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Differences in microsatellite DNA level between asthma and chronic obstructive pulmonary disease

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ABSTRACT: Previous studies have shown that microsatellite (MS) DNA instability (MSI) is detectable in sputum cells in chronic obstructive pulmonary disease (COPD) and asthma. The aim of the present study was to investigate whether asthma and COPD could be distinguished at the MS DNA level.

DNA was extracted from sputum cells and white blood cells from 63 COPD patients, 60 non-COPD smokers, 36 asthmatics and 30 healthy nonsmokers. Ten MS markers located on chromosomes 2p, 5q, 6p, 10q, 13q, 14q and 17q were analysed.

No MSI was detected in non-COPD smokers or healthy nonsmokers. A significantly higher proportion of COPD patients exhibited MSI (49.2%) compared to asthmatics (22.2%). MSI was detected even in the mild stages of COPD (33.3%) and asthma (22.2%). No relationship was found between MSI and COPD severity. The most frequently affected marker was D14S588 (17.5% in COPD and 2.7% in asthma). The markers D6S344, G29802 and D13S71 showed alterations only in COPD, and G29802 was associated with a significantly decreased forced expiratory volume in one second FEV_1 (% predicted), whereas MSI in D6S344 was associated with a significantly higher FEV_1 (% pred).

The frequency of microsatellite instability was higher in chronic obstructive pulmonary disease than in asthma, and microsatellite instability in three workers showed chronic obstructive pulmonary disease specificity. However, further studies are needed to verify the differences between chronic obstructive pulmonary disease and asthma at the microsatellite level.

KEYWORDS: Chronic bronchitis, cigarette smoking, genetic susceptibility, genomic instability, somatic mutation, sputum

Asthma and chronic obstructive pulmonary disease (COPD) are considered to be the common respiratory diseases caused by the interaction of genetic susceptibility with environmental factors [1]. COPD is a preventable and treatable disease state characterised by airflow limitation that is not fully reversible, caused primarily by cigarette smoking [2]. However, few smokers develop clinically relevant COPD, suggesting a genetically predetermined susceptibility. Severe α_1-antitrypsin deficiency is the only proven genetic risk factor for COPD; however, it is present in only 1–2% of COPD patients [3, 4]. Recently, linkage and candidate gene studies in COPD have suggested a number of candidate genes to be involved in COPD pathogenesis [4–6].

In addition, asthma is a chronic inflammatory disorder of the airways, which is associated with airway hyperresponsiveness, recurrent symptoms and reversible airflow limitation. Host and environmental factors may influence the development of asthma [7]. Recent studies have shown that there are many genes with moderate effects in the pathogenesis of asthma rather than a few major ones. Chromosomal regions likely to harbour asthma susceptibility genes have been identified [8–10].

DNA microsatellites (MSs) are one of the most abundant classes of intergenic repetitive sequences dispersed on eukaryotic genomes and contain minimal repetitive units composed (usually) of one to five base pairs. These sequences are highly polymorphic in human populations and serve as markers for human identification or pedigree analyses [11]. Many studies have shown that MSs are important for genomic stability, can affect enzymes controlling

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the cell cycle, may markedly alter transcriptional activity, or protein-binding ability, and, finally, can affect gene translation [12–14]. Their abundance and various functions and effects are associated with a very high mutation rate, as compared with the rates of point mutation at coding gene loci. MS instability (MSI) is predominantly manifested as changes in the number of repetitive units, and, because of its correlation with high mutation rates, as reported previously, has become a useful genetic tool in the identification of regions of potentially altered genes. Moreover, MSI at the level of somatic cells strongly suggests defects in cellular systems maintaining genetic information [15].

Previous studies have shown that genetic alterations in MS markers, including MSI, have been observed in several human malignant conditions [16–18] and benign diseases, such as actinic keratosis, pterygium, diabetic retinopathy, atherosclerosis, asthma, COPD, sarcoidosis, idiopathic pulmonary fibrosis and rheumatoid arthritis [19–22].

Recent studies have shown that somatic genetic alterations, such as MSI, are a detectable phenomenon in sputum cells in COPD [23, 24] and asthmatic patients [25]. It was suggested that MSI could be considered a useful marker of genetic susceptibility, indicating destabilisation of the genome at various loci [23–25].

The aim of the present study was to investigate whether there are any disease-specific MS markers that would permit distinction between asthma and COPD, and, secondly, whether MSI could be used as a genetic screening tool for further identification of chromosomal regions harbouring susceptibility genes. The results of the study support both hypotheses.

METHODS

Subjects

A total of 166 subjects were studied; 63 COPD patients (mean±SD age 68±10 yrs), 60 non-COPD smokers (age 59±15 yrs), 36 asthmatics (age 50±12 yrs) and 30 normal subjects (age 56±17 yrs) were included in the study. In the COPD group, smoking history revealed 17 current and 46 ex-smokers. Asthmatics and normal subjects were nonsmokers.

The American Thoracic Society/European Respiratory Society consensus statement [2] was used for the diagnosis and assessment of severity of COPD, and the Global Initiative for Asthma guidelines [7] for asthmatics. Patients with any upper respiratory tract infection within the 6 weeks before the study, as well those with a history of lung (or other) cancer, were excluded from the present study. The non-COPD smokers showed normal physical examination and chest radiography results, and their spirometric values were within normal limits. The normal subjects were nonasthmatic, nonatopic never-smokers who were receiving no medication (table 1).

Spirometry

Spirometry, including a bronchodilation test, was performed in all subjects using a computerised system (MasterLab 2.12; Jaeger, Würzburg, Germany) according to standardised guidelines [26].

Sputum induction

Sputum was induced *via* inhalation of a hypertonic saline aerosol, generated by an ultrasonic nebuliser (Ultraneb 2000; DeVilbiss, Somerset, PA, USA), according to standard methods [27–30]. In detail, three expiratory manoeuvres were performed 15 min after inhalation of 200 μg salbutamol and the highest value was taken as the baseline FEV_1. Subjects then inhaled the hypertonic saline aerosols for three periods of 7 min. Flow manoeuvres were performed after each inhalation. Subjects were then encouraged to cough and to expectorate sputum into a sterile plastic container, which was kept on ice. The procedure was terminated after the three periods of 7 min, if the sputum sample was of sufficiently good quality, after a fall in FEV_1 of $\geqslant$20% from baseline or if troublesome symptoms occurred. The viscid portion of the expectorated sample was separated from the sputum as described previously [31].

DNA extraction

The presence of MSI in sputum cells compared to DNA obtained from peripheral white blood cells from the same individual was investigated.

DNA extraction was carried out according to standard protocols (QIAmp DNA Blood Maxi and Mini kits; QIAGEN, Inc., Valencia, CA, USA). DNA samples were stored at -20°C.

Microsatellite markers and microsatellite instability analysis

Ten polymorphic MS markers were used to assess MSI (G29802, RH70958, D17S250, D5S207, D13S71, D14S588, D14S292, D6S2223, D6S263 and D6S344). All markers had previously been shown to be located close to genes involved in asthma and/or COPD [23, 32–39]. The sequences of the MS markers used were provided through the National Center for Biotechnology Information database [40]. The PCR technique was used to amplify DNA sequences. PCR amplifications were carried out in 50-μL final volume reaction mixtures in a PTC-100 thermal cycler (M.J. Research, Inc., Watertown, MA, USA), using the Qiagen *Taq* PCR Core Kit (QIAGEN, Inc.). Forward primers were labelled with the LI-COR IR800 fluorochrome (LI-COR, Lincoln, NE, USA). The following thermal cycling protocol was applied: 3 min at 94°C, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and 72°C for 5 min, and terminated at 4°C.

The PCR products were analysed and visualised by electrophoresis in 8% Long Ranger polyacrylamide (BMA, Rockland, ME, USA)/7 M urea sequencing gels in a LI-COR 4200 DNA sequencer, and alleles were sized using GeneProfiler version 3.54 software (Scanalytics, BS Biosciences, Rockville, MD, USA). MSI was identified by comparing the electrophoretic patterns of the MS markers of sputum DNA against peripheral blood demonstrating a shift of one or both of the alleles, thus identifying novel alleles, as indicated by an addition or deletion of one or more repeat units. Two scientists who were not aware of the clinical characteristics of the subjects performed independent readings. All MSI-positive samples were tested twice using fresh DNA, and showed 100% reproducibility.

Statistical analysis

The normality of the numerical parameters was tested using the Kolmogorov–Smirnov test. An unpaired t-test for normally

TABLE 1 Anthropometric and spirometric data for chronic obstructive pulmonary disease (COPD) patients[#], non-COPD smokers, asthmatics[¶] and healthy controls

	COPD smokers					Non-COPD smokers	Asthma[¶]	Normal subjects
	Mild	Moderate	Severe	Very severe	Overall			
Subjects n	6	15	26	16	63	60	36	30
Age yrs	55±12	64±9	73±7	69±8	68±10***	59±15	50±12	56±17
Smoking[+] pack-yrs	35±5	50±21	50±15	55±12		48±25	Nonsmokers	Nonsmokers
FEV_1 % pred	88±8	60±8	41±5	23±4	49.3±17.0[§]	85±25	78±19	92±4
FVC % pred	102±7	85±16	66±14	44±10	69±22[§]	88±10	95±20	88±4
FEV_1/FVC	70±3	60±13	55±17	38±6	62±18[§]	82±8	80±10	83±8

Data are presented as mean±SD, unless otherwise stated. FEV_1: forced expiratory volume in one second; % pred: percentage of the predicted value; FVC: forced vital capacity. [#]: four severity groups; [¶]: mild; [+]: cumulative cigarette consumption. ***: $p<0.001$; [§]: $p<0.0001$ (ANOVA between the four groups; COPD taken as one group).

and Mann–Whitney test for non-normally distributed data were used to estimate significant differences between two groups. ANOVA for normally and the Kruskal–Wallis test for non-normally distributed variables were used to compare differences among groups (COPD smokers, non-COPD smokers, asthmatics and normal subjects). The Chi-squared test was used for comparison of percentages (Yates's test). Pearson's correlation coefficient for normally and Spearman's rho for non-normally distributed variables were used to assign significant relationships. Multivariate analysis was performed in order to examine the effect of relevant covariates, such as smoking severity, and to adjust the significant associations with MSI. A p-value of <0.05 was considered significant.

Ethics

The present study was approved by Medical Research Ethics Committee of University General Hospital (Iráklion, Greece) and patients gave their informed consent.

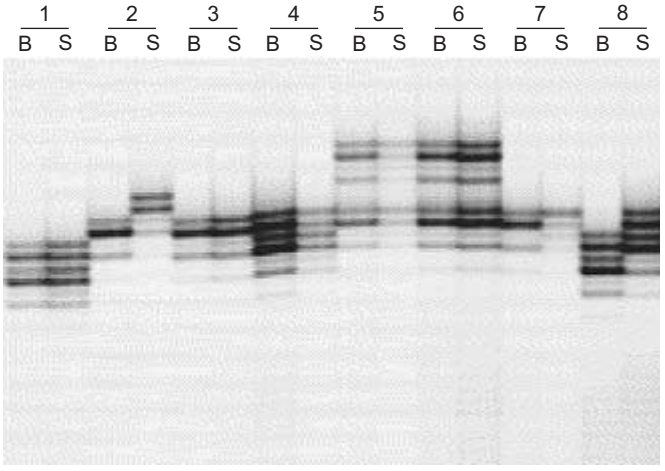

FIGURE 1. Eight representative electrophoretic profiles involving microsatellite marker D6S344. Blood (B) and sputum (S) DNA samples were obtained from chronic obstructive pulmonary disease patients (1–4) and asthmatics (5–8). Patient Nos 2 and 8 exhibit microsatellite instability; the rest show microsatellite stability.

RESULTS

Table 1 shows the anthropometric characteristics and spirometric data of COPD patients, non-COPD smokers, asthmatics and normal subjects. COPD patients are presented in groups according to the severity of the disease [2]. Figure 1 shows representative samples of MS DNA stability (MSS), as well as MSI, in the D6S344 marker. Samples taken from non-COPD smokers and normal subjects showed no MSI in any of the 10 MS markers tested. The MS marker, chromosomal location and results in COPD patients, asthma, non-COPD smokers and normal subjects are shown in table 2. A significantly higher proportion of COPD patients exhibited MSI in sputum cells *versus* blood samples compared with asthmatic patients (31 (49.2%) *versus* eight (22.2%) asthmatics ($p=0.01$; Chi-squared test)). The MSI was detected in more than one marker in the same individual (50 MSIs in 31 COPD patients). In detail, 23 COPD patients exhibited MSI in one marker, four in two markers and four patients in more than three markers. Apart from one asthmatic, who showed instability in two markers, all of the others (seven patients) showed instability in one marker.

The most frequently positive test was with the marker D14S588 (17.5% in COPD and 2.7 % in asthma ($p=0.06$; Yates-corrected Chi-squared test)). D6S344, G29802 and D13S71 showed frequent positivity, but only in COPD, whereas D6S2223 was positive in only one patient with COPD and none with asthma. No marker showed specificity for asthma (table 2). Figure 2 shows the percentage of MSI-positive cases in the four COPD severity categories according to Global Initiative for Chronic Obstructive Lung Disease guidelines [2] and in asthma. MSI was a frequent observation even in the mild COPD group (33.3%; fig. 2). The severity of COPD was not related to MSI frequency, since no significantly different percentages of patients with MSI were found in the various COPD severity groups (Chi-squared test; fig. 2). In addition, no significant relationship was found between forced expiratory volume in one second (FEV_1; % of the predicted value) and MSI frequency in the total COPD population ($p=0.5$, $r^2=0.006$; Spearman's rho).

Figure 3 shows the differences in FEV_1 (% pred) between the COPD patients who exhibited MSI in markers G29802, D6S344 and D13S71 and those who did not. A significant decrease in

TABLE 2 Microsatellite (MS) instability (MSI)-positive cases[#] according to MS marker and chromosomal region in diseases, smokers[¶] and normal controls

Marker	Chromosome	MSI n (%)			
		COPD	Asthma	Non-COPD smokers	Normal subjects
Subjects n		63	36	60	30
RH70958	2p12	3 (4.8)	1 (2.7)	0	0
D5S207	5q31.3–q33.3	1 (1.6)	3 (8.3)	0	0
D6S2223	6p21.3	1 (1.6)	0 (0)	0	0
D6S344	6p25	6 (9.5)	0 (0)	0	0
D6S263	6p23–p24.2	4 (6.4)	1 (2.7)	0	0
G29802	10q22	7 (11.1)	0 (0)	0	0
D13S71	13q32	9 (14.3)	0 (0)	0	0
D14S588	14q22.1	11 (17.5)	1 (2.7)	0	0
D14S292	14q32.1	4 (6.4)	2 (5.5)	0	0
D17S250	17q11.2–q12	4 (6.4)	1 (2.7)	0	0
Total[#]		50	9	0	0

COPD: chronic obstructive pulmonary disease. [#]: MSI was detected in more than one marker in the same individual (for further details, see Results section); [¶]: without COPD.

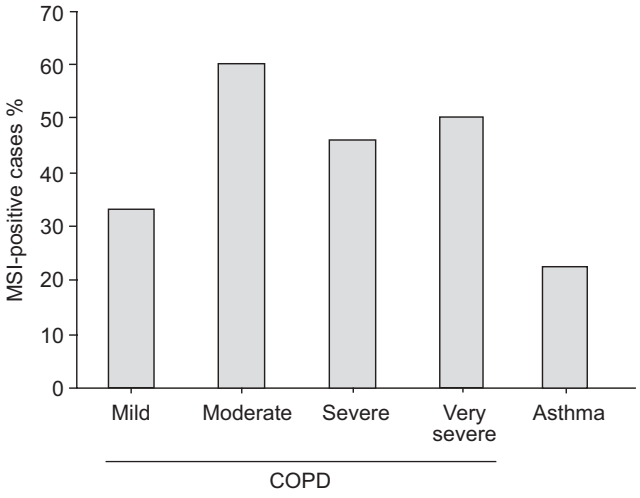

FIGURE 2. Microsatellite instability (MSI)-positive cases in the four chronic obstructive pulmonary disease (COPD) severity groups and in asthmatics. The mild COPD patients showed a similar forced expiratory volume in one second (percentage of the predicted value) to the asthmatics. No significant differences were found between the four COPD subgroups.

FEV_1 (% pred) was observed between COPD patients with MSI in the marker G29802 (Mann–Whitney test). The effect of smoking was examined using multivariate analysis, taking as a dependent variable the presence of MSI and as independent variable, the FEV_1 (% pred) and smoking severity. Both smoking severity and FEV_1 (% pred) were significantly associated with MSI in G29802. Smoking severity was more closely positively associated with MSI than FEV_1 (% pred) in G29802 ($p=0.04$). Using the same multivariate model after adjustment for smoking severity, the presence of MSI in G29802 remained significant in relation to the decreased FEV_1 (% pred; $p=0.02$). In contrast, MSI in D6S344 test associated with a significantly higher FEV_1 (% pred; $p=0.01$). No significant difference in FEV_1 (% pred) was found between patients with and without MSI in D13S71 ($p=0.5$; Mann–Whitney test; fig. 3).

DISCUSSION

It is well known that asthma shares common clinical and laboratory characteristics with COPD, making differential diagnosis extremely difficult in some cases. In sputum cells, the genetic background of both diseases was investigated at the MS DNA level, in order to find out whether COPD could be distinguished from asthma.

A limitation of the present study is the identification of the specific sputum cell subpopulation(s) that exhibit MSI. Studies currently in progress have shown that MSI is not found in the cells of haematopoietic origin, leaving the epithelial cells as the most likely candidate [41]. This may be in agreement with the potentially significant role of epithelial cells in the pathogenesis of COPD [33, 42]. Another limitation of the present study is the small number (10) of MS markers tested. However, this is the first study comparing asthma to COPD at the MS DNA level, and thus an investigation was embarked upon with only a limited number of specific MS markers.

The present results showed that 49.2% of COPD patients and only 22.2% of asthmatics exhibited MSI ($p=0.01$). These results suggest a different MSI profile in the two diseases.

A possible explanation of this discrepancy is that the burden of the oxidative stress that damages the DNA and promotes MSI is different in the two diseases. Several studies have reported that the magnitude of oxidative stress in COPD is greater than that in asthma [43–49]. Similar findings were reported in rheumatoid arthritis patients, in whom oxidative stress was correlated with MSI in synovial tissue [32]. The authors suggested that oxidative stress not only creates DNA adducts that are potentially mutagenic but also relaxes the mechanisms that limit the DNA damage by suppressing key genes of the DNA mismatch repair system [50]. Thus varying efficiency of DNA repair could be viewed as a potential determinant of disease susceptibility. The present results are in agreement with the hypothesis that acquired somatic mutations caused by cigarette smoke are the fundamental contributors to the molecular pathogenesis of COPD [33].

Three markers, namely D6S344, G29802, and D13S71, were frequently altered in COPD but not at all in asthma (table 2). This suggests that these apparently COPD-specific markers could distinguish COPD from asthma. In addition, COPD patients exhibiting MSI in D6S344 showed a higher mean FEV_1 (% pred) than those showing MSS. This may suggest a protective role of MSI in D6S344 in COPD progression or severity. Marker D6S344 is located in chromosomal region 6p25, where proteinase inhibitor (PI) 6 and 9 are also located [51]. These members of the serpin superfamily have been shown to prevent cellular damage by scavenging leaking lysosomal proteases [52]. The high FEV_1 that were associated

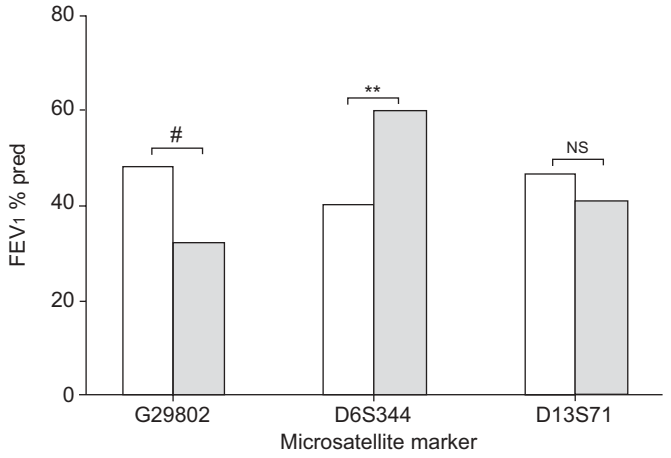

FIGURE 3. Differences in forced expiratory volume in one second (FEV_1) in chronic obstructive pulmonary disease patients exhibiting microsatellite stability (□) and instability (■) in specific markers (for further details, see Results section). % pred: percentage of the predicted value; NS: nonsignificant. #: p=0.03; **: p=0.01.

with D6S344 MSI in COPD may suggest that the observed MSI may lead to upregulation of the PI-9 gene. Similar suggestions have already been put forward in autoimmune disease, graft rejection and graft-*versus*-host disease [53].

MSI associated with the G29802 marker was colligated with more severe decline in pulmonary function in COPD patients (fig. 2). In addition, positivity in the G29802 marker was related to smoking intensity. This may therefore be an indication of acquired somatic mutations due to smoking [32]. This marker is located at the chromosomal position 10q22, where perforin is encoded. Perforin is considered the main mediator of the membranolytic action of cytotoxic CD8+ lymphocytes and is implicated in the apoptotic and destructive process leading to the development of COPD [29]. Thus, considering the low FEV_1 of the COPD patients showing MSI in G29802, it may be hypothesised that perforin expression may be increased in these patients.

MSI was detectable even in mild COPD, with an FEV_1 of $\geqslant$80%. Thus, it appears that MSI is a very early alteration of the DNA. The prevalence of MSI did not differ significantly in the four groups of COPD patients, and no relationship was found between FEV_1 (% pred) and MSI frequency in COPD. This suggests that MSI is a qualitative alteration. This is in agreement with previous reports by SIAFAKAS and co-workers [23, 30] and PARASKAKIS *et al.* [24]. In addition, it would be of interest to investigate MSI in moderate and severe asthma, since previous studies have shown that a higher frequency of genetic alterations (more than three) was associated with higher mean immunoglobulin E and blood eosinophil levels in asthmatic patients [24].

In conclusion, the present results show that there are COPD-specific MS markers in chromosomal regions 6p25, 10q22 and 13q32. The presence of MSI was not related to COPD severity. However, an association was found between two different MS markers and FEV_1. The D6S344 marker appeared to reveal a locus with potent protective effect in COPD pathogenesis, whereas G29802 was associated with the opposite. However, from the present results, it cannot be concluded that MS DNA *per se* plays a protective or promoter role in the pathogenesis of COPD. Previous studies, however, have suggested that MSs play a functional role in the genome, affecting gene expression by acting as regulatory sequences that can be recognised by transcription factors [15]. The present results are in agreement with this hypothesis, which gives a functional role to the MS DNA. The present authors speculate that MSs act as shields, protecting DNA from environmental hazards. Detecting genetic alterations at the MS DNA level could be a useful technique in the identification of the locus of potential altered genes that may play a key role in disease pathogenesis. Therefore, MSI could be a useful genetic screening tool in molecular epidemiology, identifying smokers susceptible to COPD or atopic individuals susceptible to developing asthma.

Owing to the small number of markers examined, the present results need to be confirmed in further studies with different ethnic populations (not only Greeks), requiring multicentric collaboration. Finally, the present findings highlight the importance of studying disease-associated genetic markers.

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