CEACAM6 Gene Variants in Inflammatory Bowel Disease

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Abstract

Background: The carinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) acts as a receptor for adherent-invasive E. coli (AIEC) and its ileal expression is increased in patients with Crohn’s disease (CD). Given its contribution to the pathogenesis of CD, we aimed to investigate the role of genetic variants in the CEACAM6 region in patients with inflammatory bowel diseases (IBD).

Methodology: In this study, a total of 2,683 genomic DNA samples (including DNA from 858 CD patients, 475 patients with ulcerative colitis (UC), and 1,350 healthy, unrelated controls) was analyzed for eight CEACAM6 SNPs (rs10415946, rs1805223 = p.Pro42Pro, rs4803507, rs4803508, rs11548735 = p.Gly239Val, rs7246116 = p.His260His, rs2701, rs10416839). In addition, a detailed haplotype analysis and genotype-phenotype analysis were performed. Overall, our genotype analysis did not reveal any significant association of the investigated CEACAM6 SNPs and haplotypes with CD or UC susceptibility, although certain CEACAM6 SNPs modulated CEACAM6 expression in intestinal epithelial cell lines. Despite its function as receptor of AIEC in ileal CD, we found no association of the CEACAM6 SNPs with ileal or ileocolonic CD. Moreover, there was no evidence of epistasis between the analyzed CEACAM6 variants and the main CD-associated NOD2, IL23R and ATG16L1 variants.

Conclusions: This study represents the first detailed analysis of CEACAM6 variants in IBD patients. Despite its important role in bacterial attachment in ileal CD, we could not demonstrate a role for CEACAM6 variants in IBD susceptibility or regarding an ileal CD phenotype. Further functional studies are required to analyze if these gene variants modulate ileal bacterial attachment.

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Competing Interests: U. Lass is an employee of TIB MOLBIOL Syntheselabor GmbH, which is marketing FRET probes for genotyping. However, TIB MOLBIOL Syntheselabor GmbH had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The employment of U. Lass by TIB MOLBIOL Syntheselabor GmbH does not alter the authors’ adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD), characterized by an aberrant mucosal immune response to bacteria-derived antigens in the gut of genetically susceptible hosts [1,2]. Although the exact pathogenesis of IBD still remains unsolved, current evidence indicates that defective T-cell apoptosis [3] and autophagy [4,5,6,7] as well as an impairment of intestinal epithelial barrier function [8,9] play important roles. This hypothesis is strengthened by data from genetic association studies identifying CD susceptibility genes involved in innate immunity and bacterial recognition (NOD2/CARD15) [10,11], and from genomewide association studies (GWAS), which identified susceptibility genes involved in autophagy (ATG16L1, IRGM) [4,5] and the proinflammatory IL-23/Th17 pathway [12].

While a specific causative pathogen in IBD has not been found so far [13,14], investigations of the regulatory mechanisms operating at the mucosal level suggest that regulatory cells reactive to the
Commensal intestinal microflora might play a role in cross-reactive protection toward different bacterial antigens [15]. Moreover, there is raising evidence for a major role of certain bacteria such as adherent-invasive *E. coli* (AIEC) in ileal CD [16,17,18]. Interestingly, the carcinoembryonic antigen-related cell adhesion molecule 6 (*CEACAM6*) has recently shown to act as a receptor for AIEC, supporting ileal bacterial colonization as a major pathomechanism in CD [19]. The carcinoembryonic antigen (*CEA*) family consists of two subfamilies, the CEACAM subgroup and the pregnancy specific glycoprotein (PSG) subgroup [20,21]. CEACAM family members were found to be expressed in epithelial, endothelial, and hematopoietic cells, including T-lymphocytes, natural killer (NK) cells, dendritic cells (DC) and neutrophils. They may also be useful as biomarkers in cancer since they are often over-expressed in ovarian, endometrial, breast, lung, and colon carcinomas [21,22,23]. Depending on the tissue involved, CEACAMs are transmitting signals that result in a variety of effects including regulation of the cell cycle, tumor suppression, angiogenesis, lymphocyte activation and adhesion [22,23,24,25,26,27,28,29]. CEACAM1, CEACAM5, and CEACAM6 represent three of the CEACAM subfamily members expressed in intestinal epithelial cells. There is increased expression of CEACAM5 and CEACAM6 at the apical surface of the ileal epithelium in CD patients [19]. Moreover, ileal lesions in CD patients were found to be colonized by pathogenic AIEC [19], strengthening the hypothesis that an abnormal intestinal expression of CEACAM6 in CD patients is associated with an increased colonization of AIEC via type 1 pil expression inducing gut inflammation [18]. AIEC adhere to and invade intestinal epithelial cells [30] resulting in AIEC accumulation in macrophages leading to high amounts of TNF-α [31], thereby perpetuating intestinal inflammation.

Given the potential implication of dysfunctional CEACAM6 expression in the pathogenesis of IBD, we aimed to analyze the role of *CEACAM6* SNPs in IBD susceptibility. A total of eight single nucleotide polymorphisms (SNPs) were analyzed in a large German cohort of CD and UC patients. Five SNPs in the *CEACAM6* gene (rs10415946, rs4803507, rs4803508, rs2701, rs10416839) were selected from the data of the international HapMap project covering the *CEACAM6* gene plus 10 kbp flanking the centromeric and telomeric end of the gene, respectively. Additional selection criteria for the SNPs were a minor allele frequency of at least 5% and a r² of 1. The SNPs rs4803507 and rs4803508 are localized in intron 2, rs2701 is localized within exon 6 encoding the 3' untranslated region, while the SNPs rs10415946 and rs10416839 are within the 5' and the 3' flanking region, respectively. Additionally, the coding variants rs1805223=p.Pro42Pro (exon 2), rs11548735=p.Gly239Val and rs7246116=p.His260His (exon 4) were investigated for which allele frequencies are published und which display a minor allele frequency of at least 5% in the Caucasian population. The structure of the *CEACAM6* gene and the localization of the SNPs investigated in the presented study are shown in figure 1. Considering the abnormal expression of *CEACAM6* in the ileal epithelium of CD patients and its role as receptor for ileal AIEC [19], we also analyzed for a potential association with an ileal CD phenotype and investigated potential gene-gene interactions with the *NOD2* gene, which has been shown to be a strong predictor of ileal CD, as well as with other CD susceptibility genes such as *IL23R* and *ATG16L1*.

**Materials and Methods**

**Ethics statement**

The study was approved by the local Ethics committee of the Ludwig-Maximilians-University of Munich (Department of Medicine) and adhered to the ethical principles for medical research involving human subjects of the Helsinki Declaration. Prior to the study, we obtained written, informed consent from all patients included.

**Study population and characterization of disease phenotype**

The study population comprised 858 patients with CD, 475 patients with UC, and 1350 healthy, unrelated controls of Caucasian origin. The study was approved by the local Ethics committee of the Ludwig-Maximilians-University of Munich (Department of Medicine) and adhered to the ethical principles for medical research involving human subjects of the Helsinki Declaration. Prior to the study, we obtained written, informed consent from all patients included. The phenotypic assessment was performed blind to the results of the genotypic data and included demographic data and clinical parameters (behaviour and anatomic location of IBD, disease-related complications, previous surgery or immunsuppressive therapy) which were recorded by investigation of patient charts and a detailed questionnaire. The diagnosis of CD or UC was based on established international guidelines including endoscopic, radiological, and histopathological criteria [32,33]. Patients with CD were assessed according to the Montreal classification [33] based on age at diagnosis (A), location (L), and behaviour (B) of disease. In patients with UC, anatomic location was also assessed in accordance to the Montreal classification [33], using the criteria ulcerative proctitis (E1), left-sided UC (distal UC; E2), and extensive UC (panscolitis; E3). Patients with indeterminate colitis were excluded from the study. The demographic and phenotypic data of the IBD study population are summarized in Table 1.

**DNA extraction and genotyping of the *CEACAM6* variants**

Blood samples were taken from all participants of the study and genomic DNA was isolated from peripheral blood leukocytes using the DNA blood mini kit from Qiagen (Hilden, Germany).
according to the manufacturer’s guidelines. Eight CEACAM6 SNPs (rs10415946, rs1805223 = p.Pro42Pro, rs4803507, rs4803508, rs11548735 = p.Gly239Val, rs7246116 = p.His260His, rs2701, rs10416839) were genotyped by PCR and melting curve analysis using a pair of fluorescence resonance energy transfer (FRET) probes in a LightCycler® Instrument (Roche Diagnostics, Mannheim, Germany) as described in previous studies [34,35,36]. The total volume of the PCR was 5 μl containing 25 ng of genomic DNA, 1× Light Cycler 480 Genotyping Master Mix (Roche Diagnostics), 2.5 pmol of each primer and 0.75 pmol of each FRET probe (TIB MOLBIOL, Berlin, Germany). In the case of rs1805223, rs4803507 and rs4803508, the concentration of the forward primer, and in the case of rs10415946 and rs2701, the concentration of the reverse primer, were reduced to 1.25 pmol.

Two SNPs were analyzed in a multiplex reaction, the combinations were: rs10415946+rs4803507, rs1805223+rs4803507, rs11548735+rs7246116 and rs2701+rs10416839. For the combination rs11548735+rs7246116 only one primer pair was used. The PCR comprised an initial denaturation step (95°C for 10 min) and 45 cycles (95°C for 10 sec, 60°C for 10 sec, 72°C for 15 sec).

Details on the melting curve analysis and on the PCR used for sequencing were published in previous studies [34,35,36]. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced by a commercial sequencing company (Sequiserve, Vaterstetten, Germany). All sequences of primers and FRET probes and primer annealing temperatures used for genotyping and for sequence analysis are given in Tables 2 and 3.

### Table 1. Demographic and phenotypic disease characteristics of the study population.

|                         | Crohn’s disease | Ulcerative colitis | Controls |
|-------------------------|-----------------|-------------------|----------|
|                         | n = 858         | n = 475           | n = 1350 |
| Gender                  |                 |                   |          |
| Male (%)                | 45.3            | 47.9              | 62.6     |
| Female (%)              | 54.7            | 52.5              | 37.4     |
| **Age (yrs)**           |                 |                   |          |
| Mean ± SD               | 40.2 ± 13.2     | 42.4 ± 14.4       | 45.8 ± 10.7 |
| Range                   | 11–81           | 7–86              | 18–71    |
| **Body mass index**     |                 |                   |          |
| Mean ± SD               | 23.1 ± 4.2      | 23.9 ± 4.1        |          |
| Range                   | 13–40           | 15–41             |          |
| **Age at diagnosis (yrs)** |               |                   |          |
| Mean ± SD               | 27.7 ± 11.8     | 32.0 ± 13.3       |          |
| Range                   | 1–78            | 9–81              |          |
| **Disease duration (yrs)** |              |                   |          |
| Mean ± SD               | 11.9 ± 8.6      | 10.5 ± 7.7        |          |
| Range                   | 0–44            | 1–40              |          |
| **Positive family history of IBD (%)** | 16.0 | 16.1 |
| **Disease localization (Crohn’s disease)** | 764* |          |
| L1 (ileal)              | 113             |                   |          |
| L2 (colonic)            | 97              |                   |          |
| L3 (ileocolonic)        | 554             |                   |          |
| L4 (upper GI)**         | 88              |                   |          |
| **Disease behaviour (Crohn’s disease)** | 754* |          |
| B1 (non-stricturing, non-penetrating) | 187 |          |
| B2 (stricturing)        | 208             |                   |          |
| B3 (penetrating)        | 359             |                   |          |
| **Disease extent (Ulcerative colitis)** | 260* |          |
| E1 (proctitis)          | 24              |                   |          |
| E2 (left-sided UC)      | 96              |                   |          |
| E3 (pancolitis)         | 140             |                   |          |

Disease localization and disease behaviour for Crohn’s disease and the disease extent in ulcerative colitis are given according to the Montreal classification of inflammatory bowel diseases.

*Given is the number of patients for which the corresponding disease phenotype information was available.

**Additional upper GI involvement.

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Genotyping data of the three main CD-associated NOD2 variants p.Arg702Trp (rs2066844), p.Gly908Arg (rs2066845), and p.Leu1007fsX1008 (rs2066847) were available from previous studies [34,37]. Similarly, for epistasis analysis genotype data for the main CD-associated IL23R variants (rs1004819, rs7517847, rs10489629, rs2201841, rs11465804, rs11209026 (p.Arg381Gln), rs1343151, rs10889677, rs11209032, rs1495965) and ATG16L1 SNPs (rs13412102, rs12471449, rs11209032, rs1441090, rs2298556, rs221879, rs3792106, rs4663396) were available from previous studies [34–36].

RNA isolation and quantitative PCR
Total RNA was isolated from five intestinal epithelial cell (IEC) lines (DLD-1, HCT116, HT-29, SW480, T84) as indicated with the Qiagen RNeasy Kit and was reverse transcribed using Roche Transcriptor First Strand cDNA Synthesis Kit. Quantitative PCR was performed with SYBR Green PCR Master Mix from Roche on a LightCycler480 instrument. The following primers were used for amplification: CEACAM6 forward 5′-CACAACCTGGATCTCACACTTGCTC-3′; CEACAM6 reverse 5′-GAGGCGTGAGGATCCATTTCTC-3′; β-actin forward 5′-GGCCCGAGATCACATGTCT-3′; β-actin reverse 5′-CACTCCAGAGGACTTTTC-3′. β-actin expression was used to normalize gene expression in the respective samples.

### Statistical analyses
Each genetic marker was tested for Hardy-Weinberg equilibrium in the three subgroups of the study population. Fisher’s exact test was used for comparison between categorical variables, while Student’s t test was applied for quantitative variables. Single-marker allelic tests were performed with Pearson’s χ² test. All tests were two-tailed, considering p-values<0.05 as significant. Odds ratios were calculated for the minor allele at each SNP. For multiple comparisons, Bonferroni correction was applied where

## Table 2. Primer sequences used for genotyping of CEACAM6 variants.

| Polymorphism | Primer sequences | FRET probe sequences |
|--------------|------------------|---------------------|
| rs10415946 = p.Arg702Trp | F: AGCCCTGTTGATGGTATCCAC<br>A: AGTCTGGGGTTCCTCAA | TGGATTACCCCCAGAAGAFL<br>LC670-4GGTTGACAGATGCTTTGGGCTTAG |
| rs1805223 = p.Pro42Pro | F: CCACCTTGAATGAGTTGCC<br>A: CGATTTGGGACGCTTGGT | GAATCCAGCCGTTCCTGATAGFL<br>LC670-4GGGAAGGAGGATCTTTC |
| rs4803507 = p.Gly908Arg | F: GCCATGTTCCCTTCTTATGTA<br>A: TTTTTCTAAATGGAGTGAGGGT | GAATCCACACACTGCACTACC-FL<br>LC640-AGTTATGATTACAGAAAGACTACTCCAGC |
| rs4803508 = p.Leu1007fsX1008 | F: CCTGCTCCCCCTCTCGTGCT<br>A: TTTTTCTAAATGGAGTGAGGGT | CTGGTAAAAAGATCACTCCCFL<br>LC610-GGCGGCTTCAGTAGCTCCTG |
| rs11548735 = p.Gly239Val | F: TGGTGGAGCTTACGAGGGTTG<br>A: TATGGGCTGGCCATATAGG | CCAAGTGGCCACCACTCATG |
| rs724616 = p.His260His | F: TGGTGGAGCTTGAGGGTTG<br>A: TATGGGCTGGCCATATAGG | CCAAGTGGCCACCACTCATG |
| rs2701 | F: AAGATGCTCAAACAGACTCTCTCA<br>A: AAGTCCACACTCTGAAAGACC | CAGATAGATCTGACCCTGTAATAGFL<br>LC610-ACCCTTGAGTCTACCTGCTGTTGAATACT |
| rs10416839 | F: CTTTCAGCTATTATGTTGGCTACATT<br>A: AAAAAACAGCAGATTATAGTACAG | CCCAGTGGCCACCATGAGAGCATC |

Note: FL: Fluorescein, LC610: LightCycler-Red 610; LC640: LightCycler-Red 640. The polymorphic position within the sensor probe is underlined. A phosphate is linked to the 3′-end of the acceptor probe to prevent elongation by the DNA polymerase in the PCR.

## Table 3. Primer sequences used for the sequence analysis of the CEACAM6 variants.

| Polymorphism | Primer sequences |
|--------------|------------------|
| rs10415946 | TGCAGAAAGAACAATTCAGAATCTTA<br>CTTGGGTCTGTCAGCACC |
| rs1805223 = p.Pro42Pro | GGGTGAAGAGACCTGCTCAG<br>CGCCTTTGTACCAGCTGTAAC |
| rs4803507 | ACGTTGCTTCTAATTTGGCA<br>GAAAAGTTTGTCAGGAGTTTAGACC |
| rs4803508 | CCTGCTCCCCCTCTCGTGCT<br>ATGGGTGATGATGGGACTTC |
| rs11548735 = p.Gly239Val, rs724616 = p.His260His | TGGTGGAGCTTACGAGGGTTG<br>CTGGTAAAAAGATCACTCCCFL<br>LC640-4GTAGTCTGACCAGATGCTGTTGAATACT |
| rs2701 | AAGATGCTCAAACAGACTCTCTCA<br>AAGTCCACACTCTGAAAGACC |
| rs10416839 | CTTTCAGCTATTATGTTGGCTACATT<br>A: AAAAAACAGCAGATTATAGTACAG |

Note: FL: Fluorescein, LC610: LightCycler-Red 610; LC640: LightCycler-Red 640. The polymorphic position within the sensor probe is underlined. A phosphate is linked to the 3′-end of the acceptor probe to prevent elongation by the DNA polymerase in the PCR.
Table 4. Associations of *CEACAM6* gene markers in the case-control association studies.

| SNP        | Minor allele | Crohn's disease n = 858 MAF/HWE p value OR [95% CI] | Ulcerative colitis n = 475 MAF/HWE p value OR [95% CI] | Controls n = 1350 MAF/HWE | HapMap-CEU** n = 120 MAF |
|------------|--------------|-------------------------------------------------|-------------------------------------------------|-----------------|-----------------|
| rs10415946 | G            | 0.383/0.506 0.21 1.08 [0.96–1.23]               | 0.338/0.113 0.16 0.89 [0.76–1.04]               | 0.364/0.906 0.440 |
| rs1805223  | A            | 0.309/0.679 0.21 1.09 [0.95–1.24]               | 0.264/0.235 0.12 0.87 [0.74–1.03]               | 0.291/0.262 0.342 |
| rs4803507  | A            | 0.307/0.803 0.24 1.08 [0.96–1.24]               | 0.269/0.454 0.26 0.90 [0.76–1.06]               | 0.290/0.234 0.280 |
| rs4803508  | A            | 0.377/0.266 0.25 0.95 [0.84–1.08]               | 0.406/0.622 0.35 1.07 [0.92–1.25]               | 0.389/0.606 0.358 |
| rs11548735 | T            | 0.399/0.885 0.62 0.97 [0.86–1.10]               | 0.422/0.44 1.06 [0.92–1.24]                     | 0.407/0.612 0.422 |
| rs724616   | T            | 0.359/0.653 0.24 1.08 [0.95–1.23]               | 0.371/0.11 1.14 [0.97–1.32]                     | 0.341/0.545 0.292 |

Minor allele frequencies (MAF), p-value for deviation from Hardy-Weinberg equilibrium (HWE), allelic test P-values, and odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted for both the CD and UC case-control cohorts. Measurements for linkage disequilibrium (LD) are provided in Tables 11, 12 and 13.

*monomorphic SNP.

**The MAFs in the HapMap-CEU population (= Utah residents with Northern and Western European ancestry) are derived from the NCBI SNP database (available under http://www.ncbi.nlm.nih.gov/snp).

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Table 5. Haplotypes of *CEACAM6* SNPs in Crohn's disease (CD) case-control sample and omnibus p-values for association with CD susceptibility.

| Haplotype combination | omnibus p-value |
|-----------------------|-----------------|
| rs10415946-rs1805223  | 0.46            |
| rs1805223-rs4803507   | 0.40            |
| rs4803507-rs4803508   | 0.84            |
| rs4803508-rs11548735  | 0.63            |
| rs11548735-rs7246116  | 0.85            |
| rs7246116-rs2701      | 0.78            |
| rs2701-rs10416839     | 0.36            |
| rs10415946-rs1805223-rs4803507 | 0.40     |
| rs1805223-rs4803507-rs4803508 | 0.58   |
| rs4803507-rs4803508-rs11548735 | 0.74   |
| rs4803508-rs11548735-rs7246116 | 0.63   |
| rs11548735-rs7246116-rs2701 | 0.74   |
| rs7246116-rs2701-rs10416839 | 0.36   |
| rs10415946-rs1805223-rs4803507-rs11548735 | 0.70  |
| rs1805223-rs4803507-rs11548735-rs7246116 | 0.67  |
| rs4803507-rs4803508-rs11548735-rs7246116-rs2701 | 0.59 |
| rs4803508-rs11548735-rs7246116-rs2701-rs10416839 | 0.21 |
| rs10415946-rs1805223-rs4803507-rs11548735-rs7246116 | 0.70  |
| rs1805223-rs4803507-rs11548735-rs7246116-rs2701-rs10416839 | 0.66 |
| rs4803507-rs4803508-rs11548735-rs7246116-rs2701-rs10416839 | 0.39 |
| rs10415946-rs1805223-rs4803507-rs11548735-rs7246116-rs2701-rs10416839 | 0.39 |

Given are the omnibus p-values for the *CEACAM6* haplotype combinations regarding CD susceptibility.

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### Table 6. Haplotypes of CEACAM6 SNPs in ulcerative colitis (UC) case-control sample and omnibus p-values for association with UC susceptibility.

| Haplotype combination | omnibus p-value |
|-----------------------|-----------------|
| rs10415946-rs1805223  | 0.50            |
| rs1805223-rs4803507   | 0.60            |
| rs4803507-rs4803508   | 0.73            |
| rs4803508-rs11548735  | 0.50            |
| rs11548735-rs7246116  | 0.65            |
| rs7246116-rs2701      | 0.55            |
| rs2701-rs10416839     | 0.60            |
| rs10415946-rs1805223-rs4803507 | 0.50 |
| rs1805223-rs4803507-rs4803508 | 0.82 |
| rs4803507-rs4803508-rs11548735 | 0.58 |
| rs4803508-rs11548735-rs7246116 | 0.50 |
| rs11548735-rs7246116-rs2701 | 0.54 |
| rs7246116-rs2701-rs10416839 | 0.54 |
| rs10415946-rs1805223-rs4803507-rs11548735 | 0.87 |
| rs1805223-rs4803507-rs4803508-rs11548735 | 0.87 |
| rs4803507-rs4803508-rs11548735-rs7246116 | 0.76 |
| rs4803508-rs11548735-rs7246116-rs2701 | 0.55 |
| rs7246116-rs2701-rs10416839 | 0.39 |
| rs10415946-rs1805223-rs4803507-rs4803508-rs11548735-rs7246116 | 0.87 |
| rs1805223-rs4803507-rs4803508-rs11548735-rs7246116-rs2701-rs10416839 | 0.48 |
| rs10415946-rs1805223-rs4803507-rs4803508-rs11548735-rs7246116-rs2701 | 0.87 |
| rs1805223-rs4803507-rs4803508-rs11548735-rs7246116-rs2701-rs10416839 | 0.49 |
| rs10415946-rs1805223-rs4803507-rs4803508-rs11548735-rs7246116-rs2701-rs10416839 | 0.82 |

Given are the omnibus p-values for the CEACAM6 haplotype combinations regarding UC susceptibility.
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### Table 7. Associations of CEACAM6 gene markers with the anatomic location of Crohn’s disease (CD) according to the Montreal classification [33].

| Anatomic location | rs10415946 | rs1805223 = p.Pro42Pro | rs4803507 | rs4803508 | rs11548735 = p.Gly239Val | rs7246116 = p.His260His | rs2701 | rs10416839 |
|-------------------|------------|-------------------------|----------|----------|--------------------------|-------------------------|--------|------------|
| L1 (ileal) n = 113| 0.320      | 0.321                   | 0.559    | 0.876    | 0.961                    | *                       | 0.997  | 0.566      |
| L2 (colonic) n = 97 | 0.854     | 0.988                   | 0.970    | 0.611    | 0.751                    | *                       | 0.684  | 0.580      |
| L3 (ileocolonic) n = 554 | 0.451     | 0.759                   | 0.803    | 0.425    | 0.684                    | *                       | 0.691  | 0.114      |
| Any ileal involvement (L1+L3) n = 667 | 0.373 | 0.586                   | 0.689    | 0.620    | 0.771                    | *                       | 0.744  | 0.117      |

*P*-values are depicted for the CD case-control cohorts.
*There were no carriers of the minor allele of rs7146116 in the CD and control cohort.
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indicated. Interactions between different polymorphisms were tested using logistic regression in R using the number of minor alleles as predictor variable, therefore implementing an Armitage test of trend. Data were evaluated by using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) and R-2.4.1. (http://cran.r-project.org). For haplotype analysis, PLINK v 1.06 (http://pngu.mgh.harvard.edu/~purcell/plink/) was used running a sliding window approach with variation of the window size from 2 to 8 included markers and using the option “hap-logistic”. Linkage disequilibrium (LD) was also analyzed using PLINK.

Results

CEACAM6 variants are not associated with IBD susceptibility

The genotyping success rates were at least 99% for all eight SNPs tested and were comparable between the controls and the CD and UC patients groups. In all three subgroups (CD, UC, and controls), the allele frequencies of the CEACAM6 SNPs rs10415946, rs1805223 = p.Pro42Pro, rs4803507, rs4803508, rs11548735 = p.Gly239Val, rs7246116 = p.His260His, rs2701, rs10416839 were in accordance with the predicted Hardy-Weinberg equilibrium (Table 4). Overall, we observed no significant differences in the frequencies of the investigated CEACAM6 SNPs in CD and UC patients compared to healthy controls (Table 4) implicating no significant association of CEACAM6 variants and IBD susceptibility. Only two patients (both with UC) were minor allele carriers of the rare CEACAM6 SNP rs7246116 = p.His260His, therefore not allowing a comparative analysis of this SNP regarding CD susceptibility.

CEACAM6 haplotypes are not associated with CD and UC susceptibility

Considering recent evidence showing that certain CEACAM6 haplotypes modulate susceptibility to bacterial infections [38], we next performed a detailed haplotype analysis in our IBD cohort. However, as shown in Tables 5 and 6, we could not demonstrated significant associations of CEACAM6 haplotypes with CD and UC susceptibility.

The CEACAM6 variants are not associated with an ileal disease phenotype in CD patients

Since CEACAM6 has recently shown to act as a receptor for AIEC, thereby promoting bacterial colonization in ileal CD [19], we further investigated whether CEACAM6 SNPs are associated with ileal disease in CD patients. Based on a phenotype analysis according to the Montreal classification of IBD [33], the detailed phenotypic data available from a subcohort of 667 CD patients was analyzed for disease localization. None of the investigated CEACAM6 SNPs was associated with ileal or ileocolonic CD disease.

Table 8. Analysis for gene-gene interactions between CEACAM6 and NOD2 variants regarding susceptibility to Crohn’s disease (CD).

| CEACAM6 SNPs | NOD2 SNPs | rs10415946 | rs1805223 | rs4803507 | rs4803508 | rs11548735 | rs7246116 | rs2701 | rs10416839 |
|--------------|-----------|------------|-----------|-----------|-----------|------------|-----------|--------|------------|
| rs2066844    | p.Arg702Trp | 0.56       | 0.37      | 0.39      | 0.31      | 0.62       | *         | 0.65   | 0.77       |
| rs2066845    | p.Gly908Arg | 0.06       | 0.28      | 0.28      | 0.54      | 0.35       | *         | 0.43   | 0.58       |
| rs2066847    | p.Leu1007F1X1008 | 0.93       | 0.80      | 0.97      | 0.51      | 0.71       | *         | 0.56   | 0.82       |

p-values for epistasis analysis between CEACAM6 and NOD2 SNPs in the CD case-control sample.

*There were no carriers of the minor allele of rs7146116 in the CD and control cohort.

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Table 9. Analysis for gene-gene interaction with CEACAM6 and IL23R variants regarding susceptibility to Crohn’s disease (CD).

| CEACAM6 SNPs | IL23R SNPs | rs10415946 | rs1805223 | rs4803507 | rs4803508 | rs11548735 | rs7246116 | rs2701 | rs10416839 |
|--------------|------------|------------|-----------|-----------|-----------|------------|-----------|--------|------------|
| rs1004819    | 0.13       | 0.31       | 0.29      | 0.50      | 0.46      | *          | 0.52    | 0.95   |
| rs7517847    | 0.67       | 0.20       | 0.31      | 0.17      | 0.23      | *          | 0.27    | 0.81   |
| rs10489629   | 0.64       | 0.09       | 0.11      | 0.97      | 0.79      | *          | 0.71    | 0.98   |
| rs2201841    | 0.52       | 0.42       | 0.40      | 0.61      | 0.50      | *          | 0.46    | 0.63   |
| rs11465804   | 0.20       | 0.13       | 0.19      | 0.59      | 0.26      | *          | 0.32    | 0.76   |
| rs11209026   | 0.08       | 0.05       | 0.08      | 0.35      | 0.28      | *          | 0.35    | 0.91   |
| rs1343151    | 0.35       | **0.04**   | 0.06      | 0.83      | 0.57      | *          | 0.60    | 0.64   |
| rs10899677   | 0.55       | 0.48       | 0.44      | 0.77      | 0.66      | *          | 0.62    | 0.51   |
| rs11209032   | 0.43       | 0.52       | 0.53      | 0.87      | 0.99      | *          | 0.89    | 0.19   |
| rs1495965    | 0.47       | 0.66       | 0.58      | 0.73      | 0.61      | *          | 0.84    | 0.32   |

p-values for epistasis between CEACAM6 and IL23R SNPs in the CD case-control sample. After Bonferroni correction, the association highlighted in bold did not remain significant.

*There were no carriers of the minor allele of rs7146116 in the CD and control cohort.

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Table 10. Analysis for gene-gene interaction between CEACAM6 and ATG16L1 variants regarding susceptibility to Crohn’s disease (CD).

| CEACAM6 SNPs | ATG16L1 SNPs | rs10415946 | rs1805223 | rs4803507 | rs4803508 | rs11548735 | rs7246116 | rs2701 | rs10416839 |
|--------------|--------------|------------|-----------|-----------|-----------|------------|-----------|---------|------------|
| rs13412102   | 0.89         | 0.89       | 0.72      | 0.89      | 0.55      | *          | 0.55      | 0.79    |
| rs12471449   | 0.49         | 0.44       | 0.40      | 0.74      | 0.58      | *          | 0.56      | 0.04    |
| rs6431660    | 0.53         | 0.66       | 0.50      | 0.87      | 0.98      | *          | 0.93      | 0.66    |
| rs1441090    | 0.56         | 0.32       | 0.26      | 0.78      | 0.89      | *          | 0.95      | 0.27    |
| rs2289472    | 0.67         | 0.65       | 0.49      | 0.67      | 0.86      | *          | 0.83      | 0.57    |
| rs2241880    | 0.84         | 0.74       | 0.57      | 0.64      | 0.81      | *          | 0.83      | 0.58    |
| rs2241879    | 0.93         | 0.81       | 0.68      | 0.74      | 0.73      | *          | 0.72      | 0.56    |
| rs3792106    | 0.67         | 0.52       | 0.44      | 0.50      | 0.69      | *          | 0.73      | 0.83    |
| rs4663396    | 0.81         | 0.93       | 0.80      | 0.56      | 0.85      | *          | 0.90      | 0.15    |

p-values for epistasis between CEACAM6 and ATG16L1 SNPs in the CD case-control sample. After Bonferroni correction, the association highlighted in bold did not remain significant.

*There were no carriers of the minor allele of rs7146116 in the CD and control cohort.

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Figure 2. Analysis of CEACAM6 gene expression and CEACAM6 gene variants in intestinal epithelial cell (IEC) lines. (A) Total RNA isolated from IEC lines as indicated was reverse transcribed and was analyzed for CEACAM6 gene expression by quantitative PCR. T84 cells express CEACAM6 at the highest level followed by HT-29 cells, and intermediate CEACAM6 expression was found in SW480 and DLD-1 cells. CEACAM6 expression was close to the detection limit after 40 PCR cycles in HCT116 cells (note the logarithmic scale on the y-axis). CEACAM6 expression was normalized to b-actin expression in the respective cDNA samples. (B) Genomic DNA was isolated from IEC lines and 8 SNPs in CEACAM6 were analyzed as indicated by DNA sequencing. The respective alleles for these SNPs in each cell line are depicted in the table. This analysis revealed that T84, the cell line with the highest CEACAM6 expression, and HCT116 cells, the cell line with the lowest CEACAM6 expression, are the only IEC lines with unique genotypes for certain CEACAM6 SNPs (depicted in bold italic). DLD-1 and SW480 cells have identical genotypes for all SNPs analyzed and nearly identical CEACAM6 expression levels.

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(Table 7). However, we have to acknowledge that the sample size has limited power to detect weak disease associations. For example, based on 667 patients with a L1/L3 disease phenotype, a minor allele frequency of 0.40 and an OR of 1.1, the power is 28.88% to detect an effect on a significance level of 5% (Genetic Power Calculator, http://pngu.mgh.harvard.edu/~purcell/gpc/).

**Analysis for gene-gene interaction with CD-associated NOD2, IL23R and ATG16L1 variants**

Given the raising evidence for a key role of CEACAM6 in the complex interaction of the mucosal immune system and intestinal bacteria, we next analyzed for potential epistasis between CEACAM6 SNPs (rs10415946, rs1805223 = p.Pro42Pro, rs4803507, rs4803508, rs11548735 = p.Gly239Val, rs7246116 = p.His260His, rs2701, rs10416839) and the three main CD-associated NOD2/CARD15 variants p.Arg702Trp (rs2066844), p.Gly908Arg (rs2066845), and p.Leu1007HisX1008 (rs2066847) which have previously shown to be strongly associated with CD and ileal disease localization. However, there was no evidence for epistasis between the CEACAM6 SNPs and the three analyzed NOD2/CARD15 variants (Table 8).

Recently, we demonstrated an association of the IL23R SNP rs1004819 (TT homozygous carriers) with ileal CD [34]. Therefore, we also analyzed for potential gene-gene interaction rs1004819 (TT homozygous carriers) with ileal CD [34].

In addition, novel findings indicate a major role for ATG16L1 in Paneth cell development in the terminal ileum. Therefore, we also analyzed for potential epistasis between the CEACAM6 SNPs and the major CD-associated IL23R variants. However, we did not find evidence for epistasis between CEACAM6 and IL23R regarding CD susceptibility (Table 9).

In summary, our study represents the first detailed analysis of CEACAM6 SNPs regarding disease susceptibility and phenotypic consequences in IBD patients. Compared to previous GWAS, our study has a more complete coverage of the CEACAM6 gene region (see details in Table 14). Overall, we did not observe a significant influence of the investigated CEACAM6 SNPs on CD and UC susceptibility. Moreover, a detailed haplotype analysis did not reveal significant associations with IBD susceptibility. CEACAM6 has recently shown to act as a receptor for AIEC suggesting an important role in bacterial colonization of the ileal mucosa in CD patients [19]. However, none of the investigated CEACAM6 SNPs was associated with ileal or ileocolonic CD.

Interestingly, a recent study demonstrated that the defect in CEACAM family members in intestinal epithelial cells isolated from CD patients appears to be related to the aberrant nuclear localization of the transcription factor SOX9 [39] which regulates cell proliferation and is required for Paneth cell differentiation in SW400 and DLD-1 cells showed similar, intermediate expression (Fig. 2A).

**Table 12. Analysis for linkage disequilibrium between CEACAM6 SNPs in patients with ulcerative colitis.**

| rs10415946 | rs4803507 | rs4803508 | rs2701 | rs10416839 |
|------------|-----------|-----------|--------|------------|
| rs10415946 * | * | * | * | * |
| rs4803507 0.65/0.96 | * | * | * | * |
| rs4803508 0.12/0.58 | 0.09/0.59 | * | * | * |
| rs2701 0.12/0.53 | 0.10/0.57 | 0.80/0.95 * | * | * |
| rs10416839 <0.01/0.03 | <0.01/0.13 | 0.25/0.85 | 0.31/0.90 * | |

Values are given as r^2/D^-measurements. doi:10.1371 journal.pone.0019319.t012

**Table 13. Analysis for linkage disequilibrium between CEACAM6 SNPs in controls.**

| rs10415946 | rs4803507 | rs4803508 | rs2701 | rs10416839 |
|------------|-----------|-----------|--------|------------|
| rs10415946 * | * | * | * | * |
| rs4803507 0.68/0.98 | * | * | * | * |
| rs4803508 0.15/0.63 | 0.09/0.60 * | * | * | * |
| rs2701 0.14/0.60 | 0.09/0.57 | 0.81/0.94 * | * | * |
| rs10416839 0.01/0.12 | <0.01/0.06 | 0.23/0.84 | 0.29/0.90 * | * |

Values are given as r^2/D^-measurements. doi:10.1371 journal.pone.0019319.t013

**Discussion**

In summary, our study represents the first detailed analysis of CEACAM6 SNPs regarding disease susceptibility and phenotypic consequences in IBD patients.
the intestinal epithelium [40,41]. However, ileal CD is characterized by a specific decrease in Paneth cell alpha-defensins and defective Paneth cell-mediated host defense [42] which has been linked to the NOD2 genotype [43], although this finding is opposed by the results of a recent study [44], and additional modifiers of Paneth cell function such as XBP1 are involved [45]. Therefore, one might speculate whether the role of CEACAM6 in ileal bacterial colonization is regulated via SOX9 expression implicating defective Paneth cell function in patients with small bowel CD. Given the association of defense secretion with the NOD2 genotype [43] and the findings of numerous previous studies including studies from our IBD center demonstrating ileal disease localization in CD patients with NOD2 mutations [37,46,47], we also tested for potential gene-gene interaction of CEACAM6 and NOD2. However, we found no evidence for epistasis between these two genes regarding CD susceptibility. Further functional studies analyzing the complex interaction of intestinal CEACAM6 expression and bacterial adherence in the gut particularly of CD patients carrying CEACAM6 variants will therefore be required. Given the important role of ATG16L1 in Paneth cell development of the terminal ileum and the role of IL23R in the development of proinflammatory Th17 cells, we also analyzed for epistasis of these two genes with CEACAM6 but were unable to find evidence for significant epistasis of these genes regarding CD susceptibility.

Interestingly, a recent study indicated that CEACAM6 and a regulatory element near the 3’ end of CEACAM3 are associated with disease severity in patients with cystic fibrosis [48]. However, a previous study in IBD patients suggested that heterozygous carriers of the AF308 mutation in the CFTR gene, the main susceptibility gene for patients with cystic fibrosis, might exert a protective effect in CD [49], suggesting opposing effects of genetic risk loci for cystic fibrosis and IBD.

In the meta-analysis of Barrett et al. [50], a SNP (rs4807569) within the chromosomal region 19q13, in which the CEACAM6 gene is located, was weakly associated with CD, but this association could not be confirmed in a replication cohort. In the recent meta-analysis of Franke et al. [51], two SNPs (rs736289 and rs281376) within this region were strongly associated with CD. However, the distance between these SNPs and the CEACAM6 gene is 9 and 7 megabases, respectively, and thus, the disease causing variant within this region remains to be identified.

CEACAM6 is also a major target gene for Smad3-mediated TGF-β signaling [52]. Since Smad3 differentially regulates the induction of regulatory and inflammatory Th17 cell differentiation [53], which are key players in the IBD pathogenesis [54], further investigations analyzing Th17 cell differentiation in IBD patients carrying CEACAM6 variants might also be of high interest. Moreover, very recent evidence from studies in mice demonstrated that colonization of the small intestine with a single commensal microbe, segmented filamentous bacteria (SFB), is sufficient to induce Th17 cells in the lamina propria [55]. These SFB adhere tightly to the surface of epithelial cells in the terminal ileum of mice with Th17 cells but are absent from mice that have few Th17 cells [55]. Further studies will have to characterize if SFB adherence is mediated (similar to AIEC adherence) by CEACAM family members.

In summary, we performed the first systemic analysis of CEACAM6 gene variants in IBD patients. Despite the great importance of CEACAM6 as receptor for AIEC on the ileal mucosa of CD patients, we were unable to demonstrate a specific role of CEACAM6 variants in IBD susceptibility. Furthermore, there was no evidence for an association with ileal CD or for epistasis with NOD2, IL23R, and ATG16L1 variants in CD susceptibility. Further functional studies will be necessary to elucidate how CEACAM6 gene variants may modulate bacterial colonization in IBD patients. Even if this study was unable to find a role for CEACAM6 gene variants in IBD susceptibility, the CEACAM6 protein is likely to be an important mediator of the pathogenesis of CD [56].

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This work contains parts of the unpublished degree theses of C. Fries.

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| Chromosomal position (bp) of the CEACAM6 SNP | Position in the CEACAM6 gene | CEACAM6 SNPs analyzed in our study | CEACAM6 SNPs covered by the Illumina Hap300 chip | CEACAM6 SNPs covered by the Affymetrix 500k chip |
|---------------------------------------------|-------------------------------|----------------------------------|-----------------------------------------------|-----------------------------------------------|
| 46,948,446                                  | upstream                      | rs3764577                        |                                               |                                               |
| 46,950,899                                  | upstream                      | rs10415946                       |                                               |                                               |
| 46,952,409                                  | intragenic                    | rs1805223 = P42P                 |                                               |                                               |
| 46,953,560                                  | intragenic                    | rs3790518                        |                                               |                                               |
| 46,954,095                                  | intragenic                    | rs1160653                        |                                               |                                               |
| 46,954,731                                  | intragenic                    | rs379020                         |                                               |                                               |
| 46,955,390                                  | intragenic                    | rs4803507                        |                                               |                                               |
| 46,956,489                                  | intragenic                    | rs4803508                        |                                               |                                               |
| 46,957,729                                  | intragenic                    | rs11548735 = G239V               |                                               |                                               |
| 46,957,793                                  | intragenic                    | rs7246116 = H260H                |                                               |                                               |
| 46,962,846                                  | intragenic                    | rs10413359                       |                                               |                                               |
| 46,966,939                                  | intragenic                    | rs2701                           |                                               |                                               |
| 46,970,128                                  | downstream                    | rs6508997                        |                                               |                                               |
| 46,972,172                                  | downstream                    | rs10416839                       |                                               |                                               |

*Position of the CEACAM6 gene on chromosome 19: 46,951,341 bp to 46,967,953 bp. doi:10.1371/journal.pone.0019319.t014
Author Contributions
Conceived and designed the experiments: JG JD SB. Performed the experiments: JG CF JD. Analyzed the data: JG SP CW BM-M JD DC SB. Contributed reagents/materials/analysis tools: JG JS CF CT SP MW FB.

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