CARD15/NOD2 polymorphisms in a Swedish population with oral Crohn's disease.

CARD15/NOD2 polymorphisms in Oral Crohn's disease

**Keywords**

Mucosal diseases, Orofacial granulomatosis, Arg704Trp, clinical characterisation.

Gita Gale¹, Elham Rekabdar², Åsa Torinsson Naluai², Robert Saalman³, Bengt Hasséus¹ and Mats Jontell¹

¹Dept. of Oral Medicine and Oral Pathology, Institute of Odontology, ²Genomics Core Facility and ³Dept. of Gastroenterology, Hepathology and Nutrition, Queen Silvia’s Child- and Youth Hospital, The Sahlgrenska Academy, University of Gothenburg, Göteborg, Sweden

**Corresponding author**

Mats Jontell  
Department of Oral Medicine and Pathology, Institute of Odontology, Sahlgrenska Academy, University of Gothenburg, PO Box 450, 405 30 Göteborg, Sweden.  
Tel: +46 31 786 31 33  
E-mail: jontell@odontologi.gu.se

**Date of Submission**
Abstract

**Background:** Polymorphism in the CARD15/NOD2 gene has been associated with Crohn’s disease. At present no similar studies have been carried out in patients with orofacial granulomatosis and oral Crohn’s disease.

**Objective:** To study CARD15/NOD2 polymorphisms in a Swedish patients with orofacial granulomatosis and oral Crohn’s disease.

**Methods:** Eleven patients with orofacial granulomatosis and 9 with oral Crohn’s disease were included. DNA were extracted from buccal swabs and examined for CARD15/NOD2 variants Arg702Trp, Gly908Arg and Leu1007fsinsC, all previously linked to Crohn’s disease.

**Results:** Four out of the 9 oral Crohn’s disease patients had the Arg702Trp variant, making a total allele frequency of 22%. None of the patients with orofacial granulomatosis carried this genetic variant. The Gly908Arg and Leu1007fsinsC variants were not found in any of the patients.

**Conclusion:** Oral Crohn’s disease may show a different genetic polymorphism compared to orofacial granulomatosis.
Introduction

Orofacial granulomatosis (OFG) is an oral disease where clinical characteristics are dominated by lip swelling, tag formation, cobblestone, angular cheilitis, vertical lip fissures and ulcerations of the oral mucosa. Histologically OFG is characterised by non-caseating granulomas with multi-nucleate giant cells, lymphangiectasia and perivascular lymphocytic infiltration (Wiesenfeld et al., 1985, Freysdottir et al., 2007, Patel et al., 2010). However, the absence of granuloma is not uncommon and inflammatory infiltrate may be the only finding to support the histopathological diagnosis (Hegarty et al., 2003, Tilakaratne et al., 2008). The clinical and histopathological features of OFG may also be observed as part of systemic diseases as Crohn’s disease and sarcoidosis. It is still enigmatic whether OFG may exist as a single entity or if it is always linked to clinical or subclinical variants of these systemic diseases. The association with systemic disease is supported by previous studies where approximately 60% of patients with OFG with no symptoms from the remaining gastrointestinal tract (GI) showed inflammatory changes after an extended examination (Scully et al., 1982, Sanderson et al., 2005). The diagnostic terms oral Crohn’s disease and oral sarcoidosis have been suggested when both oral and systemic disorders are present and OFG should exclusively be used when only oral conditions are observed (Hegarty et al., 2003). Most likely, the lack of an ambiguous terminology stems from that the disease engages different GI compartments over time and with different intensity of the inflammatory reaction.

Both hypersensitivity reactions of type I and IV may contribute to the development of OFG. CD20 positive B-cells expressing IgE have recently been characterized in biopsies from OFG lesions (Freysdottir et al., 2007, Patel et al., 2010). The contribution of type I hypersensitivity is also supported by the capacity of food constituents, like benzoates and cinnamon, to induce contact urticarial reaction within 60 minutes after exposure (Fitzpatrick et al., 2011). OFG
patients have been reported to have an atopic constitution in 60% of the cases, which supports an involvement of immediate hypersensitivity in the pathogenesis (James et al., 1986). However, type I hypersensitivity cannot be the only explanation to the development of OFG. Characterisation of the cellular infiltrate cytokines, and chemokine receptors in OFG lesions has revealed a dominans of CD4 expressing cells and increased levels of INF-γ, IL-10 och RANTES which supports a Th1 immune response (Freysdottir et al., 2007). Exposure to different antigens at different time points may be one explanation to why both immediate and delayed hypersensitivity have a role in the pathogenesis in OFG. This is supported by our previous study on liver transplanted children who developed an OFG-like condition with lip swelling, mucosal tag formation and angular cheilitis associated with a Th1 response, but also developed an acute angioedema following exposure to food constituents (Saalman et al., 2010).

The impact of genetic factors to the development of OFG has been studied in parallel with environmental factors like exposure to different food constituents. Associations to different HLA-molecules have been reported in several studies. Using serological techniques, it has been shown that patients with OFG were positive for HLA-A2 and HLA-A11 alleles in 50% and 25% respectively (Gibson & Wray, 2000). In a similar study, alleles HLA-B16 and HLA-Cw3 were over expressed compared to regional population (Stosiek et al., 1987). However, a consistent HLA relationship between OFG and CD has not been shown which has been interpreted as OFG is genetically different from CD (Satsangi et al., 1994, Gibson & Wray, 2000). The two studies where HLA-associations to OFG have been studied (Stosiek et al., 1987, Gibson & Wray, 2000), comprise small patient materials and it is difficult to know to what extent specific HLA alleles contribute to the development of OFG.
Thus, available scientific studies do not point at a single factor that can explain the development OFG in all patients who have incurred this disease. Studies comprising large patient material stem primarily from Great Britain (Wiesenfeld et al., 1985, Campbell et al., 2011), Ireland (Fitzpatrick et al., 2011) and Northern Ireland (Armstrong et al., 1997) while small materials and case presentations come from other parts of the world. It is important to compare OFG populations from different geographic areas to be able to find out to what extent different factors contribute to the aetiology of OFG. Therefore the objective of this study was to compare results from OFG patients of Celtic descent with a Scandinavia population. Candidate genes that substantially enhance the risk of CD in several different populations, i.e. variants of the caspase-activated recruitment domain 15 (CARD15) were also studied in our patient material. Contrary to most other studies, this study had a prospective approach with an identical electronic form to gather clinical and genetic data (Jontell et al., 2005).

**Patients and methods**

The Department of Oral Medicine and Pathology, Sahlgrenska Academy, Gothenburg University and Department of Pediatrics, The Queen Silvia Children’s Hospital, Sahlgrenska University Hospital in Gothenburg have collaborated for the last 10 years on oral manifestations of inflammatory bowel diseases. Through this collaboration, the two Departments have established a national resource for patients with OFG. In this study, a total of 22 patients with OFG (n=13) and oral Crohn’s disease OCD (n=9) were included. Predetermined medical data were first registered in medical records and later on added to the electronic form to create a common database (Jontell et al., 2005). The electronic form was developed for registration of all data related to oral conditions including clinical photos.
The diagnosis of OFG was primarily based on clinical characteristics. All patients presented with a swollen lower or/and upper lip. At least one of the following characteristics was seen in addition to swollen lips; intraoral tags, often found in the retromolar region, linear mucosal ridges in the vestibulum, buccal “egg in the basket”– appearance, or gingival enlargement with an erythematous granular appearance. In a majority of patients, representative incisional biopsy was performed of intraoral lesions.

Crohn’s disease (CD) was diagnosed according to clinical, endoscopic, radiological and histological criteria, by gastroenterological expert. The CD endoscopic diagnosis was established if one of the following three conditions should be present: (i) Intestinal longitudinal ulcer or deformity induced by a longitudinal ulcer or cobblestone patter. (ii) Intestinal small aphthous ulcerations arranged in a longitudinal fashion for at least three months, plus noncaseating granuloma. (iii) Multiple small aphthous ulcerations in both the upper and lower digestive tract, not necessarily with longitudinal arrangement, for at least three months, plus noncaseating granulomas.

To perform genetic studies, sampling of buccal epithelial cells was done using Isohelix SK1 Buccal Swabs. The buccal swabs were sent to Eppendorf AG, Hamburg, Germany for DNA extraction according to standard techniques. PCR and genetic analysis were carried out at Genomic Core Facilities, Sahlgrenska Academy, Gothenburg University, Gothenburg. DNA was isolated from buccal swabs by using the DNA isolation kit (Agowa, Germany) according to manufacturer’s protocol. For DNA amplifications, 384 wells reaction plates (twin.tec PCR plate 384, Eppendorf AG, Hamburg, Germany) were set up using Biomek® FX (Beckman Coulter, Bromma, Sweden), an automated workstation. Amplification performed by Touch-Down-PCR (TD-PCR) was performed in GeneAmp® PCR System 9700 (Applied Biosystems) in 5 µl reactions containing 2.5 ul AmpliTaq Gold® 360 Master Mix (Life
Technology, Applied Biosystems), 0.4 ul of each primer (Forward and Reverse) (10µM) and 20 ng genomic DNA; 0.7 ul H₂O. The sequences of the PCR primers are listed in Table 1.

The PCR products were purified using magnetic beads (Ampure, Agencourt, USA) in the automated workstation Biomek® NX (Beckman Coulter, USA) and eluted in 15 µl H₂O. Sequence-PCR, using BigDye® Terminator v 3.1 Cycle Sequence Kit (Life technology, Applied Biosystems), was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) according to standard procedures in 10 µl reactions containing 6 µl template (TD-PCR product); 0,25 µl BDT v.3.1; 1.875 µl BDT Buffer (5X); and 1 µl Primer (1.6 µM) (Forward or Reverse) for each regions or exons.

The sequence-PCR products were purified using magnetic beads (CleanSeq, Agencourt) in the automated workstation Biomek® NX (Beckman Coulter) and eluted in High Dye Formamide. The sequence reaction products were loaded on a 3730 DNA Analyzer (Applied Biosystems) and the results were analysed using the software program Sequencing Analysis (v. 5.2, Applied Biosystems) and SeqScape (v.2.5, Applied Biosystems). Studies with available allele and/or genotype frequencies for CARD15/NOD2 variants Arg702Trp, Gly908Arg and Leu1007fsinsC, all linked to CD, were included.

Results

CARD15/NOD2 polymorphisms

CARD15/NOD2 polymorphisms were identified in four of the patients who participated in this study. All four patients were diagnosed with Crohn’s disease involving both the oral and gastrointestinal compartments (oral Crohn’s disease). These two patients carried a single copy of the Arg702Trp variant (4/9; 44%) while the remaining five patients with the same diagnosis had the wild-type NOD2/CARD15. In a previous study of 192 Swedish healthy blood donors, six (6/192; 3.1%) individuals carried a single copy of the Arg702Trp variant.
None of the 11 patients with OFG had any of the CARD15/NOD2 mutations.

### TABLE 1. Primers used for PCR amplification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer length (bp)</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>EX4-1F</td>
<td>20</td>
<td>CAGCCCATTTGTCTGGTTAGG</td>
</tr>
<tr>
<td>EX4-1R</td>
<td>21</td>
<td>GAACTTGAACCTGTCAAAAGCC</td>
</tr>
<tr>
<td>EX4-2F</td>
<td>18</td>
<td>GCTGTTGCGCTGATGGTG</td>
</tr>
<tr>
<td>EX4-2R</td>
<td>20</td>
<td>ACCTTGGAAGCTGAGTCTTG</td>
</tr>
<tr>
<td>EX4-3F</td>
<td>17</td>
<td>GGGTCCCCAAAGACCAC</td>
</tr>
<tr>
<td>EX4-3R</td>
<td>18</td>
<td>GTCTGGCACTCAGCCAGC</td>
</tr>
<tr>
<td>EX4-4F</td>
<td>18</td>
<td>GAAAGACAGCAGCGTGG</td>
</tr>
<tr>
<td>EX4-4R</td>
<td>20</td>
<td>CTCGGTGCTCCCACCTTAG</td>
</tr>
<tr>
<td>EX8-F</td>
<td>20</td>
<td>CTGCAGAGGGAGGAGGACTG</td>
</tr>
<tr>
<td>EX8-R</td>
<td>20</td>
<td>AAGACCTTCAGAAGTGCC</td>
</tr>
<tr>
<td>EX11-F</td>
<td>23</td>
<td>AACTCCTGACGTCTCTTTAACTG</td>
</tr>
<tr>
<td>EX11-R</td>
<td>18</td>
<td>CTGCCATTTCCTCTCTCCCC</td>
</tr>
</tbody>
</table>

**Discussion**

The most significant result of this study is the different mutation pattern in NOD2/CARD15 gene of orofacial granulomatosis (OFG) and oral Crohn’s disease (OCD). OFG is defined as a granulomatous disease confined to the oral cavity while patients with OCD have Crohn’s disease in addition to an oral component similar to what is seen in OFG patients. It has previously been questioned if OFG and OCD represent different disease entities or if they signify the same disease with just different involvement of the gastrointestinal tract (Sanderson et al., 2005, Savage et al., 2004). Raised serum IgA antibodies to *Saccharomyces cerevisiae* have been found in patients with intestinal involvement but not in OFG alone.
(Savage et al., 2004) pointing at different pathogenic mechanisms of the two conditions. Our genetic analysis suggests that OFG and OCD may have different genetic polymorphism, which may account for the different phenotypic characteristics.

Previous observations have pointed to a genetic linkage of Crohn’s disease (CD) to chromosome 16. The significant gene appears to be NOD2/CARD15 where variations in Arg702Trp (exon 4), Gly908Arg (exon 8) and 3020insC (exon 11) seem to play an important role. NOD2/CARD15 has the ability to activate nuclear factor κB (NF-κB) in the presence of microbes and mutations in this gene inhibits this activation. As microbes have been suggested to have a role in the pathogenesis of OFG and OCD (Ivanyi et al., 1993, Apaydin et al., 2004, Savage et al., 2004), our finding of an Arg702Trp mutation is of interest. Antibodies against mycobacterial stress protein have been found in patients with OCD (4/4) but only in 50% of the patients with OFG (3/6). This finding in addition to what has been reported by Savage et al., i.e. that serum IgA response to S. cerevisiae were raised only in in patients with intestinal involvement but not in OFG alone (Savage et al., 2004), supports that OFG and OCD may be separate disease entities. Our finding of a NOD2/CARD15 gene mutation in patients with OCD but not in OFG patients further nourishes this hypothesis.

Following the identification of CARD15/NOD2 gene polymorphisms associated with CD (Ogura et al., 2001, Hugot et al., 2001) the understanding of genetics of this disease has during recent years made considerable progress. An association to ileal disease (Weersma et al., 2009, Mendoza et al., 2003) and the inverse to colonic disease (Lesage et al., 2002, Ogura et al., 2001, Esters et al., 2004) are the most prevailing findings. Patients with OFG and OCD have not been subjected to similar studies regarding CARD15/NOD2 gene polymorphisms. However, three studies of the association between OFG and HLA have been published but do
not show a strong link between HLA and OFG (Gibson & Wray, 2000, Satsangi et al., 1994, Stosiek et al., 1987). Thus, previous studies do not provide strong evidence to confirm a genetic background of OFG and OCD. Although our study comprises a very small number of patients it is the first to support that OFG and OCD may be genetically different. The low number of CARD15/NOD2 mutations in Swedish CD twins and healthy controls (Halfvarson et al., 2005) and in other Northern European populations (Russell et al., 2008, Bairead et al., 2003, Helio et al., 2003) even indicates that OCD may have a different polymorphism than CD. In a Swedish CD twin study of CARD15/NOD2 polymorphism (Halfvarson et al., 2005), Arg702Trp variant was identified in 3 (heterozygotes) out 38 CD patients making a total allele frequency of 3.9%. In comparison, six healthy blood donors out of 192 carried a single copy of the Arg702Trp variant. Consequently, the total allele frequency was 1.6% in the healthy controls. In our study the total allele frequency in the OCD group was considerably higher and calculated to 22%. None of the 13 OFG patients carried this genetic variant. This finding supports that OCD has a genetic polymorphism different from OFG and CD in a Swedish population.

References


