IFRD1 gene polymorphisms are associated with nasal polyposis in cystic fibrosis patients*

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Rhinology 53: 359-364, 2015

DOI:10.4193/Rhino14.229

***Received for publication:**

September 14, 2014

Accepted: May 8, 2015

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Abstract

Background: Nasal polyposis (NP) is an inflammatory disease of the upper nasal airways frequently present in CF patients. Interferon-Related Developmental Regulator 1 (IFRD1) gene was reported as a possible modifier of CF lung disease severity. Three IFRD1 SNPs were analyzed to investigate a possible effect on the development of NP in CF patients.

Methods and Patients: The DNA of 143 patients with CF (40 with and 103 without NP) was purified from peripheral blood samples. IFRD1 SNPs (rs7817, rs3807213, rs6968084) were genotyped by restriction enzyme analysis.

Results: The T allele of the common polymorphism rs7817 and the rs7817-rs3807213 haplotype were associated with NP (p=0.002 and 0.004 respectively).

Conclusions: These results showed the association of the IFRD1-rs7817 polymorphism with NP in CF patients.

Key words: cystic fibrosis, nasal polyposis, IFRD1, modifier genes

Introduction

Cystic fibrosis (CF, OMIM#219700) is a severe autosomal recessive disease caused by mutations in the CF trans-membrane conductance regulator (CFTR) gene (7q31-q32). The CFTR gene encodes a protein expressed in many epithelial cells where the CFTR protein works mainly as a cAMP-regulated chloride channel. So far, almost 2000 mutations have been identified, but the functional relevance of only a minority of them is known [(1)]. CFTR mutation frequency varies from population to population. F508del mutation accounts for about two-thirds of mutated alleles in northern European and North American populations. Worldwide, no other single mutation accounts for more than approximately 5% of CFTR mutations [(2)].

CF most commonly manifests with chronic obstructive lung disease, bacterial infections of the airways and sinuses, fat maldigestion due to pancreatic exonic insufficiency, male infertility due to obstructive azoospermia and elevated sweat chloride concentration [(3)]. Other clinical manifestations or complications, variably present in CF patients, include nasal polyposis, meconium ileus, distal intestinal obstruction syndrome, CF liver disease and CF related diabetes. Studies on genotype-phenotype correlation are still inconclusive, with the notable exception of pancreatic status, while other clinical manifestations, in particular pulmonary disease, appear to be highly variable [(4)]. CF is characterized by a broad range of clinical variability in the severity and rate of disease progression of the involved organs, even among patients carrying identical CFTR mutations [(5)] or between siblings, as first demonstrated in CF patients homozygous for the F508del mutation [(6,7)]. The phenotype variability seems to be due to non-CFTR genetic variants, acting as modifier genes, and/or environmental influences that contribute to the heterogeneity of lung-disease severity. These modifier genes are likely to be involved in host defense, inflammation, epithelial repair, mucin production and airway responsiveness [(8)]. Several studies have focused the attention on immune and/or inflammatory genes as possible candidates [(9)].

Nasal polyposis (NP) is an inflammatory disease of the upper nasal airways with a variable clinical course. Typically, NP presents with edematous semi-translucent grape-like growths originating in the ostieomeatal complex [10]. The polyps extend into the nasal cavity resulting in nasal blockage and restricted airflow to the olfactory region. NP affects 1-4% of the general population worldwide [11], whereas the prevalence has been estimated to be 6-48% in patients with CF [12]. Little is known about the pathophysiological bases accounting for the development of NP in CF and its correlation with CFTR mutations [13,14]. Many factors seem to contribute to the etiopathogenesis of this multi-component condition, including chronic inflammation [11,15].
Few studies reported the role of the Interferon-Related Developmental Regulator 1 (IFRD1, OMIM#603502) gene (7q22-q31) as a possible modifier of CF lung disease severity though the regulation of the neutrophil effector function [16-18]. IFRD1 mediates the transcriptional activity of NFκB p65 in neutrophils becoming a key player in the inflammatory response. The interaction between airway epithelial and neutrophils [19] contributes to the generation and release of a series of pro-inflammatory cytokines that sustain an inflammatory reaction which in turn may trigger polyp formation [20].

Three Single Nucleotide Polymorphisms (SNPs) (rs6968084, rs3807213, rs7817) have been described in association with more severe lung phenotypes in CF patients [16]. These SNPs contain predicted binding sites for microRNAs as well as predicted splice enhancer sites, and could thereby modify this flogistic mechanism [18]. In this study, these three SNPs are analyzed to investigate a possible association with the development of NP in patients with CF.

Materials and methods

Subjects

Caucasian patients carrying class I and II CFTR mutation [5] were enrolled by the Veneto Regional Cystic Fibrosis Centre of Verona, Italy. Exclusion criteria were immunodeficiency, congenital mucociliary complaints, noninvasive fungal balls, invasive fungal disease, and systemic vasculitic or granulomatous diseases. Forty-two patients were F508del homozygotes, 62 were F508del compound heterozygotes and 39 compound or homozygotes for mutations other than F508del (R1162X, W1282X, N1303K, 2183delAA, G542X, 1717-1G>A). The average age was 34.5 yrs ± 7.6 in 71 men and 33.8 yrs ± 8.1 in 72 women (Table 1). Forty patients had nasal polyps diagnosed according to the criteria of the European Position Paper on Rhinosinusitis and Nasal Polyps [20]. Patients had lung function (Forced Expiratory Volume in 1 second or FEV1% predicted) measured by spirometry according to the criteria of the American Thoracic Society [21]. The latest available measurement was considered. Presence of allergy and type 2 diabetes were collected from clinical records. Fifty blood-donor healthy subjects were randomly enrolled to act as controls. The average age was 40.5 yrs ± 8.7 in 29 men and 33.2 yrs ± 11.4 in 21 women.

DNA analysis

Genomic DNA was extracted from peripheral blood samples by a salting out standard method [22].
Three IFRD1 SNPs (rs7817, rs3807213, rs6968084) were genotyped by PCR and restriction enzyme analysis. Primer pairs were specifically designed to amplify regions containing these polymorphisms by the use of specific software (Primer 3, http://frodo.wi.mit.edu/primer3/, and Oligo): rs6968084: F 5'ATACTCCAACTGGATCTTCTTT 3' – R 5'ATAACAAGATGGTGCACTCC 3'; rs3807213: F 5'TTGGAGGTATATAGAACTCAC 3' – R 5'TATAAAAAACCTAGGAGATG 3'; rs7817: F 5'TAGAAGCAAATGTCGAGATAAGA 3' – R 5'CAGAACTCACCAGTTTCAATAGT 3'.
Restriction analysis were performed with Fnu4HI (New England Biolabs, Beverley, MA, USA) for rs3807213, (New England Biolabs, Beverley, MA, USA) BsrGI for rs6968084, and with BstNI (New England Biolabs, Beverley, MA, USA) for rs7817. Positive and negative mutation controls were included in each round of PCR and restriction analysis.

Statistical analysis

Mean difference between groups was analysed with Student's t-test. When not normally distributed, variables were logarithmically transformed. Values were reported as mean ± standard deviation (SD). Odds Ratio (OR) was reported with a 95% interval of confidence (95% CI). Association between SNPs and NP was tested using logistic regression model. Fisher's exact test was used to examine the significance of the association in 2×2 and 2×3 contingency tables. Logistic regression (LR) was performed to analyze the association of the outcome (polyposis) with multilevel variables (genotypes). The regression model was fitted, at a genotype level, assuming the following genetic models: general model (genotype is considered as a categorical variable of 3 levels) and multiplicative (the risk of disease is multiplicative with the risk allele dosage). The Likelihood Ratio Test (LRT) was used to check the departure of the general model from a multiplicative model. The statistical analysis was managed with the software R version 2.15.2 (www.r-project.org). SNP association analysis and Hardy-Weinberg equilibrium (HWE) was performed with the R package 'genetics' version 1.3.8.1. Haplotype estimation, frequencies (through the expectation-maximization algorithm) and association analysis were performed with the R package 'haplo.stats' version 1.6.8. Low frequency haplotypes (estimated frequency < 0.01) were grouped in a common rare haplotype category and then tested for association. Bonferroni correction for multiple comparisons was applied when required. Associations were considered statistically significant with a p-value < 0.05.

Table 1. Clinical features of the CF patients.

	NP (40)	non-NP (103)	p-value
Sex (M/F)	17/23	54/49	0.26
Age (yrs±sd)	35.4 ± 6.2	33.9 ± 8.4	0.25
M	37.6 ± 6.0	35.4 ± 13.2	0.34
F	33.7 ± 6.0	33.5 ± 9.2	0.99
FEV1% predicted	61.8 ± 24.7	58.6 ± 27.4	0.39
M	52.0 ± 21.3	62.5± 24.9	0.1
F	69.4 ± 25.0	53.3 ± 28.2	0.02
Allergy (n)	11	17	0.2
Diabetes (n)	4	13	0.76
Infection duration (yrs±sd)	10.4 ± 8.4	7.7 ± 8.3	0.04

FEV1% predicted: Predicted Forced Expiratory Volume in 1 second; yrs: years; sd: standard deviation; NP: nasal polyposis; n: number of subjects.

Results

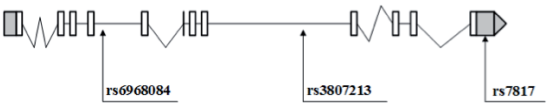
Clinical data

No age Lung function capacity (FEV1% predicted) difference was observed between NP and non-NP patients as a group (p=0.39, t-test), while a significant difference was present for females only (p=0.02) (Table 1). There was no difference in the prevalence of NP between males and females (p=0.26, Fisher's test) even when considering the CFTR mutations (data not shown). There was no statistical significant difference in the prevalence of NP among F508del homozygous patients (11/42), F508del heterozygous patients (20/62) and non-F508del CF patients (14/39) (p=0.66, Fisher's test) (data not shown). The age range of the subjects was 20-44 years old (25th percentile=29, median=35, 75th percentile=40).

SNP analysis

All CFTR patients were genotyped for three SNPs (rs6968084, rs3807213 and rs7817) of the IFRD1 gene. The genotype frequency of each of the 3 SNPs were in HWE. Information about the position and allele frequency of the three SNPs is reported in Figure 1.

No statistically significant association was found for rs6968084 and rs3807213. A significant association was found between rs7817 (genotype and allele) and NP in CF patients (Table 2). The association between polyposis and the rs7817 genotypes, observed though logistic regression, was tested to determine the proper genetic model. The general model was compared to the multiplicative model and the LRT did not detect a significant difference between the 2 models (p=0.30). Therefore, the general model was then used to describe the association of



SNP	bp	A (%)	a (%)	AA (%)	Aa (%)	aa (%)	HWE
rs6968084	112097121	C (93)	T (7)	86	14	NA	1
rs3807213	112105754	A (62)	C (38)	38	47	15	0.86
rs7817	112115658	C (50)	T (50)	24	53	23	0.61

Figure 1. Scheme of the IFRD1 gene, characteristics and location of the 3 single nucleotide polymorphisms in CFTR patients.

the SNPs with NP. A 4-fold higher probability of NP in patients with rs7817-CT genotype and a 7.3-fold higher in patients with rs7817-TT genotype was observed when compared to CC patients (Table 2).

A significant association was found between FEV1% predicted and rs7817. Patients with genotype rs7817-CT showed a higher FEV1% predicted value when compared to rs7817-CC patients (64.3±26.3 vs. 51.6±24.7 respectively, p=0.01, t-test), while the FEV1% predicted in patients with genotype rs7817-TT (54.4±27.8) was not statistically different from the rs7817-CC ones (p=0.37, t-test) (results not shown). To study the effect of the rs7817 SNP in a non-CFTR population, we performed an analysis between the CTFR patients with NP and controls, and between CFTR patients without NP and controls. A significant association was observed between CFTR patients with NP and controls (CT: OR=4.9, 95% CI 1.27 – 16.5, p=0.022; TT: OR=7.6, 95% CI 1.17 – 18.0, p=0.038) but not between CFTR patients without NP and controls. The controls were in HW equilibrium (p=0.25).

Haplotype analysis

We further investigated the association of NP with haplotypes formed by the 3 SNPs. The possible haplotypes, considering the patients analyzed, were computed and their frequency described in table 3. Only the haplotypes with a frequency in the study population higher than 0.01 were tested for the analysis. As baseline, the haplotype with the highest frequency was used (CCC, freq=0.37). The CAT haplotype showed a higher probability of NP (OR=2.63, 95% CI: 1.39-5.08, p=0.004, LR) when compared to CCC (Table 3). While the CCC haplotype occurs at a frequency of 0.24 in NP patients and 0.41 in non-NP patients, the CAT haplotype occurs at a frequency of 0.52 and 0.32 respectively. A further investigation was performed to check whether the probability is effectively given by the combination of the three alleles or there is a major effect of only one specific allele. A new set of haplotypes was estimated combining rs7817 with either rs3807213 or rs6968084. The logistic regression showed association only for haplotype rs3807213-rs7817 (p=0.004). The

Table 2. Analysis of the correlation between IFRD1 polymorphism rs7817 and nasal polyposis in CF patients.

		polyposis			
		Yes (%)	No (%)	OR (95% CI)	p-value
genotype	CC	3 (8)	30 (30)	Baseline	
	CT	22 (57)	51 (52)	4.05 (1.13-14.49)	0.026
	TT	14 (36)	18 (18)	7.38 (1.88-29.13)	0.003
allele	C	28 (33)	111 (56)	Baseline	
	T	50 (64)	87 (44)	2.27 (1.33-3.85)	0.0028

The analysis was performed on 143 subjects. The number of subjects and the percentage, in brackets, is reported. OR: odds ratio; 95% CI: 95% interval of confidence. The CC genotype and C allele are considered as baseline in the respective analysis.
rs7817 Risk Allele = T

analysis on the 2-SNP haplotypes showed similar results compared to the ones with 3 SNPs (Table 3). Using CC as baseline (freq=0.37, OR=0.12, 95% CI: 0.05-0.32, p=0.00001) only AT is statistically significant (OR=2.63, 95% CI:1.40-4.93, p=0.004).

Discussion

Little is known about the molecular mechanisms behind the development of nasal polyps. This study selected subjects with class I–II CFTR mutations as the phenotype is associated with a more severe disease compared to the other classes [4]. People with CF seem to have a higher probability to develop nasal polyposis. In particular, Jorissen et al. [23] reported a correlation between F508del homozygous and presence of polyps although this study did not observe such correlation.
An unregulated inflammatory response in the epithelium seems to play an important role in the pathogenesis of this multicomponent disease [24,25]. NP is characterized by increased inflammatory cell infiltration, cytokine production and abnormal tissue remodeling [26]. The role of IFRD1 as a modifier of CF lung disease has already been shown [16] but no association between IFRD1 and the development of NP in CF has previously been reported.

Polymorphisms and NP

We observed a linear increment in the probability of developing NP with the rs7817-T allele dosage (genotype CT OR=4.05; genotype TT OR=7.38, see Table 2). For the same SNP, we observed a significant increment of the FEV1% predicted value in subjects with rs7817-CT genotype when compared with rs7817-CC. This result can be compared with the study of Gu et al. [16] which reported that the heterozygote genotype rs7817-CT was associated with lower lung function than homozygote -CC and -TT. Our results, taken together with Gu et al.'s study seem to indicate a variability in the development of diseases either in the upper (NP) or lower (lung function) respiratory tract depending on the genotype. IFRD1 SNP interaction with respiratory epithelia and neutrophils might modulate lung diseases as well as NP development in CF [16]. However, this theory remains controversial as some studies reported no association between the inflammation pattern and the presence of NP [27,28].

Haplotype and NP

As reported in Table 3, the haplotypes carrying the rs7817-T

Table 3. Analysis of the association between IFRD1 haplotypes and nasal polyposis in CF patients.

rs6968084	rs3807213	rs7817	OR (95% CI)	p-value	freq	polyposis	
					Overall (143)	Yes (40)	No (103)
C	C	C	Baseline		0.37	0.24	0.41
C	A	C	1.39 (0.56-3.42)	0.47	0.11	0.1	0.11
T	A	T	2.53 (0.82-7.74)	0.1	0.06	0.08	0.06
C	A	T	2.63 (1.39-5.08)	0.004	0.4	0.52	0.35
	C	C	Baseline		0.37	0.24	0.41
	A	C	1.39 (0.56-3.42)	0.47	0.11	0.1	0.11
	A	T	2.63 (1.40-4.93)	0.004	0.47	0.61	0.41

The analysis was performed on 143 subjects. OR: odds ratio; 95% CI: 95% interval of confidence; freq: haplotype frequency in the study population, overall and divided into subjects with (Yes) and without (No) polyposis. The haplotype CCC and CC are considered as baseline in the respective analysis.

allele, showed NP risk similar to what observed for rs7817-T allele alone. This similarity could indicate that the risk effect is essentially associated with the rs7817 polymorphism. We questioned whether there was an influence of either or both the other SNP alleles. A Linkage Disequilibrium (LD) test highlighted a strong LD between rs3807213 and rs7817. In fact, we noticed that filtering by the rs7817-TT genotype, all the subjects had all rs3807213-AA genotypes.

Possible IFRD1 role in CF-NP

Neutrophils express IFRD1 protein. Its interaction with histone deacetylase (HDAC) enzymes modulates cell differentiation and oxidative stress mediating the transcriptional activity of NF-κB p65 [18]. Hector et al. [29] observed an up-regulation of IFRD1 expression in human CF neutrophils and found it linked to reactive oxygen species (ROS) production. Blanchard et al. [17] reported similar results as well as decreased IFRD1 protein in CF airways epithelial cells implying that IFRD1 expression and functionality could regulate CF airway inflammation. These results seem to highlight a different regulation of IFRD1 gene expression and protein transcription in the two different tissues. The change from a cytosine to a tymine in the rs7817 polymorphism occurs in the 3' UTR region of the IFRD1 gene [30]. The rs7817 SNP contains a target site for microRNA-577 (miR-577), expressed mainly in neutrophils, and a predicted site for splice enhancement [16,18]. Presently, there is no evidence whether this SNP could alter gene expression or whether the microRNA could alter the protein level. Nevertheless, two studies reported an up-regulation of miR-577 in lung cancer cells [31] and in esophageal squamous cell carcinoma [32] and an inverse correlation to proteins involved in cell proliferation. The rs7817 SNP might affect the contribution of IFRD1 in the inflammatory response pathway leading to cell inability to face oxidative stress [18]. As reported by Dagli et al, [33] the level of ROS in polyp tissue was higher than in control tissue. Cell stress and an increment in epithelial damage could then facilitate the development of polyposis in the upper airway. Further investigation should be performed to test IFRD1 polymorphism association in subjects without CF but with NP, healthy subjects and parents of CF children.

Conclusion

This is the first study that analysed the association between rs6968084, rs3807213, rs7817 IFRD1 polymorphisms and NP in CF patients and healthy controls. Although the etiology needs to be further studied, this study provides more information on the importance of genetic factors. The study supports the hypothesis that IFRD1 is a modifier gene in CF and suggests that IFRD1 polymorphisms may play a role in the risk of developing NP in CF. The analysis of the association of IFRD1 haplotypes with rs7817 together with other polymorphisms might further explain the variability of the disease development.
The assessment of modifiers of CF phenotype is not routinely used to individualize clinical and therapeutic strategies. We envision that testing for the IFRD1 rs7817 polymorphism could identify CF patients with a higher probability to develop NP, and provide a pre-symptomatic evaluation. This information could modulate the frequency of assessments by specialists and improve prevention offering the opportunity to facilitate early treatment, preventative medicine, preemptive selection of efficacious drugs, and more accurate estimation of risk.

Authorship contribution

Study design: AB, ARLP, FB, MDB. Study conduct: AB, ARLP, FB. Data collection: ARLP, CC, FB, MDB, LX. Data analysis: AB and GM. Data interpretation: AB, GM, ARLP. Drafting manuscript: AB. Revising manuscript content: AB, ARLP, FB, CC, PFP, GM, CB. Approving final version of the manuscript: AB, ARLP, FB, CC, MDB, LX, PFP, GM, CB.

Conflicts of Interest

All authors declare no conflict of interest.

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