ROLE OF GENETIC SUSCEPTIBILITY TO LATENT ADENOVIRAL INFECTION AND DECREASED LUNG FUNCTION

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Abstract

Background—Latent adenoviral infection may amplify cigarette smoke-induced lung inflammation and therefore play an important role in the development of chronic obstructive pulmonary disease (COPD). Adenoviruses can evade the human immune response via their 19-kDa protein (19K) which delays the expression of class I human leukocyte antigen (*HLA*) proteins. The 19K protein shows higher affinity to *HLA-B7* and *A2* compared with *HLA-A1* and *A3*. The receptor for adenovirus (*CXADR*) and integrin β5 (*ITGB5*) are host factors which might affect adenovirus infection. Therefore, we investigated the contribution of *HLA, CXADR*, and *ITGB5* genetic variants to the presence of the *E1A* gene and to level of lung function.

Methods—Study subjects were assayed for *HLA-B7, A1, A2* and *A3* by PCR-based assays using allele-specific primers. Polymorphisms of the *CXADR* and *ITGB5* genes were genotyped by PCR-based restriction fragment length polymorphism assays. Detection of adenoviral *E1A* gene was performed by a real-time PCR TaqMan assay.

Results—*E1A* positive individuals have a lower FEV_1 compared with *E1A* negative individuals. However, there was no significant difference in *E1A* positivity rate between the high (*HLA-B7* and *A2*) and low (*HLA-A1* and *A3*) 19K affinity groups. There was also no significant difference in FEV_1 level between each affinity group. There was no significant difference in *E1A* positivity rate or lung function among the *CXADR* and *ITGB5* genotypes.

Conclusions—Genetic variants in *HLA, CXADR* and *ITGB5* do not influence latent adenoviral infections and are not associated with COPD.

INTRODUCTION

Cigarette smoke-induced inflammation in the airways and lung parenchyma plays an important role in the development of chronic obstructive pulmonary disease (COPD). Inflammation is the basis of the proteolytic destruction of the parenchyma which causes loss of lung elastic recoil and the pathologic lesion of emphysema as well as the remodeling process which narrows the bronchioles (1). These lesions induce changes in lung function that include decreased

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expiratory flow rate, increased airway resistance, hyperinflation and abnormal gas exchange. However, there is considerable variability in the degree of airflow obstruction induced by cigarette smoking (2), and only 10-15% of chronic smokers develop COPD (3). These observations indicate that additional risk factors must contribute to the development of COPD.

Several studies have shown that childhood respiratory illness is an important risk factor for COPD (4,5). Adenovirus causes bronchiolitis, pneumonia and respiratory failure in children (6-8), and persists as a latent infection in the tonsils (9,10), peripheral blood lymphocytes (11) and lung (12). Latency is due to integration of viral genes into human genomic DNA (13). We have used animal experiments to explore the possible consequences of latent adenoviral infection and showed that latent adenovirus 5 infection amplified the cigarette smoke-induced inflammatory responses in the lung in a guinea pig model (14). We have previously shown that adenoviral E1A protein can regulate host gene expression by interacting with cellular transcription factors and thus increase synthesis of inflammatory mediators (15, 16). For example, E1A regulates the ICAM-1 promoter through its proximal NF-κB binding site (17). We also showed that COPD patients have more adenoviral *E1A* DNA in their lung tissue than non-obstructed controls matched for age, sex and smoking history (12) and that expression of this viral gene in lung epithelial cells (18) is increased in those with severe emphysema (19). Since this increase in the *E1A* gene from the left end of the viral genome is found in the absence of an excess of the *E3* viral gene from the right end (12), this suggests that the left-hand portion of the viral DNA is integrated into the host genome. These findings indicate that integrated adenoviral DNA, particularly the *E1A* gene, could have several important consequences relevant to the pathogenesis of COPD.

Adenovirus is able to evade the immune system to establish long-term infection in the host. It is known that adenoviral 19K protein binds human leukocyte antigen (HLA) class I molecules and retains them in the endoplasmic reticulum, thus preventing their presentation of viral antigenic peptides at the cell surface (20,21) and consequently cytotoxic T cell recognition of adenovirus-infected cells is averted (22). In a previous study, the affinity of the 19K protein for class I molecules was shown to depend on the *HLA* allele involved (23). Specifically, *HLA-A2.1* and *B7* bind very well to 19K, whereas 6-to 30-fold less 19K was associated with *HLA-A1, A3* and *Aw69* (23). These data suggest the possibility that individuals who have *HLA-A2* and *B7* alleles may be less able to mount an immune response against adenovirus infection than individuals with *HLA-A1, A3* and *Aw69* alleles, and therefore may be more susceptible to latent infection, amplification of lung inflammation and hence decreased lung function. Therefore, the first aim of this study was to investigate whether specific *HLA* types were related to the presence of adenoviral *E1A* gene in the lung and to decreased lung function among smokers.

If adenoviral infection is a co-factor in the development of COPD then genetic variation in host susceptibility to adenovirus infection and/or persistence could be a determinant of disease. The human cellular receptor for Group B Coxsackieviruses and type 2 and 5 adenoviruses (CXADR) (24) may influence susceptibility to viral infection (25) and polymorphisms in the *CXADR* gene could alter its affinity for the virus or the level of the receptor on the cell surface.

Virus internalization into the target cells requires subsequent interactions between the viral coat (or capsid) proteins that form the penton base and cell surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (26). These integrins, in particular $\alpha_v\beta_5$, mediate adenovirus internalization into the cells (27). The level of $\alpha_v\beta_5$ integrin was shown to predict the susceptibility of cells in culture to adenovirus infection (28). Therefore, polymorphisms that affect the level or amino acid sequence of the α_v or β_5 polypeptides may modulate susceptibility to adenovirus infection. The α_v and β_5 polypeptides are encoded by the integrin α_v (*ITGAV*) and β_5 (*ITGB5*) genes, respectively. Therefore, the second aim of this study was to determine whether specific

polymorphisms of the *CXADR* and *ITGB5* genes affect the presence of the *E1A* gene in the lungs of patients who have COPD and their level of lung function.

MATERIAL AND METHODS

Subjects

The subjects for this study were recruited from patients admitted to St. Paul's Hospital to undergo lobar or lung resection surgery for a localized lung cancer. All patients gave informed consent and completed an interviewer-administered questionnaire regarding smoking history, occupational exposure to dust or fumes and respiratory symptoms. All patients were Caucasians. Forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and FEV_1/FVC ratio were calculated. Patients in whom the lung lesion was obstructing a segmental or larger bronchus were excluded from the study as this may influence lung function. Any patients who had functional or pathologic evidence of a process other than those associated with COPD and non-smokers were also excluded from this study.

DNA for genotyping was extracted from frozen lung tissue or paraffin-embedded lung tissue using a standard phenol/chloroform protocol or from blood using the QIAamp DNA BloodMaxi kit (QIAGEN Inc. Mississauga, Ontario).

For the analysis of *HLA* alleles, a total of 170 subjects, of whom 93 (55%) were male and 77 were female (45%) with a median age of 64 years (range, 25 to 84 years) were included in the study. There were 89 DNA samples from frozen lung tissue and 81 DNA samples from blood. Additional DNA samples were available to the investigators from paraffin-embedded lung tissue but these could not be reliably assayed for *HLA* type. The frequencies of each *HLA* type are shown in Table 1. The estimated allele frequencies of each *HLA* group predicted by assuming Hardy-Weinberg equilibrium were similar to the reported Caucasian allele frequencies (29).

For the analysis of *CXADR* and *ITGB5* polymorphisms, a total of 312 subjects, of whom 198 (63.5 %) were male and 114 were female (36.5 %) with a median age of 63.4 years (range, 25 to 84 years) were included in the study. We had 231 DNA samples from paraffin-embedded or frozen lung tissue and 81 DNA samples from blood available. The genotype frequencies of each polymorphism are shown in Table 2.

This study was approved by the University of British Columbia/Providence Health Care Research Ethics Board. All subjects provided written informed consent for the study.

HLA-B7, A2, A2 and A3 genotyping

The subjects were genotyped by PCR assays using published *HLA* group-specific primers (30,31). Of the five *HLA* types previously tested for affinity to the 19K protein (23), *HLA-Aw69* was excluded from this study because of its low allele frequency in Caucasians. For the amplification of *HLA-B7*, the sense and anti-sense primers were located in exon 3 of the *HLA-B* gene and amplified a 119bp PCR product. The sense primer was 5'-CAA GTG GGA GGC GGC CCG TGA-3' and anti-sense primer was 5'-TGG TAC CAG CGC GCT CCA GCT-3'. For the amplification of *HLA-A2*, the sense and anti-sense primers were located in exon 3 of the *HLA-A* gene and amplified a 136bp PCR product. The sense primer was 5'-GGA GCC CCG CTT CAT CGC A-3' and anti-sense primer was 5'-CTC CCC GTC CCA ATA CTC CGG A-3'. For the amplification of *HLA-A1* group of alleles, the sense and anti-sense primers were designed by us to provide more specific amplification of the *HLA-A1* group. Primers were designed from exon 3 of the *HLA-A* gene (http://www.ebi.ac.uk/imgt/hla/) which was amplified as a 101bp PCR product. The sense primer was 5'-GCG GAC ATG GCA GCT CAG AT-3' and anti-sense primer was 5'-CGG AGC CCG TCC ACG CAC C-3'. For the amplification of

the *HLA-A3* group, the primers were located in exon 3 of the *HLA-A* gene and amplified a 230bp product. The sense primer was 5'-GGT TCT CAC ACC ATC CAG ATA-3' and anti-sense primer was 5'-CCA CTC CAC GCA CGT GCC A-3'. In order to verify that all samples contained amplifiable DNA, internal control primers that amplified a 394bp fragment of the *CXADR* gene (exon 2) (32) were added in each reaction mixture. The control sense primer was 5'-CTG GGC ATC TCT TGA GTT TGG A-3', and the anti-sense primer was 5'-ACT GGC AAG GTG ATG GAC ACA T-3'.

Optimized PCR conditions for *HLA-B7* typing were as follows: the PCR reaction mixture in a final volume of 20 μl consisted of 100 ng genomic DNA, 1.5 mM MgCl$_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.4 μM of each *HLA-B7* primer and 0.5 μM of each control primer, and 0.5 U DNA *Taq* Polymerase (Hotstar Taq®, Qiagen Inc, Mississauga, Ontario). PCR conditions for cycling were optimized using the touch down method (33) as follows: initial denaturation step at 95°C for 15 min, followed by 5 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 45 s, followed by 10 cycles with a decreased annealing temperature of 67°C, 20 cycles at 66°C and a final extension for 10 min at 72°C. Optimized PCR conditions for *HLA-A2* typing were the same as for *HLA-B7* except that 0.15 μM *HLA-A2* primers were used. Optimized PCR conditions for *HLA-A1* typing were the same as for *HLA-B7* except that 0.1 μM of each *HLA-A1* primer and 1.0 μM of each control primer were used and 5 cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 45 s, followed by 10 cycles with a decreased annealing temperature at 69°C, 20 cycles at 68°C replaced the respective steps of the *HLA-B7* PCR. Similarly, optimized PCR conditions for *HLA-A3* typing were the same as for *HLA-B7* except that 0.5 μM of each *HLA-A3* primer and 0.1 μM of each control primer were used and after the denaturation step PCR followed with 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 45 s and a final extension for 10 min at 72 °C. All PCR amplification were performed in a PCR EXPRESS Thermal Cycler (Thermo Hybrid, Ashford, Middlesex, UK), and PCR products were loaded on 2% agarose gels stained with ethidium bromide and visualized in ultra-violet illumination.

CXADR genotyping

Putative polymorphisms of *CXADR* on chromosome 21q11.2 were identified in the NCBI database and five single nucleotide polymorphisms (SNPs) were present in the coding region. Genotyping of these putative SNPs revealed that only one (a synonymous SNP in codon 282) was polymorphic in 30-50 DNA samples. The subjects were genotyped by PCR-based restriction fragment length polymorphism assays. The primers for the exon 7 polymorphism in codon 282 (rs437470) were designed from the *CXADR* gene sequence (GenBank accession # AF242865). The sense primer was 5'-CAG GCT CTT ATC CAT GAT TC-3' and the anti-sense primer was 5'-AAT CAT CAC AGG AAT CGC AC-3'. The primers produced an amplified product of 321 base pairs (bp). PCR was carried out in a 10 μl volume reaction mixture containing 100 ng of genomic DNA, 0.5 units of *Taq* DNA polymerase (Hotstar Taq®, Qiagen Inc.), 1.5 mM (for blood and frozen lung tissue DNA) or 3.0 mM (for paraffin tissue DNA) MgCl$_2$, 0.5 μM of each primer and 200 μM dNTPs. Amplification conditions were as follows: an initial denaturation step at 95°C for 15 minutes, 30 cycles (for blood and frozen tissue DNA) or 40 cycles (for paraffin tissue DNA) of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, followed by one incubation at 72°C for 10 minutes. Restriction enzyme digestions were performed in a total volume of 20 μl with 10 units of *Aci*I (New England Biolabs, Mississauga, Ontario) at 37°C overnight. This enzyme produced digestion products of 219 and 102 bp if the G allele was present and left an uncut product of 321 bp in the case of the A allele.

We also chose one intronic SNP (rs764657) for genotyping; the selection being based on the ability to use a RFLP assay for the SNP and a high minor allele frequency. The primers for this intron 3 *CXADR* polymorphism were designed from the GenBank sequence AF242864.

The sense primer was 5'-CAT GAT GTG GCT CAG TTC AC-3' and anti-sense primer was 5'-TCT CTT CGG CAA TCA CTT CC-3'. The primers produced an amplified product of 249 bp. PCR was carried out in a 20 μl volume reaction mixture containing same reagents as described above. Amplification conditions were also the same as before except that 40 cycles were used for all samples. Restriction enzyme digestions were performed in a total volume of 10 μl containing 10 units of *Alu*I (New England Biolabs) at 37°C for overnight. This enzyme produced cut bands of 217 and 32 bp if the T allele was present and an uncut band of 249 bp if the C allele was present. All digested PCR products were loaded on 2% agarose gels stained with ethidium bromide and visualized with ultra-violet illumination.

ITGB5 genotyping

Putative polymorphisms of the *ITGB5* gene on chromosome 3q21 were also found in the NCBI database and six SNPs were in the coding region. We performed direct sequencing for all six potential SNPs using 10 DNA samples and three were shown to be polymorphic. We found one potentially functional SNP which causes a substitution of amino acid 477 from asparagine to serine (rs2291087). The other two SNPs were silent mutations, and of these, we chose one SNP (rs2291088) located in codon 473 for genotyping. The primers for the 473 polymorphism were designed from the *ITGB5* gene sequence (GenBank accession# NM_002213). The sense primer was 5'-AGC AGA CAC ACG GAG CAT GT-3' and the anti-sense primer was 5'-CCT GGC ACA GGA GAA GTT GT-3'. The primers produced an amplified product of 369 bp. PCR was carried out as described for the *CXADR* intron 3 polymorphism except that the annealing steps were performed at 57°C. Restriction enzyme digestions were performed in a total volume of 10 μl containing 10 units of *Hha*I (New England Biolabs) at 37°C overnight. This enzyme produced cut bands of 251 and 118 bp if the C allele was present and an uncut band of 369 bp for the T allele.

The sense primer for the 477 polymorphism was the same as that for the 473 polymorphism and the anti-sense primer was 5'-TCT GGT ACA CGC TCT GGT TC-3'. The primers produced an amplified product of 229 bp. PCR was carried out as described for the 473 polymorphism. Restriction enzyme digestions were performed in a total volume of 10 μl containing 10 units of *Msp*A1I (New England Biolabs) at 37°C for overnight. This enzyme produced cut bands of 128 and 101 bp if the G allele was present and an uncut band of 229 bp from the A allele. Digested PCR products were loaded on 2% agarose gels stained with ethidium bromide and visualized with ultra-violet illumination.

Detection of the adenoviral *E1A* gene

Real-time PCR detecting a portion of the *E1A* coding region was performed using a TaqMan assay. The primers were designed from the sequence of the type 5 adenoviral *E1A* gene (GenBank Acc. No. AY147066). The sense and anti-sense primer sequences were 5'-GGA GGT GAT CGA TCT TAC CT-3' and 5'-CCG TAT TCC TCC GGT GAT AA-3' with an amplified PCR product size of 143bp. A TaqMan probe was designed using the Primer Express software (Applied Biosystems, Foster City, CA). The sequence of TaqMan probe was 5'-CTG GCT TTC CAC CCA-3', with 6-carboxyfluorescein (6-FAM) fluorescent dye on the 5' end and a non fluorescent quencher, and a minor groove binder on the 3' end. PCR was carried out in a 10 μl volume using a 384-well plate containing 0.5 μM of each primer, 100 nM of TaqMan probe, 5 μl of 2X TaqMan Universal Mastermix (Applied Biosystems) containing uracil-DNA glycosylase, and 50 ng of each DNA sample. A blank (no DNA template included) was added in each reaction. Serial ten-fold diluted (10^1-10^5 copies/μl) type 2 adenovirus DNA (GIBCO BRL, Burlington, Ontario) was used for making a standard curve. The PCR reactions were performed in an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems). After uracil-DNA glycosylase digestion (50°C for 2minutes) and enzyme activation (95°C for 10 minutes), 48 two-step cycles were performed; 15 second denaturation at 95°C followed by

1 minute annealing and extension at 60°C. After PCR, the fluorescence of 6-FAM was measured in each well using the ABI PRISM® 7900 HT system. The quantification was repeated in order to obtain four data points for each sample. Normalization of the amount of DNA template was achieved by amplification of the beta-actin gene and standard curves for this assay were made by the serial dilution of human genomic DNA (Applied Biosystems). The final copy numbers of the *E1A* gene were adjusted according to these data.

Statistical analysis

The results are presented for each *HLA* (*HLA-B7, A2, A1*, and *A3*) group; i.e. individuals either heterozygous or homozygous for each *HLA* allele. Differences in positivity for *E1A* were assessed by Fisher's exact test between the 19K high affinity (*HLA-A2* and *B7*) and low affinity (*HLA-A1* and *A3*) groups. Differences of *E1A* positivity rate between *CXADR* and *ITGB5* genotypes were assessed by χ^2 or Fisher's exact tests as appropriate. Associations were also analyzed by logistic regression to adjust for potential confounding factors, i.e. age, sex, and smoking history (pack years). Linkage disequilibrium estimation was done using the Arlequin software package (34). Differences in FEV_1 level were assessed by unpaired t tests. All tests were performed using the JMP Statistics software package (SAS Institute Inc.). All continuous variables were expressed as mean±SEM.

RESULTS

Detection of the adenovirus *E1A* gene by TaqMan PCR assays

The 231 DNA samples derived from paraffin-embedded or frozen lung tissue were analyzed for the presence or absence of the *E1A* gene by TaqMan real-time PCR. In total, 19 samples (8.2 %) showed a positive PCR for the *E1A* gene in at least one of the five reactions whereas the remaining 212 samples (91.8 %) were not positive in any reaction. A summary of the *E1A* positive samples is shown in Table 3.

Comparison of FEV_1 level between *E1A* positive and negative groups

Individuals who showed at least one positive *E1A* PCR were classified into an *E1A* positive group (n=19) and their mean FEV_1 level was compared with the *E1A* negative group (n=212). The mean FEV_1 level of the *E1A* positive group was significantly lower than the *E1A* negative group (62.2±4.8 vs. 72.5±1.4; p<0.04). The p value after adjustment for age, sex, and smoking history (pack years) still showed a borderline value (p=0.06).

We also investigated whether COPD patients had a higher positive rate of *E1A* compared with patients who had normal lung function. Individuals who had an FEV_1<80% predicted and FEV_1/FVC<70% were classified as having COPD (n=151) and those with an FEV_1>85% predicted and FEV_1/FVC>75% were classified as non-obstructed (n=57). Using these criteria 23 out of 231 individuals with intermediate levels of lung function were excluded. The COPD group had a higher *E1A* positive rate of 10.6% (16/135) compared with the controls with 3.5% (2/55), but this difference was not statistically significant (adjusted p=0.18).

Comparison of *E1A* gene positivity among the *HLA* groups

Contrary to our hypothesis, the *HLA-A1* or *A3* (low 19K affinity) positive groups had a higher positive rate for *E1A* (20.0% and 4.6%, respectively) compared with *B7* or *A2* (high 19K affinity) positive groups (0%, 2.5%, respectively). However, none of the differences between the groups were statistically significant. A subset of the study subjects had two (e.g. *A2* and *A3*) or three (e.g. *B7, A2* and *A1*) of the *HLA* types that had previously been investigated for affinity for 19K. Therefore, we excluded these samples and compared the *E1A* positivity rate between each affinity group. Although the high 19K affinity (*A2* or *B7* positive without *A1* or

A3) group had a lower *E1A* positive rate (2.9%) compared with the low affinity group (*A1* or *A3* positive without *A2* or *B7*) (18.2%), there was no significant difference between the groups (p=0.08).

Comparison of FEV$_1$ level among the *HLA* groups

FEV$_1$ values for *HLA* type are presented in Table 4. Since this table includes the individuals who were double or triple positive for the *HLA* types of interest, we also compared the FEV$_1$ level between each affinity group as described above (Table 5). There was no significant difference in FEV$_1$ value between the high 19K affinity group (*A2* or *B7* positive without *A1* or *A3* positive) and low affinity group (*A1* or *A3* positive without *A2* or *B7* positive) (p=0.78).

CXADR and *ITGB5* polymorphism detection and genotyping

Direct sequencing of the *CXADR* exonic and promoter regions in 10 DNA samples did not reveal additional polymorphisms to those in the NCBI database. There was strong but not complete linkage disequilibrium between the two *CXADR* polymorphisms (D'=0.97, r^2=0.20, p<0.0001). There was no significant linkage disequilibrium between the two *ITGB5* polymorphisms even though they are only 11 bp apart (D'=0.32, r^2=0.001, p=0.53).

Comparison of *E1A* positivity rate among *CXADR* and *ITGB5* genotypic groups

Positivity for the *E1A* gene in groups of subjects divided by the different SNP genotypes is presented in Table 6. For the statistical analysis, individuals who were heterozygous and homozygous for the minor allele were combined into the same group since there were too few individuals to analyze each group separately. However, there were no significant differences in *E1A* positivity between any genotypic groups. We also performed two-point haplotype analysis for both genes but no significant association with *E1A* positivity was found (data not shown).

Comparison of FEV$_1$ level among *CXADR* and *ITGB5* genotypic groups

The mean FEV$_1$ was also compared in each *CXADR* and *ITGB5* genotypic group. However, we found no significant difference in FEV$_1$ between any genotypic group (data not shown). We also performed two-point haplotype analysis for both genes but no significant association was found (data not shown).

DISCUSSION

We have previously shown that latent adenovirus 5 infection amplifies cigarette smoke induced inflammatory responses in the lung in a guinea pig model (14). We found that adenoviral *E1A* gene which integrates into human DNA (13) is expressed in human lung cells (18). This viral protein can interact with cellular transcription factors and thus regulate host gene expression of inflammatory mediators such as interleukin-8 and intracellular adhesion molecule-1 (15,16). In addition, we previously found that COPD patients have higher copy number of the adenoviral *E1A* gene in their lung compared with non-obstructed controls matched for age, sex, and smoking history (12). These findings suggest that latent adenovirus infection, and specifically the *E1A* gene, amplifies the chronic inflammatory response that contributes to the development of COPD in smokers.

One purpose of the current study was to investigate whether the presence of adenoviral *E1A* gene was related to FEV$_1$ % predicted among smokers. In this study, we used a real-time PCR method employing a TaqMan probe to detect the *E1A* gene. In total, we could detect the *E1A* gene in 19 DNA samples (8.2 %) and found that these individuals had a lower FEV$_1$ percent predicted (62.2±4.8) than the *E1A* negative group (72.5±1.4). Our real-time PCR result

suggests that the presence of adenoviral *E1A* gene in human lung DNA results in lower FEV_1 in smokers and supports findings of our previous semi-quantitative analysis (12). These current results are also in keeping with the report of increased adenoviral E1A expression in severe emphysema (19).

Viruses such as adenovirus have mechanisms for evading the immune response to establish long-term infection in the host. These strategies for immune evasion include inhibition of various steps in the class I MHC assembly pathway. HLA molecules bind foreign antigens and present them to T cells that subsequently differentiate into cytotoxic T cells or helper T cells by recognition of the antigen-HLA complex. This mechanism is central to the elimination of virus infected cells. However, in the case of adenovirus, the 19K protein is retained in the endoplasmic reticulum via its short cytoplasmic tail (35,36) and its luminal domain binds specifically to heavy chains of HLA (37). As a result, the intracellular transport, terminal glycosylation, and cell surface expression of class I MHC is dramatically inhibited. The intracellular transportation of class I MHC in the endoplasmic reticulum also requires the cooperative function of several co-factors such as transporter associated with antigen processing (TAP) (38). The 19K protein also binds TAP and acts as a TAP inhibitor preventing class I MHC and TAP association (39). Interestingly, the results of a previous study showed that the strength of interaction between 19K and different *HLA* types may influence the susceptibility of individuals to persistent infections (23). *HLA-A2* and *B7* bound with high affinity to 19K, whereas *HLA-A1* and *A3* were associated with 6-to 30-fold less 19K (23). Therefore, we hypothesized that individuals who have *HLA-B7* and *A2* would have more integrated adenoviral DNA than *HLA-A1* and *A3* positive individuals. This integrated DNA could result in increased E1A synthesis and therefore increased airway inflammation.

Based on these data, we investigated whether specific *HLA* types affect the level of integrated adenoviral *E1A* gene and also affect the level of FEV_1. We did not find any association between specific *HLA* types and the *E1A* positivity rate, or the level of FEV_1 and our findings suggest that the *HLA* types we studied are not related to latent adenoviral infection and subsequent airway obstruction. With any negative study it is important to calculate the power of the experimental design to detect a difference. Therefore, we performed a post-hoc power analysis of our study design. Given the numbers in this study we should have been able to detect a relative risk of ≥6.8 for *HLA-A2/B7* as a risk factor for *E1A* positivity and a difference in mean FEV_1 ≥9.8% predicted between the *HLA-A2/B7* and *HLA-A1/A3* groups (given α=0.05 and β=0.80 for two sided tests). Therefore, the major weakness of this study is the possibility that the lack of association was due to type 2 error.

Products of *HLA-A/B* alleles that are bound by the 19K protein are thought to allow immune evasion. However, is it also possible that by promoting immune evasion, viral replication will be allowed to proceed so that the infected host cell is ultimately killed and this may result in decreasing *E1A* gene retention by that cell.

HLA molecules play key roles in the immune response and have been identified as susceptibility or resistance genes for a number of diseases. There have been few studies which have investigated the potential association between *HLA* and COPD. Kauffmann and colleagues demonstrated a significant increase of *HLA-B7* in non smokers who had low FEV_1 levels compared with heavy smokers who had high FEV_1 levels (odds ratio 3.8, P value=0.05) (40). This result suggests that *HLA-B7* might be one of the risk factors for COPD. Maranetra and colleagues reported a significant increase in *HLA-Bw60* frequency in a group of COPD patients with low ventilatory drive (odds ratio 42, P value=0.03) (41). This finding suggests that *HLA-Bw60* might be related to ventilatory response to CO_2 in COPD.

HLA typing was performed in a Japanese patient group who had diffuse panbronchiolitis (DPB) which is a distinctive form of COPD of unknown etiology (42,43). The results demonstrated strong association between *HLA-Bw54* and DPB (relative risk=13.3 and OR=3.4 compared with the control group). Although these specific *HLA* types have been shown to be candidates for COPD and related phenotypes, the biological mechanism(s) by which *HLA* variants could contribute to susceptibility has not been sufficiently studied.

We also hypothesized that polymorphisms in specific human genes could influence susceptibility to latent adenovirus infection. We investigated two candidate genes which might affect latent adenoviral infection and the subsequent development of COPD. Adenovirus infection is initiated by attachment of the viral fiber knob to CXADR, a 46-kDa integral membrane glycoprotein (24) that is expressed in a wide range of human and murine cell types (44). The gene that encodes *CXADR* consists of seven exons that are distributed over an area of 54 kb and produces a 365 amino acid protein (32). The integrins, a family of heterodimeric transmembrane glycoproteins, are the major cell surface receptors responsible for cell adhesion to matrix (45,46). The adenoviral coat protein, penton base, binds to cell surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. In this study, we genotyped two SNPs in *CXADR* and two in *ITGB5*. We did not find any obviously functional SNPs in the *CXADR* gene although in the *ITGB5* gene we found one amino acid changing SNP. There were no associations of any of the SNPs with $E1A$ positivity or FEV_1 level. In this study, we did not analyze the *ITGAV* gene polymorphisms which might also affect the viral internalization into the cells. Further study is needed to clarify the correlation between SNPs of this gene and *E1A* positivity.

In summary, our data show that *E1A* positive individuals have a lower FEV_1 compared with *E1A* negative individuals. This result supports the suggestion that the presence of the *E1A* gene in lung tissue DNA affects lung function in smokers. We investigated the contribution of specific *HLA* types to adenoviral mediated COPD. Our finding of a lack of association indicates that *HLA-B7, A1, A2*, and *A3* types do not substantially influence latent adenoviral infections and are not associated with COPD. In addition, polymorphisms of the *CXADR* and *ITGB5* genes did not affect the presence of adenoviral *E1A* gene.

Acknowledgments

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Table 1

Genotyping results for each *HLA* group.

HLA group	Total (n)	Positive (%) / Negative (%)	Allele frequency[*]	Reported allele frequency (Caucasians)[**]
HLA-B7	170	40 (24) / 130 (76)	0.13	0.10 – 0.15
HLA-A2	170	73 (43) / 97 (57)	0.24	0.20 – 0.28
HLA-A1	170	42 (25) / 128 (75)	0.13	0.13 – 0.18
HLA-A3	170	46 (27) / 124 (73)	0.15	0.11 – 0.15

[*] Allele frequencies were estimated assuming Hardy-Weinberg equilibrium.

[**] From Imanishi et al. (28).

Table 2

Genotype frequencies of *CXADR* and *ITGB5* polymorphisms.

Gene	Polymorphism	Genotype	n (%)
CXADR	Exon 7	AA	238 (81.5)
		AG	49 (16.8)
		GG	5 (1.7)
	Intron 3	TT	123 (40.9)
		TC	130 (43.2)
		CC	48 (15.9)
ITGB5	Codon 473	CC	208 (70.7)
		CT	72 (24.5)
		TT	14 (4.8)
	Codon 477	AA	278 (94.6)
		AG	15 (5.1)
		GG	1 (0.3)

Table 3

E1A positivity in replicate real-time PCRs.

Number of replicate samples with *E1A* positive PCR	Number of individuals	Mean (±SD) number of copies of *E1A* (per 50 ng lung DNA)
5/5	2	18.9
4/5	2	1.1
3/5	1	8.9
2/5	8	4.5 (±6.6)
1/5	6	0.5 (±0.4)

Table 4

Comparison of FEV$_1$ levels among each *HLA* group.

	HLA-B7 (+) n=40	*HLA-A2* (+) n=73	*HLA-A1* (+) n=42	*HLA-A3* (+) n=46
FEV$_1$ level	82.5±3.1	78.7±2.3	75.9±3.0	82.8±2.9

Values are expressed as mean±SEM percent predicted. There were no significant differences between the groups.

Table 5

Comparison of FEV$_1$ levels between the 19K high affinity (*B7* or *A2*) and low affinity (*A1* or *A3*) *HLA* groups.

	HLA-B7 or *A2* n=56	*HLA-A1* or *A3* n=45	P value
FEV$_1$ % predicted	77.4±2.3	76.5±2.6	0.78

Values are expressed as mean±SEM percent predicted.

Table 6

E1A positivity in different genotypic groups.

Gene	Polymorphism	Genotype	E1A positive N (%)	E1A negative N (%)	P value[*]
CXADR	Exon 7	AA	16 (9.2)	158 (90.8)	0.45
		AG	2 (5.9)	32 (94.1)	
		GG	0 (0.0)	3 (100)	
	Intron 3	TT	7 (8.0)	81 (92.0)	0.90
		TC	7 (7.5)	87 (92.5)	
		CC	4 (10.5)	34 (89.5)	
ITGB5	Codon 473	CC	15 (9.9)	136 (90.1)	0.10
		CT	2 (3.9)	50 (96.1)	
		TT	0 (0.0)	10 (100)	
	Codon 477	AA	17 (8.5)	183 (91.5)	0.91
		AG	1 (8.3)	11 (91.7)	
		GG	0 (0.0)	1 (100)	

*
adjusted for age, sex, and smoking history (pack years) and grouping rare homozygotes with heterozygotes.