

# Microsatellite DNA Instability in Nasal Polyposis

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**Objective:** Genetic alterations, such as microsatellite instability (MSI) and loss of heterozygosity (LOH), have been detected in various inflammatory diseases, providing evidence that acquired somatic mutations might play a role in the aetiopathogenesis of chronic inflammatory conditions. The aim of this study is to assess the presence of MSI and/or LOH in nasal cytology of patients with nasal polyps.

**Study Design:** Prospective case-controlled basic science experiment utilizing human blood and human nasal brush samples.

**Methods:** Nasal brush samples and peripheral blood from 12 patients with nasal polyps were analyzed. DNA was extracted and analyzed for MSI and LOH using the following microsatellite markers: D2S2113, D6S344, D6S1002, D11S1253, D11S480, USAT24G1, and D13S273, harboring potential susceptibility genes for nasal polyposis. Microsatellite DNA analysis was also performed in 7 control subjects.

**Results:** MSI or LOH were revealed in 3 specimens of the nasal polyps group. Among these there were 2 cases of LOH, one for marker D11S1273 and one for D13S273, and one case of MSI in marker USAT24G1. Each one of these alterations was detected in a different patient. None of the control subjects exhibited any genetic alterations in the 7 markers tested.

**Conclusions:** This is the first time that microsatellite genetic alterations are reported in nasal disease. The presence of such alterations suggests that acquired genomic somatic mutations might play a role in the pathogenesis of nasal polyps.

**Key Words:** Loss of heterozygosity, microsatellite instability, nasal polyps, somatic mutations.

## INTRODUCTION

Sinonasal polyposis is a chronic inflammatory disease process characterized by the appearance of protruding polypoid tissue swellings from the mucosa of the nose and paranasal sinuses.<sup>1</sup> In the general population its prevalence is 0.5%–4%, comprising one of the most common chronic diseases in the upper respiratory tract. In addition, nasal polyposis has a major impact on the patients' quality of life. Tests using disease independent questionnaires have shown that the quality of life in patients with nasal polyps is worse compared to subjects suffering from hypertension, migraine or head and neck cancer.<sup>2</sup> Despite such facts, the etiology of nasal polyposis still remains largely unknown.<sup>3</sup>

The genetic background of nasal polyposis has been the focus of many recent studies.<sup>4–17</sup> Nevertheless, the role of acquired somatic mutations in the pathogenesis of nasal polyposis has not received much attention. This kind of genetic disorder has been shown to be present in other chronic inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma.<sup>18–20</sup> Acquired somatic mutations are sporadic changes in genes or gene regulatory regions that occur spontaneously but only rarely and dramatically increase in frequency in tissues exposed to repeated exogenous mutagenic insults. Somatic mutations do not affect the germ line and are not heritable, although the susceptibility to acquiring such mutations might be controlled by inherited genes.<sup>18</sup>

Genomic microsatellites are repetitions of simple 1–6 bp nucleotide sequences that have been detected in both prokaryotic and eukaryotic cells. They are present in both coding and noncoding regions of each chromosome. Nevertheless, they constitute a rather large fraction of the noncoding DNA.<sup>21,22</sup> Microsatellites are stably inherited and, therefore, well preserved from one generation to the next. In addition, microsatellites are unique to every individual and identical in cells from

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TABLE I.  
Anthropometric Characteristics of the Groups Studied.

	NP group	Control group
Number of patients	12	7
Ages	29–64 (mean 44.7)	24–44 (mean 38.4)
Males / females	8 / 4	4 / 3

NP= nasal polyposis.

different tissues in the same individual.<sup>23</sup> Because of such features, microsatellites have been used in a wide range of scientific fields, genome mapping, forensic DNA studies, degraded DNA studies, population genetics, and management of biological resources.<sup>20,23</sup>

Loss of heterozygosity (LOH) and microsatellite instability (MSI) are microsatellite genetic alterations that have been initially reported in a number of human malignancies.<sup>20,24–26</sup> As already mentioned, during recent years such phenomena have also been detected in various benign diseases of the lungs, but also in nonrespiratory diseases, including actinic keratosis, pterygium, atherosclerosis, and rheumatoid arthritis.<sup>27</sup> MSI and LOH have been proposed as an important genetic screening tool, reflecting the inactivation of the human DNA mismatch repair (MMR) system.<sup>19,20</sup> The assessment of MSI and LOH in peripheral tissue samples offers a reliable mean for the study of acquired mutations.<sup>19</sup> This is accomplished with the use of microsatellite markers targeting specific chromosomal loci containing genes that are known or suspected to be implicated in the pathogenesis of a disease. Such markers are used to compare DNA extracted from a tissue that is used as index, for example, blood, and DNA from a target tissue, such as the lower respiratory tract, in the form of sputum or the upper airway in the form of a nasal brush sample.<sup>20,24</sup>

Although microsatellite genetic alterations have been reported in many inflammatory conditions, they have never, to our knowledge, been detected in diseases of the nose.<sup>24,28</sup> The aim of the present study is to test the hypothesis whether acquired somatic genome mutations play a role in the pathogenesis of nasal polyposis by assessing the presence of MSI and/or LOH in the nasal cytology of patients with this disease.

## MATERIALS AND METHODS

### Subjects

A total of 12 asthmatic patients with nasal polyposis, aged 29–64, were studied. All patients had bilateral nasal polyps on endoscopy with stage equal or worse than 2, according to the Lildhold clinical scoring system.<sup>29</sup> In addition, all patients were lifelong nonsmokers with a negative history of aspirin sensitivity but with a previous diagnosis of bronchial asthma of more than 1-year duration. Bronchial asthma was verified in all cases with either a reversibility test (increase of forced expiratory volume in 1 second (FEV1)  $\geq 12\%$  predicted after inhalation of four puffs of 100 mg salbutamol) or a median daily variation in peak expiratory flow rate (PEF)  $\geq 15\%$  for  $\geq 4$  days a week for at least the previous 4 weeks.

A control group was also studied, consisting of 7 individuals with negative history and clinical examination for nasal

polyps. All control subjects were lifelong nonsmokers and had a positive history of bronchial asthma of more than one year duration. Verification of asthma was made as with the previous group. Anthropometric features for both groups are shown on Table I. No subjects with a history of systemic steroid use within the last 6 weeks prior to examination were included in the study. No exclusion criteria regarding recent local therapy for the upper or lower airway were followed. An informed consent was signed by all subjects participating in the study.

### Methods

Nasal cytological specimens and peripheral blood samples were obtained from all subjects. A standard brush method was used for nasal cytology and was performed by the same doctor in all cases.<sup>30,31</sup> Blood and nasal samples were stored at  $-70^{\circ}\text{C}$  for DNA extraction. DNA from nasal cytological material and peripheral blood was extracted with the QIAamp (Qiagen Extraction Kits, QIAamp DNA Blood Maxi and Mini Kits, QIAGEN Inc., Hilden, Germany) tissue and blood kits, respectively, following the manufacturer's instructions. Samples of DNA were stored at  $4^{\circ}\text{C}$ .

Seven polymorphic microsatellite markers were used to reveal MSI and/or LOH. The selection of the chromosomal regions that were studied was based on previous knowledge that these regions contain genes controlling the expression of proteins involved in the pathogenesis of nasal polyps.<sup>3,32–37</sup> The microsatellite markers used were the following: D2S2113, D6S344, D6S1002, D11S1253, D11S480, USAT24G1, and D13S273 (Table II). Sequences of the microsatellite markers were provided through the NCBI database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

The polymerase chain reaction (PCR) technique was used to amplify DNA sequences. PCRs were performed in 50  $\mu\text{L}$  final volume reaction mixtures in a PTC-100 thermal cycler (M.J. Research Inc., Watertown, MA), using the Qiagen Taq PCR Core Kit (QIAGEN Inc., Hilden, Germany). Forward primers were labeled with the Licor IR800 fluorochrome. Reactions were denatured for 5 min at  $95^{\circ}\text{C}$  and the DNA was subsequently amplified for 30 cycles at  $95^{\circ}\text{C}$ ,  $55^{\circ}\text{C}$  and  $72^{\circ}\text{C}$  during each step. The PCR products were analyzed and visualized by electrophoresis in 8% Long Ranger polyacrylamide (BMA, Rockland, ME) 7 M urea sequencing gels in a Licor 4200 DNA sequencer (Lincoln, NE). MSI and/or LOH was identified by comparing electrophoretic patterns of the microsatellite markers of DNA of nasal specimens versus peripheral blood demonstrating a shift of one or both of the alleles, thus, generating novel alleles as indicated by an addition or deletion of one or more repeat units. All MSI and/or LOH positive samples were tested twice using fresh DNA, showing 100% reproducibility.

### Statistics

Clinical characteristics are presented as mean. The statistical software SPSS Version 15 (SPSS In., Chicago, IL) for Microsoft Windows was used for the analysis. A *P* value of  $<.05$  was considered statistically significant.

## RESULTS

Mean age in the nasal polyposis group was 44.7 and 38.4 in the control group. Male to female ratio was 2 (8 males/4 females) in the study group and 1.3 (4 males/3 females) in the control group (Table I). No statistically significant differences regarding the severity of bronchial asthma were noted among the 2 groups. DNA

TABLE II.

List of Microsatellite DNA Markers Used in this Study With Corresponding Chromosomes and Candidate Genes that are Implicated in the Pathogenesis of Nasal Polyposis.

Markers	Chromosomes	Candidate genes
D2S2113	2	IL-1 $\beta$
D6S344	6	TNF $\alpha$
D6S1002		PAF-2
D11S1253	11	Mammoglobin gene family
D11S480		P2RY6
USAT24G1	13	TNF SF13B
D13S273		

IL = interleukin; TNF = tumor necrosis factor; PAF = platelet-activating factor.

was successfully extracted in satisfactory quantities from all cytological samples. MSI or LOH were revealed in 3 specimens of the nasal polyposis group. Among these there were 2 cases of LOH, 1 for marker D11S1273 and 1 for D13S273, and 1 case of MSI in marker USAT24G1. On the other hand, none of the control subjects exhibited any genetic alterations in the 7 markers tested. Detailed results regarding the nasal polyposis group are found on Table III. Figure 1 shows a case of LOH in marker D13S273 and an MSI case in marker USAT24G1.

## DISCUSSION

Studying the genetic basis of a disease is of fundamental importance in medicine, as it offers better insight into the pathogenesis and opens new, disease-specific, therapeutic trends. This study is the first to evaluate acquired somatic mutations at the microsatellite DNA level in patients with nasal polyps, providing evidence that such mutations might play a role in the pathogenesis of chronic inflammatory conditions.

Microsatellites are often located in or near important gene loci, allowing them to be used as markers for

disease and providing information about individual gene status. This may be accomplished by assessing allelic imbalance or LOH of a particular gene by analysing microsatellites that are located in specific loci near or in the gene. Furthermore, mutations within microsatellites have been described due to defective DNA repair mechanisms resulting in MSI.<sup>23</sup> This has been implicated in the aetiopathogenesis of several hereditary and nonhereditary conditions.<sup>23,25,26</sup> There are several ways of analysing microsatellites, but regardless of the method used, microsatellite analysis is considered an important tool in molecular biology and has provided new information in many diseases.<sup>23</sup>

The human DNA mismatch repair (MMR) system corrects single-base mismatches and small insertion or deletion loops that occur during DNA replication.<sup>38,39</sup> The MMR system functions through the interactions between several proteins, such as hMSH2, hMSH3, hMSH6, hMLH1, hPMS2, and hMLH3.<sup>22</sup> Oxidative stress, on the other hand, is a state in which the production of reactive oxygen species exceeds the capacity of the antioxidant defense system in cells and tissues.<sup>40</sup> Chronic inflammatory diseases create significant oxidative stress for cells and tissues.<sup>40,41</sup> It has been proposed that the inactivation of the MMR function in response to oxidative stress may be responsible for the MSI seen in non-neoplastic diseases associated with chronic inflammation.<sup>41</sup> Moreover, the reduced MMR activity as a result of oxidative stress may cause somatic mutations in hMSH3, hMSH6, and hPMS2 genes. Such an effect could subsequently augment microsatellite instability in other target genes that also contain microsatellites in their coding regions.<sup>41</sup> These target genes could be implicated in the pathogenesis of various diseases. If oxidative stress, which is a well-known feature of nasal polyposis,<sup>42,43</sup> can lead to microsatellite genetic alterations, then it would probably be responsible for the presence of MSI and/or LOH in nasal polyposis. Such a hypothesis remains to be tested in future studies.

TABLE III.

Detailed Results Regarding the Nasal Polyposis Group are Shown, According to Each Microsatellite Marker Used.

Patients	D5S207	D6S1002	D6S344	D11S480	D11S1253	D13S273	USAT24G1
1	NO	NO	NO	NO	NO	NO	NO
2	NO	NO	NO	NO	NO	NO	NO
3	NO	NO	NO	NO	NO	NO	<b>MSI</b>
4	NO	NO	NO	NO	NO	<b>LOH</b>	NO
5	NO	NO	NO	NO	NO	NO	NO
6	NO	NO	NO	NO	NO	NO	NO
7	NO	NO	NO	NO	<b>LOH</b>	NO	NO
8	NO	NO	NO	NO	NO	NO	NO
9	NO	NO	NO	NO	NO	NO	NO
10	NO	NO	NO	NO	NO	NO	NO
11	NO	NO	NO	NO	NO	NO	NO
12	NO	NO	NO	NO	NO	NO	NO

NO = negative for either microsatellite instability (MSI) or loss of heterozygosity (LOH).

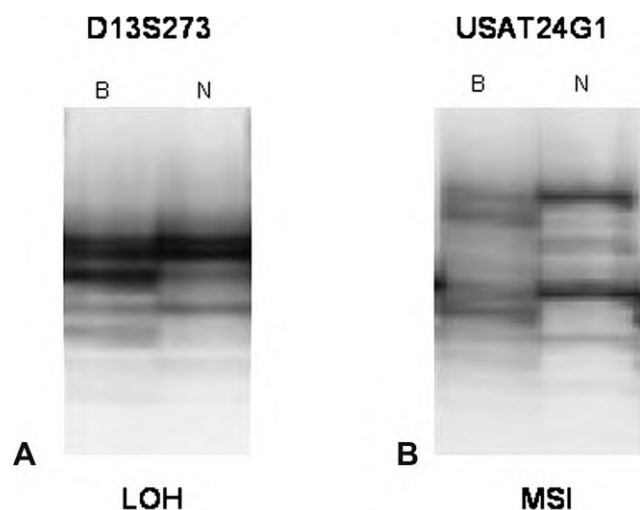


Fig. 1. Representative electrophoretic patterns of (A) loss of heterozygosity (LOH) in the marker D13S273 and (B) microsatellite DNA instability (MSI) in the marker USAT24G1. Results are shown for three subjects, one with LOH, one control (panel A) and one with MSI (panel B). B = blood-extracted DNA; N= nasal brush specimen-extracted DNA.

As previously mentioned, all patients in the study group were known asthmatics. For this reason, all subjects in the control group were also recruited according to bronchial asthma status. Although MSI has been previously detected in the lower airways of patients with bronchial asthma, this disease should not be considered responsible for positive MSI in the nose because none of the control subjects exhibited any microsatellite alterations. Moreover, in a previous study conducted by our lab to assess MSI in nasal cytologic samples of patients with allergic rhinitis, results were negative in all cases, although many subjects suffered from concomitant bronchial asthma.<sup>24</sup> In another previous study, the assessment of microsatellite genetic alterations in nasal and sputum samples of patients with COPD yielded positive results only in the sputum samples, suggesting that such alterations are exclusively specific for the target organ of each disease.<sup>28</sup> Nevertheless, it would be of interest to compare, in future studies, microsatellite alterations between the upper and lower airways in patients with coexisting nasal polyposis and bronchial asthma.

In the present study, the following 7 polymorphic microsatellite markers were used to assess the presence of MSI and/or LOH in nasal polyposis: D2S2113, D6S344, D6S1002, D11S1253, D11S480, USAT24G1, and D13S273. Marker D2S2113 targets a locus on chromosome 2 near the coding region for IL-1 $\beta$ , a proinflammatory cytokine that is upregulated in nasal polyposis.<sup>35</sup> D6S344 and D6S1002 correspond to chromosome 6 in a locus near coding regions for TNF- $\alpha$  and PAF-2. Both these molecules are implicated in the pathogenesis of chronic rhinosinusitis and nasal polyposis.<sup>35-37</sup> Markers D11S1253 and D11S480 correspond to loci on the long arm of chromosome 11 near the mammoglobin gene superfamily and pyrimidinergic receptor P2Y, G-protein coupled, 6, also known as P2RY6. These genes,

especially the mammoglobin gene that, interestingly, had initially been implicated in tumorigenesis have been shown to be upregulated in patients with nasal polyps.<sup>33,34</sup> Finally, markers USAT24G1 and D13S273 target loci on chromosome 13 near another one of the coding regions for TNF family (TNF SF13B), a group of cytokines that can cause apoptosis and, as mentioned, have been well-known to be implicated in the pathogenesis of nasal polyps.<sup>3,4,36</sup>

Previous studies have been conducted by our lab to reveal the presence of microsatellite genetic alterations in nasal diseases.<sup>24,28</sup> In the first study, as mentioned, the presence of MSI and/or LOH was assessed in nasal aspirates and induced sputum from patients with COPD. Results for nasal cytology were negative, supporting the theory that MSI, which was successfully detected in sputum samples of patients with COPD, is a specific finding for the lung epithelium, despite the fact that inflammation coexists in the nasal mucosa of these patients.<sup>28</sup> In the second study, microsatellite genetic alterations were investigated in nasal cytology of patients with allergic rhinitis, based on previous reports that MSI and/or LOH are detectable phenomena in sputum samples of patients with bronchial asthma, a disease very closely related to allergic rhinitis. The negative results for allergic rhinitis, nevertheless, were attributed to a previously stated scenario that microsatellite genetic alterations could be related to tissue remodeling of the lower airways of patients with bronchial asthma. Therefore, it was hypothesized that microsatellite genetic alterations would be absent in the upper airways of patients with allergic rhinitis where remodeling patterns are far less extensive.<sup>24,26</sup> If such a hypothesis is true, then it makes much sense to expect the presence of MSI and/or LOH in the upper airways of patients with nasal polyps, a disease with remodeling patterns and other histopathologic characteristics very similar to those seen in bronchial asthma.<sup>3,32</sup> The results of the present study, therefore, further strengthen this hypothesis.

In the present study MSI and/or LOH were only detected in 3 of 12 patients in the nasal polyps group and only in markers D11S1253, USAT24G1, and D13S273. The fact that microsatellite genetic alterations were detected in a small percentage of patients is perhaps related to the limited, total number of patients studied and microsatellite markers tested. In further studies currently underway by our lab, a larger quantity of microsatellite markers is utilized, thus targeting with more precision a larger number of chromosomal regions that are known or suspected to be related to the aetiopathogenesis of nasal polyposis. A larger number of subjects is also currently under exam with patients categorized according to different clinical types of polyposis, including those with and without coexistent bronchial asthma, subjects with Samter's disease, and atopic individuals. A group with patients suffering from chronic rhinosinusitis without nasal polyps has also been included in current experiments. In addition, polyp biopsy specimens are taken to compare potential microsatellite alterations to histopathology. The

assessment of MSI and/or LOH, either in general or for specific markers in patients from different groups will allow, perhaps, a better understanding of this phenomenon in nasal polyposis.

In addition to the presence or not of MSI in different clinical or histological types of nasal polyps, for the same or different microsatellite markers, other questions need also to be answered with future studies. Are there, for example, any diagnostic implications? Could different types of treatment affect the microsatellite DNA pattern? Is oxidative stress responsible for the appearance of MSI and/or LOH in nasal disease, and if yes, then why is MSI present only in some chronic inflammatory processes like nasal polyposis and not in others like allergic rhinitis? Finally, is it possible that during the course of chronic rhinosinusitis on a patient with an inherited susceptibility, the continuous effect of a mutagenic insult from a process such as oxidative stress in the nasal mucosa might lead to a vicious circle of MMR dysfunction, causing microsatellite instability that results in the dysfunction of other genes responsible, eventually, for the appearance of nasal polyps. Hopefully, future studies will provide more clues in these directions.

## CONCLUSION

This is the first time that MSI and/or LOH are reported in benign nasal disease. The presence of such alterations provides evidence that acquired somatic mutations might play a role in the pathogenesis of nasal polyps.

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