Use of sanger and next-generation sequencing to screen for mosaic and intronic APC variants in unexplained colorectal polyposis patients

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Abstract
In addition to classic germline APC gene variants, APC mosaicism and deep intronic germline APC variants have also been reported to be causes of adenomatous polyposis. In this study, we investigated 80 unexplained colorectal polyposis patients without germline pathogenic variants in known polyposis predisposing genes to detect mosaic and deep intronic APC variants.

All patients developed more than 50 colorectal polyps, with adenomas being predominantly observed. To detect APC mosaicism, we performed next-generation sequencing (NGS) in leukocyte DNA. Furthermore, using Sanger sequencing, the cohort was screened for the following previously reported deep intronic pathogenic germline APC variants: c.1408 + 731C > T, p.(Gly471Serfs*55), c.1408 + 735A > T, p.(Gly471Serfs*55), c.1408 + 729A > G, p.(Gly471Serfs*55) and c.532-941G > A, p.(Phe178Argfs*22). We did not detect mosaic or intronic APC variants in the screened unexplained colorectal polyposis patients. The results of this study indicate that the deep intronic APC variants investigated in this study are not a cause of colorectal polyposis in this Dutch population. In addition, NGS did not detect any further mosaic variants in our cohort.

Keywords Unexplained colorectal polyposis · APC · Mosaic variants · Intronic variants · Pseudoexons

Abbreviations
FAP Familial adenomatous polyposis syndrome
AFAP Attenuated FAP
NGS Next-generation sequencing
DGGE Denaturing gradient gel electrophoresis
PTT Protein truncation test
HRMA High resolution melting analysis
IGV Integrative genomics viewer
VAF Variant allele frequency

Introduction
Pathogenic germline variants in APC (MIM# 611,731) cause familial adenomatous polyposis syndrome (FAP; MIM# 175,100), a rare autosomal dominant-inherited syndrome characterized by the development of multiple colorectal adenomas and a very high risk of colorectal cancer [1–4].

In classic FAP, patients develop hundreds to thousands of colorectal adenomatous polyps, while in attenuated FAP (AFAP), patients develop fewer adenomas (< 100) at a later age than those with classical FAP [5–8]. A subset of patients with multiple colorectal adenomas and no APC germline variants have been found to carry biallelic variants in the base excision repair gene MUTYH (MIM# 604,933), causing MUTYH-associated polyposis (MAP; MIM# 608,456) [9]. In addition, a number of other genes associated with adenomatous polyposis, such as POLE, POLD1, NTHL1, MSH3 and MLH3, have recently been reported [10–13]. The detection rate of APC variants in FAP patients depends on phenotype and methods. In classic FAP, APC germline variants can be detected in up to 85% of patients [14, 15]; however, the detection rates of APC germline variants in patients with fewer colorectal adenomatous polyps (AFAP patients) are lower, ranging from 10 to 30% of patients [14, 16], suggesting that a proportion of pathogenic variants remain undetected by routine methods [17–19]. Mosaic APC variants and deep intronic variants localized in regions not covered by PCR-based diagnostics were previously identified as additional causal factors. Using RNA-based assays and next-generation sequencing (NGS), it has been shown that a proportion of variant-negative FAP patients harbor molecular changes in deep intronic regions of APC [19, 20].
studies identified deep intronic \textit{APC} variants that result in pseudoexon formation [19, 20]. Through the use of sensitive techniques, somatic \textit{APC} mosaicism has been demonstrated in a minority of adenomatous polyposis patients [21–26]. In addition, using deep sequence analysis of \textit{APC} in DNA isolated from multiple adenomas, mosaic variants were identified in 9 of 18 patients with 21 to 100 adenomas; in some of these cases, NGS also detected the variants in leukocyte DNA at low frequency [27]. In this study, we investigate the role of deep intronic germline \textit{APC} variants and mosaic \textit{APC} variants in leukocyte DNA as possible genetic causes of colorectal polyposis in a Dutch cohort of unexplained patients with more than 50 polyps.

**Materials and methods**

**Patients**

A total of 80 index patients with more than 50 colorectal polyps (Table 1) were selected from a previously described cohort [28–31]. The cohort included patients previously screened for germline mosaic \textit{APC} variants by denaturing gradient gel electrophoresis (DGGE) [17], the protein truncation test (PTT) [17] and high resolution melting analysis (HRMA) [21]. All cases tested negative for pathogenic germline \textit{APC}, \textit{MUTYH}, \textit{POLE}, and \textit{POLD1} and for \textit{NTHL1} hotspot variants. Clinicopathological data included date of birth, gender, age at diagnosis of colorectal polyps/adenomas, cumulative number of polyps, location and histology of polyps/adenomas, information on CRC and presence of polyps/CRC in first-degree family members. Since the term serrated adenomas is currently preferred over hyperplastic polyps, we lumped together polyps described as such under the term sessile serrated lesions with or without dysplasia. Three controls were included in this study. Leukocyte DNA from this cohort was available for the study. The study was approved by the medical ethics committee of Leiden University Medical Center, protocol P01-019.

**APC intronic variant screening**

Leukocyte DNA of the patients was screened for the intronic \textit{APC} variants in Table 2 using Sanger sequencing. Primers were designed using Primer3 software [http://primer3.ut.ee/](http://primer3.ut.ee/) and were obtained from Eurofins Genomics (Ebersberg, Germany). The following primers with universal M13 tails were used: c.1408 + 731C > T, c.1408 + 735A > T and c.1408 + 729A > G; forward: 5’-TGTAAGACGCCAGCTGCTCACCACATCTCAT-3’ and reverse: 5’-CAGGAACACCGTATGAGCTCTGCTGC CTTAGAAAACTG-3’. Sanger sequencing of the PCR amplified fragments was performed by Macrogen (Amsterdam, Netherlands). The sequencing results were analyzed using Mutation Surveyor software (Sofgenetics, State College PA, USA).

| Table 1 | Clinical characteristics of the colorectal polyposis patients (n = 80) |
|---------|---------------------------------------------------------------|
| Patient characteristics | Individuals % |
| Number of polyps | |
| > 100 | 29 (36.2%) |
| 50–100 | 51 (63.8%) |
| Type of polyps | |
| Adenomas | 36 (45%) |
| Mixed (Adenomas + Serrated*) | 38 (47.5%) |
| Serrated | 5 (6.2%) |
| Unknown | 1 (1.3%) |
| Age at diagnosis with polyposis | |
| ≥ 50 years | 49 (61.3%) |
| < 50 years | 31 (38.7%) |
| Diagnosed with CRC | |
| Yes | 27 (33.8%) |
| No | 53 (66.2%) |
| Age at diagnosis with CRC | |
| > 50 | 19 (70.4%) |
| ≤ 48 | 8 (29.6%) |
| Sex | |
| Male | 53 (66.2%) |
| Female | 27 (33.8%) |
| Polyposis family | |
| Polyposis family | 29 |
| No polyposis family | 37 |
| Unknown | 14 |
| CRC family | |
| CRC family | 33 |
| No CRC family | 34 |
| Unknown | 13 |

*Sessile serrated lesions with or without dysplasia

and reverse: 5’ CAGGAACACCGTATGAGCTCTGCTGC CTTAGAAAACTG-3’. Sanger sequencing of the PCR amplified fragments was performed by Macrogen (Amsterdam, Netherlands). The sequencing results were analyzed using Mutation Surveyor software (Sofgenetics, State College PA, USA).

**Next-generation sequencing and data analysis**

Deep \textit{APC} sequencing was performed using a previously described custom \textit{APC} panel [27]. The complete sequencing panel consisted of 115 amplicons (11,216 bp), covering 99.3% of the coding regions of \textit{APC}. Libraries were prepared with Ion Ampliseq™ 2.0 Kit (Thermo Fisher Scientific, Bleiswijk, The Netherlands) according to the manufacturer’s instructions and were sequenced on the Ion Torrent Proton Platform (Thermo Fisher Scientific, Bleiswijk, The Netherlands). Sequence data were analyzed as described
Results and discussion

In this study, we attempt to identify the genetic causes of colorectal polyposis in unexplained patients with colorectal polyposis. Deep NGS of APC was performed to identify possible undetected pathogenic mosaic variants. Furthermore, APC intronic germline variants described previously [19, 20] were studied to evaluate their role. A high-risk cohort was selected for this study, consisting of 80 index patients with ≥ 50 colorectal polyps (Table 1), of whom many had a relatively early onset, which increases the probability of finding undiscovered mosaic or intronic variants. The mean age at diagnosis of colorectal polyps was 49 years (range 12–80). The majority of patients (n = 51, 63.8% with a mean age of 51 years at diagnosis) had a cumulative polyp count between 50 and 100, while 29 patients (36.2% with a mean age of 46 years at diagnosis) showed more than 100 polyps. Forty-five percent of the patients displayed only adenomatous polyps, while 47.5% of the patients displayed a mixed phenotype with adenomas and sessile serrated lesions with or without dysplasia. CRC was found in 27 patients (33.8%, with a mean age of 56 years, range 37–80). The clinical characteristics of the patients are summarized in Table 1.

First, we screened the leukocyte DNA of 80 patients for the following deep intronic heterozygous germline variants in APC: c.1408 + 731C > T, p.(Gly471Serfs*55), c.1408 + 735A > T, p.(Gly471Serfs*55), c.1408 + 729A > G, p.(Gly471Serfs*55), c.4110_4111delAA (Fig. 1) and c.4057G > T was absent were clearly present, while c.2493dupA was reported previously [27] in normal colonic mucosa and was absent in leukocyte DNA. Both positive controls, APC c.4110_4111delAA (Fig. 1) and APC c.2493dupA, were clearly present, while APC c.4057G > T was absent in the negative control. No additional APC mosaic variants were detected in our cohort. A limitation of this study is that we used only leukocyte DNA for mosaicism screening due to the scarcity of available DNA from patient adenomas. Mosaicism might remain undetectable or be overlooked if the molecular analysis is limited to blood, even when sensitive techniques are applied, due to very low or even absent presentation of the mutated allele [23, 27]. Peripheral blood cells arise from the mesoderm, and when the variant occurs after mesoderm and endoderm specification (early postzygotic mutation), the mosaicism is likely restricted to the colon and is difficult to detect the variant in leukocyte DNA [23, 27, 32, 33]. In a previous study, it was recommended to test at least two or more adenomas to detect mosaic variants [27].

A recent systematic review of current APC mosaicism studies recommends testing adenomas from the polyposis patients without APC germline variant to allow the detection of low allele frequency mosaicism as well as mosaicism
confined to colon [33]. Consequently, in our study, APC mosaic variants confined to the colon could have been missed because we could not screen the DNA from the adenomas of the patients.

In conclusion, we did not detect any of the four previously reported APC intronic variants in our cohort. We also did not detect mosaic APC variants in our cohort using deep sequencing analysis in blood. This finding suggests that the benefit of using targeted amplicon-based NGS to further scrutinize the APC gene in unexplained cases of polyposis is limited. Analyzing DNA from adenomas in addition to leukocyte DNA is recommended to detect a possible underlying mosaicism. Also, other approaches, such as whole genome sequencing or transcriptome sequencing, could be employed to detect undiscovered intronic or promoter variants or other regulatory variants.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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