rs1042522* polymorphism. Individuals homozygous for the G-allele (encoding p53^{R72}) were more susceptible to smoking-induced lung function impairment (Table 2). Among the G-allele homozygotes, smoking history was significantly associated with lower values for FEV₁ and FEV₁/FVC, whereas these associations were not significant for carriers of at least one C-allele. The interactions between the number of G-alleles and smoking were statistically significant for both post-bronchodilator FEV₁ and the FEV₁/FVC ratio ($p_{\text{int}}=0.020$ and 0.037 , respectively). Thus, the degree of protection from lung-function decline after cigarette smoke exposure appears to vary according to both the strength and nature of the p53 response.

Accelerated decline in lung function after cigarette smoking varies with the number of risk alleles

To test whether combinations of the *HDM2* and *TP53* polymorphisms influenced smoking-related lung function decline, we calculated a score based on the number of “risk” alleles (G-alleles for *HDM2*, and G-alleles for *TP53*). (There was no significant association between the *HDM2* and *TP53* variants, $\chi^2=4.54$, $p=0.34$.) Thus, an individual homozygous for both T-alleles for *rs2279744* and C-alleles for *rs1042522* had 0 risk alleles, whereas an individual homozygous for G-alleles at both loci had 4 risk alleles. Regression analyses of the association between smoking and lung function among these subgroups are shown in Table 3. There were significant interactions between smoking and the number of risk alleles for post-bronchodilator FEV₁ and FEV₁/FVC ($p_{\text{int}}=0.001$ and 0.020 , respectively). The

association between heavy smoking and decline in post-bronchodilator FEV₁ was stronger in those with more risk alleles (Fig 1). Thus, individuals with combinations of the *rs2279744* G-allele and the *rs1042522* G-allele appear to be at greater risk of developing smoking-related impairment of lung function.

There was an unexpected trend to improved lung function amongst smokers with no risk alleles (Table 3). It seems most likely that this is a chance finding since the sample was small (n=29) and the trend was not statistically significant. If this group was removed from the analysis, the interaction between the number of risk alleles and smoking remained statistically significant for FEV₁ but not for FEV₁/FVC ($p_{\text{int}}=0.008$ and 0.21, respectively).

Discussion

The observation of greater smoking-induced impairment of FEV₁ in individuals who are homozygous for the *HDM2* G-allele is consistent with its role in attenuating the p53 response. Similarly, the difference in the liability of the *TP53 rs1042522* alleles to confer susceptibility to smoking-induced lung damage may relate to their differential ability to promote the apoptotic and cell cycle arrest functions of p53. p53^{R72} (encoded by the G-allele) binds more tightly to HDM2 than p53^{P72} (encoded by the C-allele) (10), and this interaction promotes export of p53 from the nucleus (15) to the mitochondrial membrane where it mediates apoptosis (11, 12). p53^{P72} by contrast is reported to be more effective than p53^{R72} at causing cell cycle arrest (13, 14). Thus, our data showing that those with p53^{P72} alleles appear to be more resistant to smoking-induced lung damage suggest that cell cycle arrest and DNA repair are more important than apoptosis in protecting lung tissue from smoke-induced DNA damage. Although ultimately less efficient than apoptosis at removing DNA damaged cells, activation of repair by p53^{P72} would afford considerable protection from DNA damage and, at the same time, minimise tissue damage after exposure to cigarette smoke.

Lung function naturally declines slowly after reaching a peak in young adulthood. Our data suggest that the extent to which smoking accelerates this decline depends on the strength of the p53 response. However, age 32 is too young to detect serious smoking-related lung damage and the relationship between our observations and advanced smoking-related diseases such as chronic obstructive pulmonary disease and lung cancer remains unknown.

The finding that functional genetic polymorphisms in the p53 pathway moderate the effect of cigarette smoking on lung function requires replication in independent cohorts. However, we think it is unlikely that our findings are due to chance. First, the findings are biologically plausible as p53 plays a key role in regulating the cellular response to genotoxic stress. Second, the nature of the p53 responses associated with these *HDM2* and *TP53* genotypes are biologically consistent with each other, and in combination, showed the strongest association. Third, we were able to rule out the presence of gene-environment correlations; that is, there were no significant differences in the pack-year smoking histories of individuals as a function of the number of risk alleles in *HDM2* ($p=0.51$), *TP53* ($p=0.43$) or the sum of *HDM2* and *TP53* risk alleles ($p=0.95$). Fourth, measurements of lung function were made 14 years apart, which allowed us to document that genetic polymorphisms involved in p53 function moderated the effect of smoking on 'within-individual' changes in lung function over the course of young adulthood. Fifth, smoking exposure was ascertained via repeated, prospective assessments, largely negating the problems associated with retrospective recall.

In summary, we found that common polymorphisms of the gene for p53, and its principal regulator HDM2, are associated with accelerated smoking-related decline in lung function in

young adults. These data may provide an explanation for differences in the susceptibility of individuals to the adverse effects of smoking. Furthermore, they provide novel evidence that p53 can mediate physiologically adaptive responses to genotoxic insults in humans.

Methods

The Dunedin Multidisciplinary Health and Development Study is described in detail elsewhere (18-20). Briefly this is a longitudinal study of an unselected birth cohort of 1037 individuals (52% male) born in Dunedin in 1972/1973. The cohort represents the full range of socioeconomic status in New Zealand's South Island and Study members are mostly of New Zealand/European ethnicity. The cohort has been assessed repeatedly since birth. Here we report data on lung function and smoking assessed during the 14-year period between ages 18 to 32 years. The Otago Ethics Committees approved the study and written informed consent was obtained at each assessment.

Lung function

Post-bronchodilator spirometry was measured at ages 18 and 32 years using an Ohio computerized spirometer (Ohio instruments) and a SensorMedics body plethysmograph (Yorba Linda, CA), respectively. At least 3 acceptable manoeuvres were obtained with the best FEV₁ and FVC from any of the tests reported and used for calculation of FEV₁/FVC (21). Spirometry was measured 10 minutes after inhalation of 5mg/ml salbutamol nebulised for 2 minutes at age 18 and after 200 µg salbutamol via a metered dose inhaler using a large volume spacer at age 32. A portable spirometer (Spiropro, Sensormedics, Yorba Linda CA) was used to test Study members who were unable to sit in the plethysmograph or were unable to attend the research unit (n = 10 in this analysis). At each age, standing height was measured to the nearest millimetre.

Cigarette smoking

Personal smoking history was obtained from the Study members at ages 18, 21, 26 and 32 years. Cumulative smoking history to age 32 was calculated from these assessments. One pack year is defined as the equivalent of 20 cigarettes a day for one year.

Genotyping

Both polymorphisms were genotyped using primer extension-mass spectrometry (SEQUENOM, San Diego, CA). All PCR and MassEXTEND™ reactions were conducted utilising standard conditions using 2.5 ng of genomic DNA per sample (22). Primers used to genotype the 309G/T polymorphism in intron 1 of *HDM2* (c.-5+309G>T; rs2279744; g.G>T) were 5'-ACGTTGGATGTCGGAGGTCTCCGCGG-3' (forward PCR primer), 5'-ACGTTGGATGCCGACAGGCACCTGCGATC-3' (reverse PCR primer) and 5'-TCCGACCTCCCGCGCCG-3' (extension primer). The primers used to genotype the R72P polymorphism in *TP53* (c.215G>C; rs1042522; g.G>C) were 5'-ACGTTGGATGGGCCGCCGGTGTAGGAGC-3' (forward PCR primer), ACGTTGGATGCCAGGTCCAGATGAAGCTCC-3' (reverse PCR primer) and 5'-GCCAGAGGCTGCTCCCC-3' (extension primer). Automated analysis of these samples by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry was performed on a SEQUENOM–Bruker MassARRAY mass spectrometer. A randomly selected 10% of PCR amplified products from the cohort were re-genotyped using restriction fragment length polymorphism analysis with *Bst*UI (*TP53*) or *Msp*AI (*HDM2*). Results demonstrated exact concordance with those obtained with mass spectrometry.

Statistical analysis

The influence of smoking (in pack years) and *HDM2* or *TP53* genotype (coded as 0, 1, or 2 risk alleles, respectively) on spirometric lung function was assessed using linear regression with adjustments for lung function at age 18 (to adjust for any pre-existing differences in lung function), sex, and standing heights at age 18 and 32 (to adjust for sex- and height-related differences in expected lung function at each age). Interactions between genotype and the airway response to smoking were assessed by computing multiplicative genotype x smoking terms in these regression analyses. Separate analyses were repeated for each *HDM2* and *TP53* genotype. The effect of smoking on lung function was assessed amongst those with different combinations of these polymorphisms by repeating the analyses amongst those with different numbers of “risk” alleles (*HDM2* rs2279744 G-alleles and *TP53* rs1042522 G-alleles). The possibility that there may be a gene-environment correlation such that the genetic polymorphisms of *HDM2* or *TP53* influenced smoking behavior was assessed by comparing the smoking histories of the different genotypes. Analyses excluded pregnant women and were limited to Study members who reported that all four grandparents had European ethnicity. Initial analyses found no evidence that the effects of smoking on lung function differed for men and women. Hence, in subsequent analyses, sexes were grouped together with an adjustment in the models. All analyses were performed using Stata version 10 (College Station, TX).

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Figure 1. Mean percent change in FEV₁ between ages 18 and 32 according to smoking history and the number of risk alleles

White bars indicate non-smokers (no pack years, n=350), grey bars indicate light smokers (less than 5 pack years, n=97), black bars indicate heavy smokers (at least 5 pack years, n=221). The trend across risk allele groups is significant for moderate-heavy smokers ($p=0.005$) but not for non- or light smokers ($p=0.83$ and 0.12 respectively). Error bars represent standard errors of the mean.

Table 1Associations between lung function and smoking for *rs2279744 HDM2* genotypes

	Genotype	n	coefficient	(95% CI)	p	p _{int}
FEV₁ (ml)	TT	308	−0.6	−5.9 to 4.8	0.837	
	T/G	287	−3.0	−8.0 to 1.9	0.227	
	GG	73	−19.6	−30.9 to −8.2	0.001	0.004
FEV₁/FVC (%)	TT	308	−0.05	−0.12 to 0.01	0.110	
	T/G	287	−0.07	−0.14 to 0.00	0.051	
	GG	73	−0.16	−0.32 to 0.01	0.070	0.154

Analyses by regression of lung function (dependent variable) and pack-years smoking (independent variable) with adjustment for sex and lung function at age 18. Coefficients (95% confidence intervals) represent the difference in lung function associated with each pack-year of smoking (thus, for example, a 5 pack-year smoking history is associated with a mean decline in FEV₁ of 99ml in those homozygous for the G-allele). p_{int} is the p value for the interaction between the number of G-alleles and smoking.

Table 2Associations between lung function and smoking for *rs1042522 TP53* genotypes

	Genotype	n	coefficient	(95% CI)	p	P _{int}
FEV₁ (ml)	CC	48	5.9	−6.6 to 18.3	0.346	
	C/G	247	−1.7	−7.6 to 4.1	0.560	
	GG	373	−7.0	−11.6 to −2.4	0.003	0.020
FEV₁/FVC (%)	CC	48	0.00	−0.14 to 0.13	0.970	
	C/G	247	−0.05	−0.13 to 0.03	0.220	
	GG	373	−0.11	−0.18 to −0.05	<0.001	0.037

Analyses by regression of lung function (dependent variable) and pack-years smoking (independent variable) with adjustment for sex and lung function at age 18. Coefficients (95% confidence intervals) represent the difference in lung function associated with each pack-year of smoking. p_{int} is the p value for the interaction between the number of G-alleles and smoking.

Table 3

Associations between lung function and smoking according to the number of *HDM2* and *TP53* risk alleles

	No. of risk alleles	n	coefficient	(95% CI)	p	P _{int}
FEV₁ (ml)	0	29	15.7	−2.1 to 33.4	0.080	
	1	129	3.2	−5.1 to 11.5	0.446	
	2	277	−7.0	−12.3 to −1.7	0.010	
	3	189	−2.9	−9.5 to 3.8	0.395	
	4	44	−19.9	−35.4 to −4.4	0.013	0.001
FEV₁/FVC (%)	0	29	0.14	−0.03 to 0.30	0.095	
	1	129	−0.05	−0.15 to 0.06	0.403	
	2	277	−0.09	−0.16 to −0.03	0.007	
	3	189	−0.09	−0.18 to 0.00	0.060	
	4	44	−0.20	−0.44 to 0.04	0.099	0.020

Analyses by regression of lung function (dependent variable) and pack-years smoking (independent variable) with adjustment for sex, lung function at age 18. Coefficients (95% confidence intervals) represent the difference in lung function associated with each pack-year of smoking. p_{int} is the p value for the interaction between pack-years smoking and the number of risk alleles for *HDM2* GG and *TP53* GG (e.g. *HDM2* genotype TT and *TP53* genotype CC = 0 risk alleles; *HDM2* genotype GG and *TP53* genotype GG = 4 risk alleles).