Germline deletions in the tumour suppressor gene \textit{FOCAD} are associated with polyposis and colorectal cancer development

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Abstract

Heritable genetic variants can significantly affect the lifetime risk of developing cancer, including polyposis and colorectal cancer (CRC). Variants in genes currently known to be associated with a high risk for polyposis or CRC, however, explain only a limited number of hereditary cases. The identification of additional genetic causes is, therefore, crucial to improve CRC prevention, detection and treatment. We have performed genome-wide and targeted DNA copy number profiling and resequencing in early-onset and familial polyposis/CRC patients, and show that deletions affecting the open reading frame of the tumour suppressor gene \textit{FOCAD} are recurrent and significantly enriched in CRC patients compared with unaffected controls. All patients carrying \textit{FOCAD} deletions exhibited a personal or family history of polyposis. RNA \textit{in situ} hybridization revealed \textit{FOCAD} expression in epithelial cells in the colonic crypt, the site of tumour initiation, as well as in colonic tumours and organoids. Our data suggest that monoallelic germline deletions in the tumour suppressor gene \textit{FOCAD} underlie moderate genetic predisposition to the development of polyposis and CRC.

Keywords: polyposis and colorectal cancer; cancer predisposition; \textit{FOCAD}; copy number variation; gene expression

Introduction

Adenomatous polyposis, the development of numerous polyps in the colon and rectum, is strongly associated with the prevalence of heritable genomic variants. These polyps have been shown to act as precursors of \textit{in situ} carcinomas. Therefore, individuals who develop adenomatous polyposis are considered to be at an increased risk to develop colorectal cancer (CRC) [1], the second most frequent cause of cancer-related death in the Western world. In the past, several genes have been associated with a high risk for polyposis, including \textit{APC}, \textit{MUTYH}, \textit{AXIN2}, \textit{SMAD4}, \textit{BMPRIA}, \textit{STK11}, \textit{POLD1} and \textit{POLE} [2]. Although germline mutations in these genes underlie the majority of polyposis cases, approximately 20% of the cases remain unexplained [3,4]. The identification of additional heritable genomic variants will be instrumental for increasing our understanding of the molecular mechanisms underlying polyposis and CRC initiation. This, in turn, will lead to an improved clinical management of individuals and families at risk, including surgical removal of polyps at regular intervals during surveillance [5].

It is generally accepted that aberrant proliferation of epithelial cells in colonic crypts represents an initiating step in the development of polyposis and CRC, and it has been shown convincingly that so-called crypt base columnar (CBC) and +4 cells, both considered to be crypt stem cells, possess cancer-initiating potential [6–8]. Due to this potential, normal intestinal
proliferation of these epithelial cells requires strict regulation. Loss of this strict regulation may underlie the development of multiple polyps in the colon, as illustrated by familial adenomatous polyposis (FAP), juvenile polyposis syndrome (JPS) and hereditary mixed polyposis syndrome (HMPSy) [9]. FAP is caused by loss of functional expression of APC, a negative regulator of β-catenin, which results in increased activation of the WNT signalling pathway and transcriptional activation of proliferation-enhancing genes, including MYC and CCND1 [10–12]. JPS and HMPSy are both caused by decreased activation of the TGFβ signalling pathway, due to loss of functional expression of the cytoplasmic mediator gene SMAD4 and the serine-threonine kinase type I receptor gene BMPRIA [13,14]. Deregulation of both the WNT and the TGFβ signalling pathways is known to be associated with aberrant proliferation of epithelial cells in the colonic crypt. The proliferation of these cells is regulated by genes expressed in stem cell progenitor cells [15]. These latter genes may, therefore, act as potential polyposis or CRC susceptibility genes.

In the search for novel CRC susceptibility genes in unexplained CRC families, we previously screened a cohort of 41 early-onset CRC subjects with a clear positive family history of CRC for the presence of rare DNA copy number variants (CNVs). This screening effort revealed several germline CNVs in genes that are considered to be candidates for CRC susceptibility, such as PTPRJ and GREM1 [16,17]. PTPRJ was previously identified as a CRC susceptibility gene in mice [18] and loss of heterozygosity (LOH) of PTPRJ has frequently been observed in early stages of colorectal cancer development [19]. Genome-wide association studies (GWAS) have revealed that GREM1-related germline variants are associated with CRC susceptibility [20,21]. In addition, it has been found that a 40 kb germline duplication upstream of the GREM1 locus is associated with an increased expression of GREM1. This duplication was found to be recurrent in hereditary mixed polyposis patients of Ashkenazi descent [22]. These examples clearly illustrate the power of CNV screening in the identification of novel heritable genomic variants affecting the risk of developing polyposis/CRC.

Here, we show that the tumour suppressor gene FOCAD [23] (encoding Focadhesin; previously known as KIAA1797), located on 9p21.3, is recurrently affected by CNVs in early-onset/familial CRC index patients with a personal or family history of polyposis. Previous studies have suggested a relatively low overall expression of FOCAD in the colon compared with other tissues [23]. We show that FOCAD is abundantly expressed in epithelial cells within the colonic crypt and that, as such, this gene may play a role in the development of polyposis and/or CRC. The variable numbers of FOCAD-expressing cells in colonic organoids and tumours from different patients suggest a role of this gene in at least a subset of colonic tumours. Our findings indicate that intragenic deletions in FOCAD are a novel risk factor for polyposis and CRC development.

Materials and methods

Patient and control cohorts

Our initial discovery cohort, encompassing 41 patients with microsatellite-stable (MSS) early-onset or familial CRC, has been described previously [16]. For a subsequent targeted screening of the FOCAD locus, we used an independent validation cohort of 1232 patients diagnosed with early-onset and/or familial CRC from the Radboud University Medical Centre, Nijmegen, The Netherlands (n = 89), from the Universitätsklinikum Carl Gustav Carus, Dresden, Germany (n = 159) and from the Wellcome Trust Centre for Human Genetics, University of Oxford, UK (n = 984). An additional unrelated validation cohort of 38 polyposis patients from The Netherlands, who were diagnosed with at least 10 polyps and (a) developed serrated adenomas, (b) developed CRC, or (c) had a positive familial history of polyposis, was also included. In order to exclude common copy number polymorphisms, we compared the patient-derived data with CNVs reported in the Database of Genomic Variants (http://projects.tcag.ca/variation) [24], our in-house database of copy number variants obtained from healthy individuals in The Netherlands (n = 1604), another control cohort encompassing 1880 individuals from the Nijmegen Biomedical Study [25] and our in-house database of genomic variants, Genome Diagnostics, department of Human Genetics, Nijmegen (n = 9000). To determine the frequency of (highly conserved) single-nucleotide variants (SNVs) in FOCAD in subjects without a known history of polyposis/CRC, exome data from the Exome Variant Server (EVS; n = 6500) [26] and our in-house exome sequencing database (MDI; n = 2096) were retrieved. An overview of all cohorts, including the selection criteria and genomic screening techniques used, is provided in Table S1 (see supplementary material). All patient and control samples were obtained after informed consent.

Multiplex Ligation-dependent Probe Amplification (MLPA) and genomic qPCR

MLPA probes were designed for the FOCAD/miR-491 locus according to guidelines provided by MRC–Holland (Amsterdam, The Netherlands) and, subsequently, MLPA assays were performed and analysed as described previously [16]. Genomic qPCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), as described previously [16], using SYBR Green-based quantification according to the manufacturer’s protocol (Bio-Rad, Veenendaal, The Netherlands). Both MLPA and genomic qPCR primers are available upon request.

Real-time quantitative RT–PCR

Real-time quantitative reverse-transcriptase PCR (RT–PCR) was performed as described previously [27]. Briefly, cDNA was prepared from 1–2 μg RNA
Association of germline deletions in FOCAD polyposis and colorectal cancer

Statistics

A one-sided $\chi^2$ test with Yates’ correction was applied to determine the statistical significance of enrichment of FOCAD deletions in CRC patients compared to the control groups. Statistical significance of the RT–PCR results was determined using a two-tailed t-test, assuming equal variances. For both tests, the predetermined level of significance was $p = 0.05$.

Results

FOCAD deletions are recurrent in familial and early-onset CRC patients

In a previous microarray-based CNV screen of familial and early-onset microsatellite-stable (MSS) CRC patients ($n = 41$), we identified an intragenic deletion affecting the FOCAD gene locus, which encodes the potential tumour suppressor Focadhesin and miRNA miR-491, in one of the index patients (patient A) [16]. In order to assess whether an association of FOCAD deletions with CRC development could be confirmed, we screened an additional cohort of familial and early-onset CRC patients ($n = 1232$) as well as healthy controls ($n = 1880$), using targeted MLPA analysis. This screen revealed two additional FOCAD deletions in the CRC patient cohort (patients B and C), but none in the healthy control cohort (Figure 1). In addition, no FOCAD deletions were found to be reported in our in-house database of copy number variants obtained from healthy individuals in The Netherlands ($n = 1604$), whereas only a single intragenic FOCAD deletion was identified in the samples run by our array diagnostics pipeline at the department of Human Genetics, Nijmegen ($n = 9000$). Therefore, the enrichment of FOCAD deletions in CRC patients (2/1232) compared to the control group (1/12400) is significant ($p = 0.0067$). Together, these results indicate that deletions in FOCAD are rare but recurrent in familial and early-onset CRC patients.

The open reading frame is affected in all patients with FOCAD deletions

To determine the genetic boundaries of the FOCAD deletions, we mapped the deletion breakpoints in each of the three patients, using a combined MLPA and genomic qPCR-based approach. All three deletions were found to be different in size and location, encompassing exons 4–23 in patient A, 2–14 in patient B and 7–20 in patient C (Figure 2). Therefore, the miR-491 locus, located within intron 4 of the FOCAD gene, was only affected in patients A and B. All three deletions do affect the FOCAD gene: two deletions include exon 4, containing the ATG start site (patients A and B), whereas the deletion in patient C results in a frameshift in the coding sequence and, consequently, a premature translational stop. As a consequence, all identified deletions encompass several exons and disturb the open reading frame.
Figure 1. Confirmation and identification of genomic deletions in the FOCAD gene: confirmation of the genomic deletion by MLPA in germline DNA of patient A (index patient) and identification of genomic FOCAD deletions by MLPA in the germline of patients B and C (extended cohort); three controls were included in the MLPA assay; ex, exon

reading frame (ORF) of FOCAD, strongly suggesting a loss-of-function scenario.

Truncating second-hit mutations are not detected in polyps and tumours of FOCAD deletion carriers

Based on Knudson’s two-hit paradigm, we questioned whether loss of the remaining wild-type allele might be a common event in the development of polyps and tumours in FOCAD deletion carriers. Therefore, Sanger sequencing was performed to reveal the presence of truncating somatic second-hit mutations in available tumour tissues of two index patients with FOCAD deletions. Truncating somatic mutations were identified in none of three independent adenomas derived from patient C or in a tumour sample derived from patient B. These findings suggest haplo-insufficiency, rather than a classical two-hit tumour suppressor gene scenario, to be the most likely mechanism underlying CRC susceptibility in these patients.

Pathogenic FOCAD single nucleotide variants are not enriched in polyposis/CRC patients

Since all identified deletions result in a loss of function of FOCAD, we reasoned that pathogenic SNVs affecting the function of its encoded protein (Focadhesin) might also be enriched in early-onset or familial CRC patients as compared to healthy controls. No nonsense, frameshift or splice site mutations were identified in a selected cohort of 117 early-onset/familial CRC patients and 33 polyposis patients. We did, however, identify three previously unreported missense variants, p.Y759F, p.T1313A and p.S1660F (for all variants, see supplementary material, Table S3), one of which (p.S1660F) was predicted to be deleterious (see supplementary material, Table S4). However, in order to draw firm conclusions about its pathogenicity, additional (functional) evidence has to be obtained. Moreover, missense variants with similar in silico characteristics have also been reported in healthy controls (see supplementary material, Table S5).

Germline deletions of FOCAD are associated with polyposis

To reveal whether FOCAD deletion-positive patients share any phenotypic characteristics, the clinical data of our patients and their family members were collected and compared (Table 1). Patient A developed over 20 polyps and a rectal MSS carcinoma at the age of 33. He had four relatives (second- and third-degree) with CRC, but neither of his parents developed CRC (see supplementary material, Figure S3). Co-segregation analysis revealed that the same deletion was present in the germline of his mother, who was not affected by polyposis or CRC at time of last contact (age 66). Patient B was diagnosed with CRC at age 62 years and, in addition, was found to have four relatives (first-, second- and third-degree) diagnosed with CRC. Patient B developed one traditional adenoma, her sister developed three adenomas and two hyperplastic polyps and her son was diagnosed with two hyperplastic polyps (see supplementary material, Figure S3). Patient C also had a positive family history for CRC, ie three relatives (first- and second-degree) were affected (see supplementary material, Figure S3). He was diagnosed with adenomatus polyps at age 64 years and had a well-documented history of constitutive polyp development for at least 7 years. Therefore, in addition to familial CRC, these FOCAD deletion carriers appear to share a personal or family history of polyposis.

FOCAD is expressed in epithelial cells of the colonic crypt

Based on information available in public databases and published data [23], FOCAD appears to be ubiquitously expressed in almost all tissues, with the highest levels in the brain and relatively low levels in colonic
Association of germline deletions in FOCAD polyposis and colorectal cancer

Figure 2. Diagram of germline FOCAD deletions, all affecting the ORF: the upper line schematically represents the FOCAD gene, its exons (1–46) and the location of the miR-491 locus therein (intron 4); bars represent the deletions identified in each patient (A–C); deletions (bars) and truncating mutations (arrowheads) depicted in grey have been encountered in an early-onset breast cancer (BC) and three attenuated polyposis patients, respectively (see [32,33]).

Table 1. Overview of clinical phenotypes of carriers of mono-allelic FOCAD deletions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age of onset (years)</th>
<th>CRC</th>
<th>Polyposis</th>
<th>Number of relatives with CRC</th>
<th>FOCAD exons deleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Male</td>
<td>33</td>
<td>Yes</td>
<td>&gt;20 polyps</td>
<td>Son and sister with polyps</td>
<td>4–23</td>
</tr>
<tr>
<td>B</td>
<td>Female</td>
<td>62</td>
<td>Yes</td>
<td>Constitutive polyp development</td>
<td>2–14</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Male</td>
<td>64</td>
<td>No</td>
<td></td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Variable expression of FOCAD in tumours and tumouroids

To explore the expression pattern of FOCAD in CRC samples, we initially compared the expression levels of this gene in 12 matched normal and primary tumour tissues, using quantitative RT–PCR. This analysis revealed variable, but overall increased, levels of FOCAD expression in the tumours (Figure 4a). However, when expression levels were compared between organoids and patient-matched tumouroids, which are the tumourigenic equivalents of human colonic organoids originating from adenocarcinoma stem cells [30], no differences were observed. In fact, some tumouroids even showed significantly reduced expression of FOCAD compared to their matched organoids (Figure 4b). Next, we applied RNA ISH to multiple primary colonic tumour tissues and revealed that the number of FOCAD-expressing cells differs between different tumours (Figure 4c). This difference in FOCAD-expressing cells may explain the observed differences in FOCAD expression levels in tumour samples, and suggests an enrichment of a specific epithelial cell subtype in some tumours. The exact nature of these cells, however, still needs to be defined. Taken together, our results show that cells expressing FOCAD are present in most tumours and that, in accordance with the absence of second-hit mutations, complete loss of expression of FOCAD in colonic tumours is not likely to be a common event in CRC development.

Discussion

Our data show that rare intragenic deletions in the FOCAD gene recurrently occur in familial and early-onset colorectal cancer (CRC) patients and that these deletions are significantly enriched in patient cohorts compared to unaffected control cohorts (p = 0.0067). Furthermore, we noticed that germline FOCAD

deletions may be associated with a polyposis phenotype, since multiple polyps were observed in all affected individuals or their family members. This observation is in agreement with a recent report in which three individuals with attenuated polyposis were described as carrying a deletion or truncating mutation in FOCAD [32] (Figure 2). In addition, a constitutional mono-allelic deletion in the FOCAD gene has recently been reported in an early-onset breast cancer patient [33], and recurrent deletions and somatic point mutations in FOCAD have been observed in sporadic cases of other cancer types [34–37] (Figure 2; see also supplementary material, Figure S4). These somatic and constitutional deletions in FOCAD substantiate its putative role as a novel cancer (susceptibility) gene.

Polyposis/CRC susceptibility factors can be divided into very rare variants with a high penetrance [38], intermediate to rare variants with a moderate penetrance [16] and common variants with a low penetrance [39]. Extensive co-segregation analyses within the families reported here could, unfortunately, not be performed, due to a lack of material from the affected, often deceased, family members. However, since this germline deletion was also found to be present in a non-affected control and in the non-affected mother of patient A, we conclude that germline FOCAD deletions are not fully penetrant and that additional germline variants may act as modifiers, as has, for example, been reported previously for APC and MLH1 [40,41]. Indeed, our patient with the earliest age of onset (patient A, age 33 years) was found to harbour a de novo pathogenic germline mutation in the exonuclease domain of POLE (p.Leu424Val), which may explain the early age of onset compared to the other polyposis/CRC patients with a germline deletion in FOCAD. Rare variants, such as FOCAD deletions with moderate penetrances, may, however, still account for a significant number of the unexplained hereditary polyposis/CRC cases, which can now easily be identified through the availability of efficient and robust detection methods [28,42,43].

To further assess whether FOCAD may play a role in polyposis and CRC development, we determined the expression pattern of this gene in healthy colonic tissues, including in vitro-cultured organoids. Based on information available in public databases and published data [23], FOCAD appears to be ubiquitously expressed in almost all tissues, with the highest levels in brain. Here, we show that FOCAD is expressed in epithelial cells within the colon. Since normal colonic tissue contains several non-epithelial cell types, the overall expression of FOCAD is relatively low. This observation is in line with the observed relatively high expression of FOCAD.

Figure 3. Expression of FOCAD in normal colonic tissue. (A) Relative average expression of FOCAD in normal colonic tissue normalized to housekeeping gene HPRT (n = 12). (B) Expression levels of FOCAD in normal and organoid samples (average expression of 12 and 5 tissue samples, respectively). (C) RNA in situ hybridization reveals expression of FOCAD (red arrowheads) in epithelial cells within the colon; magnifications = (left) × 10; (right) × 20.
Association of germline deletions in FOCAD polyposis and colorectal cancer

Figure 4. Expression of FOCAD in colonic carcinoma tissue. (A) Expression levels of FOCAD in normal and matched tumour samples (n = 12), normalized to housekeeping gene HPRT. (B) Expression levels of FOCAD in organoid and patient-matched tumouroid (n = 5). (C) RNA in situ hybridization for FOCAD in colonic tumour samples; (upper panels) large numbers of FOCAD-expressing epithelial cells are observed in tumour sections (magnifications = ×4 and ×10); (lower panels) microarray tumour sections show that the amounts of FOCAD-expressing cells (red arrowheads) vary between colon tumours from different patients (four samples; magnification = ×10). Tumours derived from germline FOCAD deletion carriers could not be included in this analysis; **p < 0.01; n.s., not significant.

in organoids, which consist of only colonic epithelial cells. The high expression levels of FOcad in epithelial cells within the colon is also in line with its potential role as a novel polyposis/CRC susceptibility gene, since aberrant proliferation of epithelial cells in colonic crypts is considered to be an initiatory step in the development of polyposis/CRC.

All encountered deletions affect the ORF of FOcad and previously published data have shown that Focadhesin, the FOcad-encoded protein, acts as a tumour suppressor [23]. The observed germline deletions strongly suggest a loss-of-function scenario, but somatic second-hit mutations could not be identified in the tumour and adenoma samples tested (from patients B and C, respectively) and RNA ISH on sporadic colonic tumours revealed that loss of FOcad expression is not a common event in colonic tumourigenesis. These findings may point towards a haplo-insufficiency scenario. On the other hand, complete loss of FOcad expression due to homozygous FOcad deletions has been reported in glioblastomas [23] and breast cancers [36]. In addition, somatic second-hit mutations in FOcad were reported in an individual with attenuated polyposis [32]. Together, these data suggest that FOcad is a tumour suppressor gene that can be subject to either a classical two-hit or a haplo-insufficiency scenario. In the epithelial cells of the colon, loss of one allele of this gene may already underlie the development of polyposis and CRC.

The lack of enrichment of deleterious germline SNVs in the FOcad gene in our CRC cohorts is remarkable, since overall such variants are more frequent in CRC-predisposing genes as compared to deletions [42,44]. Protein truncating FOcad variants are very rare in the normal population, ie only two identical nonsense variants were identified in our in-house exome database (n = 2096) and truncating variants were only observed in a small percentage of subjects (<0.07%) listed in the ExAC database [45] (see supplementary material, Figure S4). In contrast to our results, it has been reported that targeted sequencing of 192 polyposis/CRC patients revealed potentially truncating germline SNVs in two attenuated polyposis patients [32]. We assume that our strictly selected cohort was too small and/or heterogeneous to reveal a significant enrichment of truncating variants in the FOcad gene.

Previously published data have shown that Focadhesin serves as a novel component of the focal adhesion complex [23]. Although the exact function of Focadhesin remains to be established, components of the focal adhesion complex, such as the focal adhesion kinase (FAK), have already been linked to intestinal tumourigenesis [46]. Similar to the role of FAK in intestinal tumour development, Focadhesin not only acts as a novel interaction partner for the focal adhesion complex but also exerts crucial functions in cell survival and proliferation, as illustrated by its negative effect on tumour growth [23]. Thus, like other components of the focal adhesion complex, a role of Focadhesin in the regulation of cell proliferation and, therefore, tumourigenesis is assumed.

In conclusion, we show that FOcad germline deletions are recurrent and significantly enriched in patients with a positive (familial) history of polyposis/CRC. All identified deletions affect the ORF, suggesting a loss-of-function scenario. The enhanced expression of FOcad in epithelial cells within colonic crypts suggests a regulatory role of this gene in the proliferation of potentially tumour-initiating colonic stem cells. We conclude that FOcad may serve as a novel polyposis/CRC susceptibility gene.

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Author contributions

RDaw, RV, AGvK and RPK designed the study; SNP array and CNV analysis was performed by RV, JBC and ETPV; RDAW, RV, HFF, RMdV, LV, EJK and KN performed laboratory experiments and/or analysed data; RV and LV performed second-hit screenings; RDAW, EJK and KN performed targeted resequencing; human colonoid and tumouroid culture and expression analyses were performed by HFF and MvdW under the supervision of HC; in situ hybridization was performed by HFF; CMK, HKS and NH were responsible for patient counselling and clinical data acquisition; samples of the independent validation cohort were collected and provided by HKS, LCC and IPT; samples from the Nijmegen Biomedical Study were provided by LAK and KKHA; tumour histology was evaluated by IDN; DNA isolation was performed by MvA; MJLL, NH, AGvK and RPK supervised the work; and RDAW, AGvK and RPK wrote the manuscript, with assistance and final approval from all co-authors.

References


SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supplementary material may be found in the online version of this article:

- **Figure S1.** Average depth of coverage/FOCAD amplicon, obtained with 454 targeted amplicon resequencing of 117 patients with early-onset CRC
- **Figure S2.** Average depth of coverage/FOCAD amplicon/barcode, obtained with IonTorrent targeted amplicon resequencing of 33 polyposis patients
- **Figure S3.** Pedigrees of FOCAD deletion patients
- **Figure S4.** Germline and somatic variants in the FOCAD gene
- **Table S1.** Overview of selected cohorts in our study
- **Table S2.** Quality settings applied for variant calling using the SeqNext and NextGENe software package
- **Table S3.** Variants identified by targeted amplicon resequencing of FOCAD in early-onset CRC and polyposis
- **Table S4.** In silico prediction of three previously unreported missense variants in the FOCAD gene
- **Table S5.** In silico prediction scores of potential pathogenic missense variants called in the EVS database

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