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ABSTRACT

Background and objective: Periodontitis in one of the most prevalent dental diseases. Despite numerous studies have investigated its aetiopathogenetic factors, very few works have focused on its genetic predisposition and most of them took into account only candidate genes. Therefore, we conducted a genome wide association study in an Italian isolated population aimed at uncovering genetic variants that predispose to this disorder.

Methods: Diagnosis of chronic periodontitis was made following the criteria of the American Academy of Periodontology. Patients with chronic periodontitis were grouped into different categories: slight, severe, localized and generalized. A control group composed by people without signs of periodontitis or gingivitis was defined. DNA was genotyped using 370k Illumina chips. Linear mixed model regression was used to test the association between each SNP (independent variable) and the periodontitis status (dependent variable), controlling for confounders sex, age and smoking. The genomic kinship matrix was also used as random effect.

Results: 4 SNPs on the gene EFCAB4B resulted significantly associated to localized periodontitis ($p<5x10^{-8}$), with the best hit on the rs242016 SNP ($p=1.5 \times 10^{-8}$).

Conclusions: We have identified a novel significant association between the EFCAB4B gene and localized periodontitis. These results open a new perspective in the understanding of genetic factors contributing to this common disorder.

INTRODUCTION

Periodontal disease consists in a progressively destructive change leading to loss of bone and periodontal ligament due to a chronic inflammation of the supporting tissues of the teeth. Clinically there are plaque and calculus and is characterized by pocket formation and/or recession of the gum. In the advanced stages, the tooth loses its support until to be lost.[1]

This pathologic condition affects almost half of the adult population after 30 years of age[2] and many epidemiological studies have evaluated how its prevalence is higher in male compared to female and increases with age.[3] The percentage of individuals with healthy periodontium (absence of inflammation and probing depths < 4 mm) decreases with increasing age and does not represent more than 10% of the adults.[4] In the Italian population prevalence of chronic periodontitis has been estimated around 60%, while between 10 % and 14% show either severe or advanced forms [5,6], in agreement with the prevalence in the global population.[7]

It is well known that periodontitis is a complex inflammation-based disease with a multifactorial etiology. The main cause of this inflammation is to be found in the pathogenic bacterial species that may influence the onset and progression of the disease in combination with risk factors as smoking, diabetes and stress, or a genetic predisposition.[8] Although the bacterial accumulation is the condition *sine qua non* for the insurgence of the inflammation[9], it was demonstrated that there is no correlation between the amount of plaque and the severity of periodontitis[10]. This suggests that the host response and its genetic contribution is a key factor in developing the periodontal disease. A population study in more than 10,000 Swedish twin pairs revealed that the genetic contribution to the risk of periodontal disease is 39% in women and 33% in men[11], and studies in adult twins have confirmed that the host susceptibility is influenced by genetic factors.[11,12,13]

A gene can be present in the genome in different forms defined alleles. Allelic modifications may produce normal or altered proteins resulting in any, minor or big change in their functions, or sometimes in suppression of function. A genetic polymorphism occurs when an allele is present in at least the 1% of population, and it is considered a usual variant of a gene in a population. The

most common type of genetic polymorphisms is the Single Nucleotide Polymorphisms (SNPs). The change in protein function that a polymorphism produces is normally small, but its effect can be enhanced by environmental exposures, i.e. diet, smoking, or infective factors, increasing the risk of developing a specific disease.

Most recently, genome-wide association approach was used to identify new genetic risk factors for periodontal disease. Different suggestive loci were identified[14,15,16,17], but only very few studies have shown genome-wide significant results associated to periodontal disease or to related phenotypes.[18,19,20] In Schaefer at al. association in German population between aggressive periodontitis and rs1537415 in the glycosyltransferase gene GLT6D1 was reported.[19] In another study genome-wide significant signal were detected analyzing periodontal complex traits derived from information on levels of periodontal pathogens and tissue inflammatory response (IL-1β).[18] Recently, Munz *et al*. identified new loci (SIGLEC5 and DEFA1A3) involved in innate and adaptive immunity response as susceptibility factor for aggressive and chronic periodontitis.[20]

However, to date these results on periodontitis were controversial and more studies are needed to clarify the genetic contribution on the different forms of periodontitis.

Therefore, in this study we perform a Genome Wide Association Study (GWAS) in a genetic isolate population in North Italy, aimed to identify new genes associated to different categories (slight, severe, localized, generalized) of chronic periodontal disease.

MATERIALS AND METHODS

Participants

Data of 826 individuals aged 18-89 years were collected between March 2008 to November 2008. thanks to the "Friuli Venezia Giulia Genetic Park" project, aimed at analyzing a series of villages located in the North-east of Italy (San Martino del Carso, n=232, Erto e Casso, n=378, Clauzetto, n=377, Illegio, n=340, Sauris, n= 412, and Val di Resia, n=1021). showing evidence of isolation due to geographical, historical, linguistic and/or cultural factors. A detailed description of these villages is reported in Esko et al. (2013)[21] and in Xue et al. (2017)[22]. Briefly, a strong signal for

genetic isolation and a high level of inner structure resulting in increased genetic similarity was reported. Moreover, these villages showed a divergence time from the closest general population of around 150 generations ago (~1000 years).

Participants gave written informed consent and the ethical committee of IRCCS Burlo Garofolo/ the Ethics Committee of the University of Trieste approved the study (Prot. CE/V - 78, 06/08/2007).

Periodontal status assessment

Medical family and individual history was firstly recorded. All the adults were subjected to an accurate oral examination in which periodontal examinations included measurements of periodontal Pocket Depth (PD), Plaque Index (PI), Bleeding Index (BoP), Clinical Attachment Loss (CAL) and Gingival Recession (GR) on all teeth using a periodontal probe PCP12 (Hu-Friedy, Chicago, IL, USA). Four sites on each tooth were assessed: mesial, buccal, distal and lingual. Moreover, the overall number of teeth present in the mouth was recorded. An additional x-ray examination (panoramic radiography) was collected. Wisdom teeth were excluded.

Diagnosis was made following the criteria of the American Academy of Periodontology[23]and subjects were then categorized as summarized in Table 1.

Sample Collection and phenotype definition

A common group of controls composed by people without signs of periodontitis or gingivitis was defined. Then, patients with diagnosis of chronic periodontitis were classified into different categories: “slight” (people which fell into group 3 or 4), “severe” (people which fell into group 5 or 6), “localized” (people which fell into group 3 or 5) and “generalized” (people which fell into groups 4 and 6). Finally, was defined a last group named “all” which include all people affected from chronic periodontitis (groups 3,4,5 and 6). A total number of 224 subjects were excluded, including patients with clinical signs of gingivitis (n=93), aggressive periodontitis (n=10), edentulous (n=93) and patients with incomplete data (n=28).

Genotyping and Imputation

For each sample, DNA was extracted from peripheral blood and genotyping was carried out using Illumina 370k high density SNP array (Illumina, Inc., San Diego, CA, USA). Genotype imputation was conducted after standard QC using SHAPEIT2 for the phasing step[24] and IMPUTE2 for the imputation[25] using the 1000 Genomes phase I v3 reference set[26].

Statistical Analysis

For statistical analysis, we treated the logistic trait (0= controls, 1= cases) as though it was quantitative which corresponds to the Armitrage Trend Test. We thus used a linear mixed model regression where the periodontitis status was the dependent variant while the SNP dosages were the independent variable. Sex, age and smoking status were included in the model as covariates. The genomic kinship matrix was used as random effect in order to take into account the non-independence of the samples since they are coming from an isolated population. Genomic kinship was estimated using the ibs function from the GenABEL R package. Linear regression was conducted using MixABEL and the GRAMMAR+ method.[27] We considered significant all SNPs that exhibited a p-value less than $5x10^{-8}$. Odds ratios was assessed using standard logistic regression since no method can estimate odds ratios when inbreeding is present, while standard errors for confidence intervals were derived using the corrected test statistic and the beta from the logistic regression.

RESULTS

Main features of participants are shown in Table 2. The mean age of the study sample was 51.39±16.22 (range 18-89 years). 44% (n=363) of the participants were males and 56% (n=463) were females. 20% of subjects were current smokers.

In our sample, 160 subjects without signs of periodontitis or gingivitis formed the control group, while 442 resulted affected by chronic periodontitis. Within the group of patients with chronic periodontitis were then distinguished: 273 individuals with slight periodontitis, 169 with severe periodontitis; 207 with localized periodontitis, 235 with generalized periodontitis.

Mean age differ significantly (p-value<0.05) between control group (36.87±14.04) and the group of subjects with chronic periodontitis (54.63±12.41), as well as the groups of subjects with slight (51.59±12.12), severe (59.54±11.28), localized (51.83±12.56) and generalized (57.10±11.76) periodontitis.

Case-control genome wide association analysis on all these groups of periodontitis identified 4 SNPs significantly associated only to localized periodontitis. Figure 1 shows the Manhattan plot of the results on localized periodontitis across the genome. Results and detailed information for the significant SNPs are reported in Table 3. The best hit was on rs242016 (p-value=$1.5x10^{-8}$, OR=3.7). Moreover, also rs242014 (p-value=$1.6x10^{-8}$, OR=3.7), rs10491972 (p-value=$1.7x10^{-8}$, OR=3.7 and rs242002 (p-value=$2.7x10^{-8}$, OR=3.6) reached statistical significance. All the identified SNPs fall inside the EF-hand calcium binding domain 4B (EFCAB4B) gene, localized on chromosome 12.

Figure 2 shows the regional association plot for the top hit of GWAS results.

No significant associations were found for the other sub-classifications of periodontitis (all, severe, slight, generalized).

The possible functional role of the identified SNPs was explored using Haploreg v4 [28], showing that rs242002 is located inside enhancers regions active in numerous tissues, suggesting an involvement in affecting gene expression (http://archive.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs242002).

DISCUSSION

This study investigated the association among different sub-types of chronic periodontal disease and SNPs thanks to a Genome Wide Association Study. The advantage to be run in geographically

isolated towns is that these populations are consequentially also genetically isolated; thus, they have a restricted environmental, phenotypic and genetic heterogeneity, and this help to identify the impact of genetic variants on multifactorial diseases, such as the periodontal disease. In the present study, association among SNPs and chronic periodontitis (distinguished in slight, severe, localized and generalized periodontitis) was performed, founding for the first time an association between SNPs into the EFCAB4B gene and localized chronic periodontitis.

The function of SNPs we identified is still unknown. rs242016 is a synonymous SNP, while rs242014, rs10491972, rs242002 are intron variant. Ad showed from Haploreg v4, a possible involvement of rs242002 in gene expression could be hypothesized.

Little is known also about EFCAB4B. This gene, localized on chromosome 12, codes for a protein involved in Ca++ regulation during inflammation[29]. EFCAB4B is a Ca++ binding protein highly expressed in T cells. It is a component of a ternary complex of proteins, which are essential elements in store-operated Ca++ entry through Ca++ release-activated Ca++ (CRAC) channels in immune cells[30]. The Ca++ influx across these channels is important for the activation, proliferation and cytokine production of these cells[31].

Moreover, EFCAB4B is also largely expressed in minor salivary glands, as reported in GTEX portal[32] (Supplementary figure S1). Considering the saliva itself produces proteins with immune activator/modulator properties (reviewed in Fabian TK et al 2012) [33], further studies will be needed to investigate its link with this protein.

The link between the expression of the EFCAB4B protein in these tissues and periodontitis should be investigated in further studies to find out if its presence can be useful to monitor the onset and progression of the disease.

Other GWAS studies found association between SNPs in EFCAB4B gene and other pathological inflammatory conditions such as in non-alcoholic fatty liver disease (NAFLD)[34].

Moreover, it has already been investigated if certain polymorphisms of proteins involved in the inflammatory response may increase or decrease a person's risk for periodontitis. Many are the

candidate gene studies that investigated alteration of genes encoding for inflammatory mediators that can be periodontitis-associated, such as interleukin-4, interleukin-6, tumor necrosis factor, etc.[35,36,37,38]. I.e. it was found that the polymorphism of the IL-1 gene cluster is associated with severity of periodontitis in non-smokers, and moreover it distinguished patients affected by severe periodontitis from those by mild disease[39]. Periodontal disease is also itself a risk factor for other inflammatory diseases. Chronic inflammation of the periodontal tissues continuously released into the circulation blood type inflammatory mediators important in the progression of diseases such as coronary heart disease[40], diabetes[41], pre-term birth and low birth weight infants[42].

Results of our study suggested a role of EFCAB4B gene in localized periodontitis through the possible involvement of inflammatory mechanism. It can be hypothesized that this gene can be crucial in the early stages of the periodontal disease, when it is localized, influencing the beginning of inflammation process but not when the disease becomes to be massive.

To identify susceptible individuals in the early stages of the periodontitis, when the bone loss is limited, it might be useful to the clinician to perform better preventive therapy and periodontal treatment.

Strengths and limitation of the study: The strengths of this study include that a deep and detailed periodontal examination was conducted and that patients originated from geographically isolated towns. In fact, within the limitation small sample size (for a GWAS), the advantage to conduct a GWAS in isolated populations is that could allow to detect genetic variation that could require a much larger sample size. Moreover, this study provides a potential link between an inflammatory pathology, such as periodontal disease, and this still poorly studied protein. However, this study needs to be confirmed in other populations where both periodontal status and genetic data are available to enable replication.

In conclusion, even if it is not possible at this point to use the present results to diagnose the chronic periodontitis, the present investigation for the first time reported an association between SNPs in EFCAB4B gene and chronic localized periodontitis. It suggests a possible role of this gene in the

inflammatory response underlying pathogenesis of periodontal disease and contributes to cover the lack of knowledge in its complex pathogenetic pathway. Moreover, further investigations are required to better understand the role in periodontal disease of identified SNPs and of EFCAB4B gene, as well as its link with the complexity mechanism of the inflammatory response.

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FIGURES LEGENDS

Figure 1. Manhattan plot of the genome-wide association study results.

Each dot represents a SNP, with X-axis showing chromosomal positions and Y-axis showing log10 (p-value).

Figure 2. Regional association plot for the top hit of GWAS on periodontal disease

Plot made using the tool Locus Zoom. SNPs are plotted with their P values (as –log10 values) as a function of genomic position. Estimated recombination rate are plotted to reflect the local LD structure around the associated SNPs.

Supplementary Figure S1. EFCAB4B gene expression.

TABLES

CODE	CONDITION	CRITERIA
0	Healthy	Bop<25%
1	Gingivitis	Bop>25%
2	Aggressive PD	Tooth mobility and pockets of incisors and molars, age<35
3	Chronic localized PD slight o moderate	<30% with support loss <1/3 of the root
4	Chronic generalized PD slight o moderate	>30% with support loss <1/3 of the root
5	Chronic localized PD severe	<30% with support loss >1/3 of the root
6	Chronic generalized PD severe	>30% with support loss >1/3 of the root
7	Edentulous	
8	Not evaluable	No rx, incomplete data

Table 1. Criteria of the American Academy of Periodontology followed to classify the periodontal health status of subject involved in the study.

	All (n=826)	Males (n=363)	Females (n=463)
Age (yrs) (mean±sd)	51.3±16.2	51.8±15.7	51.0±16.6
Smoking Status (n)			
Yes	164	73	91
No	662	290	372
Periodontal status (n)			
Controls (code 0)	160	58	102
All (code 3+4+5+6)	442	214	228
Slight (code 3+4)	273	131	142
Severe (5+6)	169	83	86
Localized (3+5)	207	98	109
Generalized (code 4+6)	235	116	119
Others (code 1+2+7+8)	224	91	133

Table 2. Subject characteristics and Periodontal Disease Status.

"Others" refers to subjects with gingivitis, edentulous, not evaluable or aggressive periodontitis. All these subjects were excluded from the statistical analysis.

SNP	Chr	Position	Other allele	Coded allele	MAF	N	p	OR	CI
rs242016	12	3788260	G	A	0.21	367	$1.54x10^{-8}$	3.7	2.32-6.29
rs242014	12	3789135	C	T	0.20	367	$1.63x10^{-8}$	3.7	2.32-6.29
rs10491972	12	3789949	A	G	0.20	367	$1.70x10^{-8}$	3.7	2.32-6.29
rs242002	12	3807915	G	T	0.21	367	$2.73x10^{-8}$	3.6	2.28-6.13

Table 3. Results for the SNPs in EFCAB4B gene significantly associated to localized periodontitis. The first column reports the name of the SNP, the second the chromosome number, the third the position of the polymorphism expressed in base pairs, the fourth and the fifth the alternative and the coded allele respectively. The sixth column reports the frequency of the coded allele. N represents the total number of samples used in the analysis, p the p-value, OR the odds ratio and CI the 95% Confidence Interval values.

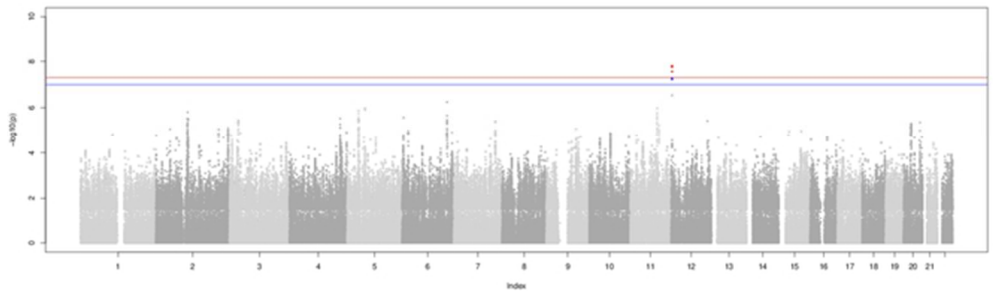

Figure 1. Manhattan plot of the genome-wide association study results.
Each dot represents a SNP, with X-axis showing chromosomal positions and Y-axis showing log10 (p-value).

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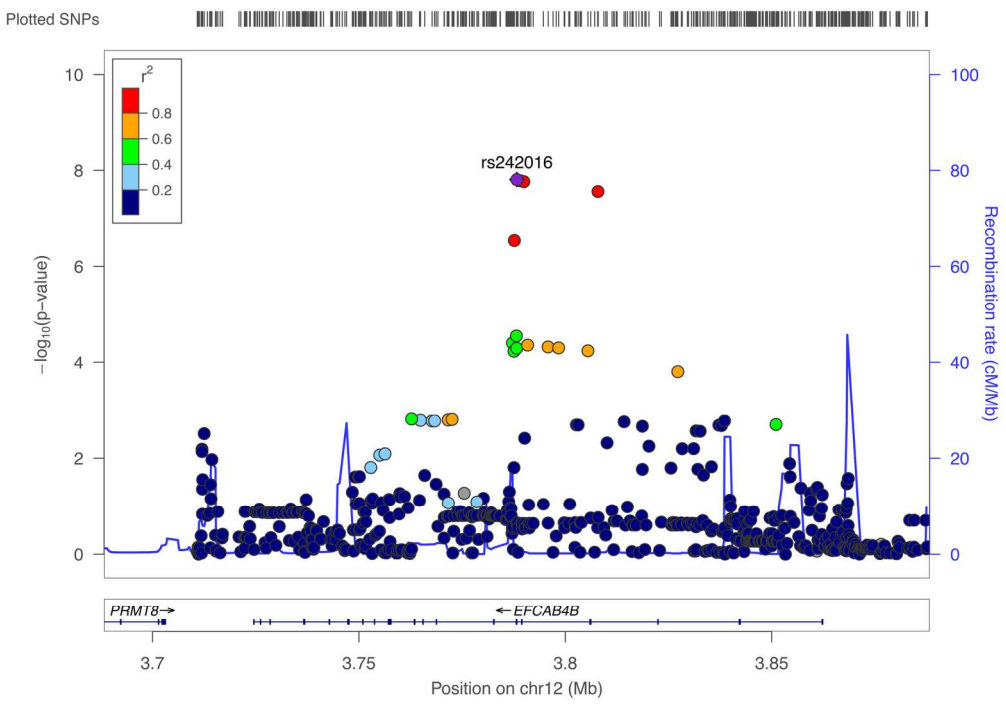
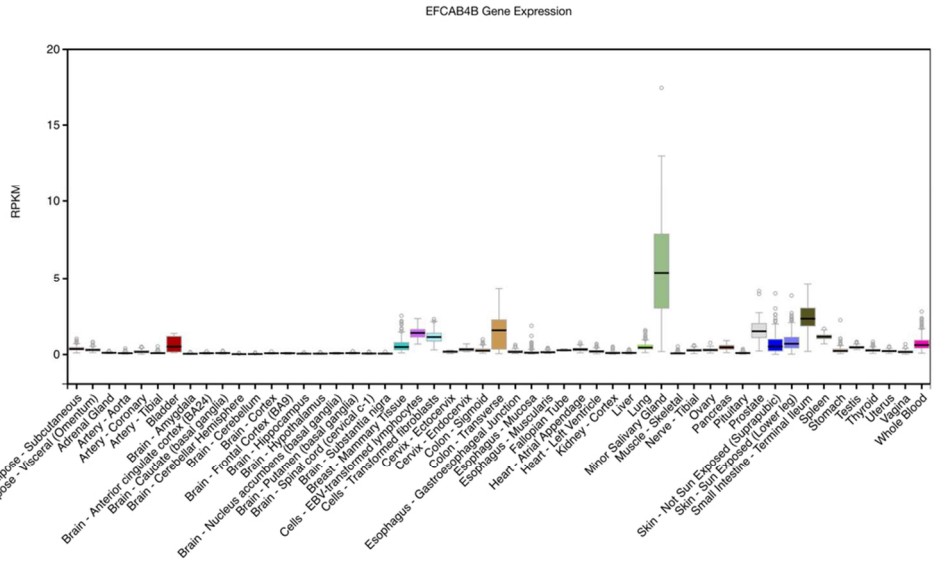


Figure 2. Regional association plot for the top hit of GWAS on periodontal disease
Plot made using the tool Locus Zoom. SNPs are plotted with their P values (as –log10 values) as a function of genomic position. Estimated recombination rate are plotted to reflect the local LD structure around the associated SNPs.

173x121mm (300 x 300 DPI)



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