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The primary aim of *World Journal of Gastrointestinal Oncology (WJGO, World J Gastrointest Oncol)* is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc.*

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Missed colorectal cancers in a fecal immunochemical test-based screening program: Molecular profiling of interval carcinomas

Manon van der Vlugt, Beatriz Carvalho, Joelle Fliers, Nahid Montazeri, Christian Rausch, Esmée J Grobbee, Manon van Engeland, Manon C W Spaander, Gerrit A Meijer, Evelien Dekker

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Abstract**BACKGROUND**

For optimizing fecal immunochemical test (FIT)-based screening programs, reducing the rate of missed colorectal cancers (CRCs) by FIT (FIT-interval CRCs) is an important aspect. Knowledge of the molecular make-up of these missed lesions could facilitate more accurate detection of all (precursor) lesions.

AIM

To compare the molecular make-up of FIT-interval CRCs to lesions that are detected by FIT [screen-detected CRCs (SD-CRCs)].

METHODS

FIT-interval CRCs observed in a Dutch pilot-program of FIT-based screening were compared to a control group of SD-CRCs in a 1:2 ratio, resulting in 27 FIT-interval CRC and 54 SD-CRCs. Molecular analyses included microsatellite instability (MSI), CpG island methylator phenotype (CIMP), DNA sequence mutations and copy number alterations (CNAs).

RESULTS

Although no significant differences were reached, FIT-interval CRCs were more often CIMP positive and MSI positive (33% CIMP in FIT-interval CRCs *vs* 21% in SD-CRCs ($P = 0.274$); 19% MSI in FIT-interval CRCs *vs* 12% in SD-CRCs ($P = 0.469$)), and showed more often serrated pathway associated features such as *BRAF* (30% *vs* 12%, $P = 0.090$) and *PTEN* (15% *vs* 2.4%, $P = 0.063$) mutations. *APC* mutations, a classic feature of the adenoma-carcinoma-sequence, were more abundant in SD-CRCs (68% *vs* 40% in FIT-interval CRCs $P = 0.035$). Regarding CNAs differences between the two groups; FIT-interval CRCs less often showed gains at the regions 8p11.22-q24.3 ($P = 0.009$), and more often gains at 20p13-p12.1 ($P = 0.039$).

CONCLUSION

Serrated pathway associated molecular features seem to be more common in FIT-interval CRCs, while classic adenoma carcinoma pathway associated molecular features seem to be more common in SD-CRCs. This indicates that proximal serrated lesions may be overrepresented among FIT-interval CRCs.

Key Words: Fecal immunochemical test-interval colorectal cancer; Mutation analysis; Colorectal cancer screening; Serrated pathway; Adenoma-carcinoma pathway

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Core Tip: Fecal immunochemical test (FIT) is effective in reducing colorectal cancer (CRC) but FIT testing is not perfect. FIT interval cancers, *i.e.* CRCs diagnosed after a negative FIT but before the next FIT invitation, still occur. Previous studies have shown that FIT sensitivity for sessile serrated lesions (SSLs) is low, but a correlation between the occurrence of FIT interval cancers and the serrated pathway has not been established. In our study, the serrated pathway-associated molecular features were more common in FIT-interval CRCs as compared to screen detected CRCs. This indicates that proximal serrated lesions may be overrepresented among FIT interval CRCs. The findings of this study can provide guidance on strategies to further improve stool-based CRC screening with incorporating biomarkers for SSLs, thereby reducing the number of screening interval CRCs.

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INTRODUCTION

Fecal immunochemical test (FIT)-based screening worldwide has had a major impact on reducing colorectal cancer (CRC)-related mortality. Despite this success, the issue of false negative tests giving rise to FIT interval CRCs (*e.g.*, CRCs diagnosed after a negative FIT but before the next FIT invitation) leaves room for improvement[1]. Monitoring the incidence of FIT-interval CRCs is a key quality indicator of a FIT-based screening program, and any CRC that occurs in spite of recent screening can be regarded as an unwanted program outcome. The sensitivity of FIT for CRC is approximately 75%-85%, which indicates that still 1 in 4-5 CRCs will be missed in any single screening round[2-4]. Possible reasons for FIT-interval CRCs are the limited sensitivity of FIT for specific molecular types of CRCs that were already present at the time of FIT-screening, or rapid progression of premalignant lesions during the interval between two screening rounds. Both of these causes may be the consequence of differences in tumor biology between FIT-interval CRCs and screen-detected CRCs (SD-CRCs). Such biological differences may translate *e.g.*, into a lower bleeding tendency of colorectal lesions or an increased progression rate.

CRC has several precursor lesions reflecting different tumor-biology. Adenomas are well-known precursors of CRC. Adenomas may follow the canonical adenoma-carcinoma sequence with *APC* and *KRAS* mutations and subsequent typical patterns of chromosomal copy number alterations (CNAs) as classic features to develop into CRC[5-7]. More recently, also sessile serrated lesions (SSLs) have been identified as precursors of CRC. SSLs may follow the serrated neoplasia pathway resulting in CRCs that are more often microsatellite instable (MSI), CpG island methylator phenotype (CIMP) high and harbor *BRAF* mutations[8,9]. As a consequence of a different tumor biology, colorectal lesions can have

different morphology. Most SSLs are non-polypoid (flat or slightly elevated) lesions located in the proximal colon, whereas adenomas can be either non-polypoid or polypoid (*e.g.*, stalked or sessile). Previous studies have shown that FIT sensitivity for SSLs is low[10]. Also, non-polypoid colorectal neoplasms (NP-CRNs) have a flat morphology and are associated with more aggressive biologic behavior when compared to polypoid precursor lesions[11]. These NP-CRNs are usually located in the proximal colon. As they are more challenging to detect during colonoscopy, these NP-CRNs are thought to be a major contributor to post-colonoscopy CRCs (PC-CRCs)[12]. In a study on molecular characterization, NP-CRNs were more often found to harbor 5q-loss and *BRAF* mutations and have less *APC* and *KRAS* mutations[11,12]. It was hypothesized that NP-CRNs bleed less intensely and/or not continuously, which could lead to a falsely negative FIT and FIT-interval CRCs.

Generating more insight in the molecular features of FIT-interval CRCs may help to optimize screening strategies, aiming to reduce their incidence. The aim of the present study therefore was to compare the molecular composition of FIT-interval CRCs to that of SD-CRCs.

MATERIALS AND METHODS

Population and study design

From 2006 onwards, two cohort studies of biennial FIT-based CRC screening have been conducted in the southwest and northwest regions of the Netherlands. After three screening rounds, these two cohorts were combined in 2014 to conduct a fourth round of FIT screening. A threshold of 10 µg hemoglobin (Hb) per gram feces was used. Screenees with a fecal Hb concentration above this threshold were referred for colonoscopy. Colonoscopies were performed according to international quality guidelines[13]. Details about the design of this study have been reported previously[2,14,15]. After finishing the fourth screening round in 2015, the total cohort was linked to the Netherlands Cancer Registry, managed by the Netherlands Comprehensive Cancer Organization (Utrecht, The Netherlands), in order to identify CRC missed by FIT testing during the three completed screening rounds including 2 years of follow up.

Definitions

All CRCs detected during colonoscopy after a positive FIT (threshold ≥ 10 µg Hb/g feces) were recorded and labeled as SD-CRC.

FIT-interval CRCs were defined as a CRC diagnosed after a negative FIT (threshold < 10 µg Hb/g feces) and before the date of the next invitation for FIT-screening[16]. If a participant had a negative FIT and was not invited for a consecutive round (for having passed the upper age limit or after moving out of the target area) but developed CRC within the 2.37 years interval (median time between invitations), this CRC was also defined as a FIT-interval CRC.

Persons who had been inconsistent in participating in FIT screening and developed CRC outside the screening interval (median 2.37 years) were not defined as an interval CRC and not included in this study. Data on tumor stage and location (at time of resection) were collected for both FIT-interval CRC and SD-CRCs. With regard to tumor location, the colon was divided into proximal (cecum, ascending, hepatic flexure, and transverse colon) and distal colon (splenic flexure, descending colon, sigmoid colon, and rectum). All cancers were staged according to the 7th edition of the American Joint Committee on Cancer[17]. In the three intervals between four screening rounds, including the 2.37 years follow up for individuals that had reached the upper age limit after any of the first three rounds, in total 27 FIT-interval CRCs were identified. Besides, a total of 116 SD-CRCs was detected in the four screening rounds. All 27 FIT-interval CRCs were included in the study, and a random sample of SD-CRCs in a 1:2 ratio to SD-CRCs, yielding a control group of 54 SD-CRCs.

Sample collection and DNA isolation

All tissue samples of FIT-interval CRCs and SD-CRCs were collected from 11 departments of pathology through the Dutch National Pathology Registry[18]. DNA from formalin-fixed, paraffin-embedded material was isolated as previously described[19]. Good quality DNA could be obtained from 25 of 27 FIT-interval CRCs and 46 of 54 SD-CRCs (see [Supplementary Figure 1](#)).

CIMP status analysis

CIMP status was analyzed in the Pathology Department at the University of Maastricht. The CIMP panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOC51*)[20] was determined by nested methylation-specific polymerase chain reaction (PCR) using sodium bisulfite modified genomic DNA (EZ DNA methylation kit (ZYMO research Co., Orange, CA, United States) as described before[21,22], and CIMP positive was defined when ≥ 3 of the 5 CIMP markers were methylated. In some samples DNA was no longer available, in other samples the analysis was performed but failed, leaving CIMP-analysis results available for 21 of 27 FIT-interval CRCs and 39 of 54 SD-CRCs (see [Supplementary Figure 1](#)).

MSI status analysis

MSI status analysis was performed using the multiplex marker PCR panel from Promega (MSI Multiplex System Version 1.2, Promega, Madison, WI, United States). When two or more markers were unstable, the sample was interpreted as MSI. All other samples were classified as microsatellite stable. In some samples insufficient DNA was left, while in others the results obtained did not meet the quality criteria, leaving results for MSI analysis available for 21 of 27 FIT-interval CRCs and 41 of 54 SD-CRCs (see [Supplementary Figure 1](#)).

Mutation analysis

For mutation analysis, targeted sequencing was performed. DNA libraries were prepared using the KAPA HyperPrep Kit (KAPA Biosystems, Wilmington, MA, United States) as described in the KAPA HyperPrep Kit protocol (KR0961-v5.16). Target enrichment was performed using a custom 48 gene xGen® Predesigned Gene Capture Pools (Integrated DNA Technologies, San Diego, CA, United States), as previously described[23]. In some samples, DNA was no longer available, and one sample was analyzed but sequencing was not sufficiently deep to draw conclusions for almost all genes, leaving mutation analysis results available for 20 of 27 FIT-interval CRCs and 42 of 54 SD-CRCs (see [Supplementary Figure 1](#)). Genes and/or samples with $\geq 50\%$ of low-quality reads, were excluded from analysis, as the results were not reliable. Mutation calling was done as previously described[23]. Raw mutation data have been deposited in the European Genome-Phenome Archive (EGA)[24], with the study ID: EGAS00001004683.

DNA copy number analysis

DNA CNAs were analyzed with low-coverage whole genome sequencing as described previously[23]. Briefly, DNA was fragmented by sonication (Covaris S2, Woburn, MA, United States) and run on the HiSeq 2500 (Illumina, San Diego, CA, United States) on a 65 basepairs single-read modus using the KAPA HyperPrepKit (KAPA Biosystems, KK8504, Wilmington, MA, United States). This yielded a coverage of 0.13x (IQR 0.12-0.14) genome coverage. To compare the frequencies of alterations in the two groups, the R-package CGHtest was used[25]. Good quality DNA copy number profiles were obtained for 19 of 27 FIT-interval CRCs and for 44 of 54 SD-CRCs (see [Supplementary Figure 1](#)). Raw DNA copy number data has been deposited in the EGA[24], with the study ID: EGAS00001004683.

Statistical analysis

Differences between groups were evaluated for statistical significance using the Chi square-test statistic, Fisher's exact test statistic, linear-by-linear test or Mann-Whitney-U test where appropriate. Two-sided *P* values < 0.05 were considered to indicate statistically significant differences. Differences between proportions were reported as mean with 95%CI.

Ethics approval and tissue handling

Ethics approval for performing FIT-based screening including linkage to the Netherlands Cancer Registry was provided by the Dutch National Health Council (WBO 2642467, 2832758, 3049078 and 161536-112008, The Hague, The Netherlands). No separate ethics approval was necessary for the additional molecular analysis, as judged by the scientific ethics board of the AMC University Hospital. Collection and use of tissue and patient data were performed in compliance with the 'Code for Proper Secondary Use of Human Tissue in the Netherlands' (www.federa.org). All authors had access to the study data and reviewed and approved the final manuscript.

Patient and public involvement

It was not appropriate or possible to involve patients or the public in the design, or conduct, or reporting, or dissemination plans of our research.

RESULTS

Clinicopathological characteristics

Demographic and clinicopathological characteristics of the patients with FIT-interval CRCs and SD-CRCs are shown in [Table 1](#). No significant differences were found between both groups. Almost 60% of all patients were men. Among the FIT-interval CRCs, 21 patients (77.8%) were estimated to have a "normal/average" socioeconomic status, compared to 33 patients (61.1%) in the SD-CRC group. In the group of FIT-interval CRCs, fecal Hb concentrations were undetectable ($n = 12$, 44%) or below the threshold ($n = 12$, 44%). For three screenees (11%), the precise level of Hb/g feces was not available. [Supplementary Table 1](#) shows per patient and per CRC type (SD-CRC or FIT-interval CRC) how many rounds of FIT participation had been completed prior to the diagnosis of CRC.

Table 1 Clinicopathological characteristics of all selected persons with fecal immunochemical test-interval and screen-detected colorectal cancers

Characteristics	FIT-interval CRC	SD-CRC	P value
Patients, <i>n</i>	27	54	
Age at diagnosis, mean (min-max)	65.9 yr (53-76)	65.2 yr (50-75)	NS
Male sex, <i>n</i> (%)	16 (59.3)	33 (61.1)	NS
Socio-economic status, <i>n</i> (%)			NS
Low	2 (7.4)	10 (18.5)	
Normal	21 (77.8)	33 (61.1)	
High	4 (14.8)	11 (20.4)	
Tumor location, <i>n</i> (%)			
Proximal	10 (37)	17 (31.5)	NS
Tumor stage, <i>n</i> (%)			
Stage I	8 (29.6)	25 (46.3)	NS
Stage II	6 (22.2)	8 (14.8)	
Stage III	9 (33.3)	20 (37.0)	
Stage IV	4 (14.8)	1 (1.9)	
Time interval between FIT and CRC			
Mean	1.4 yr		
Min-max	0.4-2.3 yr		
IQR	0.9-2.0 yr		
Mean Hb concentration last FIT in µg Hb/g feces, mean ± SD	2.7 ± 3.4 ^a	167.8 ± 168.7	< 0.001

^a3 cases missing.

All values expressed as number and % with respect to fecal immunochemical test-interval and screen-detected colorectal cancers. FIT: Fecal immunochemical test; CRC: Colorectal cancer; SD-CRC: Screen-detected colorectal cancer; IQR: Interquartile range; Hb: Hemoglobin; NS: Not significant.

MSI and CIMP analysis

MSI was more common among patients with FIT-interval CRCs compared to patients with SD-CRCs: 19% (4/21) *vs* 12% (5/41) [6.85% (95%CI: -12.7, 26.4); *P* = 0.469], respectively. Furthermore, CIMP was more prevalent in FIT-interval CRCs than in SD-CRCs, 33% (7/21) and 21% (8/39) [12.82% (95%CI: -11.36, 36.6); *P* = 0.274], respectively. However, differences for both variables did not reach statistical significance (Table 2).

Mutation analysis

Results of the mutation analysis in FIT-interval CRCs and SD-CRCs are shown in Figure 1. Of the 48 genes tested, 37 genes were mutated in at least one sample (see Figure 1). No mutations were detected in *FGFR1*, *FLT3*, *GNA11*, *GNAQ*, *HRAS*, *NPM1*, *PTPN11*, *SMARCB1*, *SMO*, *SRC* and *STK11*.

APC and *PIK3CA* were more often mutated in SD-CRC compared to FIT-interval CRC [*APC*: 68% *vs* 40%, 28.29% (95%CI: 2.53, 54.1); *P* = 0.035; *PIK3CA*: 27% *vs* 0%, 26.83% (95%CI: 13.27, 40.39); *P* = 0.011]. *KRAS* was mutated in 37% of SD-CRC compared to 30% in FIT-interval CRC [5% (95%CI: -20.57, 30.58); *P* = 0.705]. *TP53* was mutated in 51% of SD-CRC compared to 40% in FIT-interval CRC [11.22% (95%CI: -15.14, 37.58); *P* = 0.410].

The following genes were significantly more often mutated in FIT-interval CRCs; *ALK* [20% *vs* 2.4%; 17.56% (95%CI: -0.59, 35.72); *P* = 0.019], *CSF-1R* [20% *vs* 2.4%; 17.56% (95%CI: -0.59, 35.72); *P* = 0.019], *EGFR* [10% *vs* 0%; 10% (95%CI: -3.15, 23.15); *P* = 0.037], *FGFR2* [10% *vs* 0%; 10% (95%CI: -3.15, 23.15); *P* = 0.040], *MET* [10% *vs* 0%; 10% (95%CI: -3.15, 23.15); *P* = 0.040], *MPL* [15% *vs* 0%; 15% (95%CI: -0.65, 30.65); *P* = 0.010].

BRAF [30% *vs* 12%; 17.8% (95%CI: -4.64, 40.25); *P* = 0.090], *KDR* [20% *vs* 5%; 15.12% (95%CI: -3.61, 33.85); *P* = 0.063] and *PTEN* [15% *vs* 2.4%; 12.56% (95%CI: -3.78, 28.9); *P* = 0.063] mutations were more abundant in FIT-interval CRCs compared to SD-CRC but this difference did not reach statistical significance (see Figure 1).

Table 2 Microsatellite instability analysis and CpG island methylator phenotype analysis

	FIT-interval CRC	SD-CRC	P value
MSI analysis ^a			
MSI	4 (19%)	5 (12%)	0.469
MSS	17 (81%)	36 (88%)	
CIMP analysis ^b			
Methylated, positive	7 (33%)	8 (21%)	0.274
Unmethylated, negative	14 (66%)	31 (79%)	

^a*n* = 62.^b*n* = 60.

All values expressed as number and % with respect to fecal immunochemical test-interval and screen-detected colorectal cancers. FIT: Fecal immunochemical test; CRC: Colorectal cancer; SD-CRC: Screen-detected colorectal cancer; MSI: Microsatellite instability; MSS: Microsatellite stable; CIMP: CpG island methylator phenotype.

DNA copy number analysis

Figure 2 and Table 3 show the results of the DNA copy number analysis for FIT-interval CRC and SD-CRC. When comparing both groups, the only significantly different alterations were less frequent gains in FIT-interval CRCs at chromosome 8 region p11.22-q24.3 ($P = 0.009$) and more frequent gains in FIT-interval CRCs at chromosome 20 region p13-p12.1 ($P = 0.039$).

DISCUSSION

Of all interventions currently available, screening is one of the most powerful approaches for reducing CRC-related mortality[1]. Nevertheless, like all screening programs, CRC screening is facing the challenges of overdiagnosis and underdiagnosis. The latter mainly manifests as interval cancers. In the Dutch CRC screening program with a target population of 2.2M screenees, per screening round 544 interval cancers are observed, consistent with a FIT sensitivity of approximately 85% [26]. Theoretically, these FIT interval cancers consist of cancers that were present at the time the FIT was performed, but were missed, as well as cancer precursors missed at the time FIT was performed and that showed a rapid progression to a symptomatic cancer. On one hand sensitivity and specificity are simply determined by the cut off chosen, given the characteristics of the test. On the other hand, specific tumor characteristics driven by the underlying biology may differ between screened detected and interval cancers, a subject that so far has received little attention.

To address that question, the aim of the present study was to investigate the molecular characteristics between both categories. While we had access to a large well documented cohort of individuals followed over multiple screening rounds, the absolute number of interval cancers still was limited, which we consider to be the main reason why for most variables, differences were not statistically significant. Moreover, due to inherent formalin-fixed, paraffin-embedded associated artifacts, like DNA cross-links, the quality reads of some of the downstream analyses was poor and therefore some of the selected cases were further excluded from the final analysis (Supplementary Figure 1). Inherently these findings are exploratory in nature, yet they provide important indications on a major healthcare issue.

Indeed, the results of this exploratory study indicate that FIT-interval CRCs seem to more frequently carry the molecular features of the serrated neoplasia pathway and NP-CRN than SD-CRCs do. FIT interval CRCs present more often CIMP high, MSI high and carry mutations like *ALK*, *BRAF*, *CSF1R*, *EGFR*, *FGR2*, *PTEN*, *KDR*, *MET* and *MPL* compared to SD-CRC. *PTEN* and *KDR* were previously described mutations detected in SSLs with high-grade dysplasia[27]. Furthermore, *APC* and *KRAS* mutations, which classically are part of the canonical adenoma-carcinoma sequence[5-7,27], were less frequently found in FIT-interval CRCs. Regarding DNA CNAs, FIT-interval CRCs showed very similar profiles to SD-CRCs, with only differences observed in the frequency of two genomic regions, namely, less often gains at 8p11.22-q24.3, and more often gains in FIT-interval CRCs at 20p13-p12.1. So, these results suggest that FIT-interval cancers are a mixed group of classical pathway and serrated pathway cancers, although with more commonly serrated pathway features and less commonly classical pathway features (both mutations and CNAs) in comparison with SD-CRCs. The overall pattern is striking, even if the individual variables do not reach statistical significance. Levin *et al*[28] also evaluated whether FIT-interval CRCs differed from FIT-positive patients with CRC by analyzing 7 *KRAS* mutations and 10 aberrantly methylated DNA biomarkers. They did not find any differences in their DNA profiles. In our study, however we investigated other genomic features and additional genes and did find differences as

Table 3 Copy number alterations

CGH-test ^a	Chr	Type	Band	Length basepairs	Group comparison	P value ^b
2.50%	8	Gain	p11.22-q24.3	106890001	Less in FIT-interval CRCs	0.009
2.50%	20	Gain	p13-p12.1	14520001	More in FIT-interval CRCs	0.039

^aPercentage of information loss accepted at CGH-comparison test.

^bFrequency of altered region in FIT-interval CRCs compared to screen-detected CRCs.

CGH: Comparative genomic hybridization; FIT: Fecal immunochemical test; CRC: Colorectal cancer.

described above[29].

FIT is a good test to detect cancers. However, it does not perform so well in the detection of precursor lesions, in particular sessile serrated polyps. In a study that compared sensitivities of several FITs in a screening population, FIT sensitivity for SSLs of > 1 cm was only 5.1% [10]. One possible explanation might be the result of less bleeding tendency due to the low vessel density in serrated lesions in combination with their flat morphology and proximal location. Another explanation could be that serrated pathway lesions may show faster progression to cancer than classic adenomas. This indicates that in FIT-screening, a substantial number of serrated polyps will be missed, and therefore cancers derived from these precursor lesions might be overrepresented in FIT-interval CRCs.

NP-CRNs also show distinct molecular features compared to classical polypoid adenomas, are frequently located in the right colon and might bleed less [11,30,31]. In previous studies, NP-CRNs have been described as being less often APC and KRAS-mutated [11]. In a study comparing PC-CRCs and prevalent CRCs, KRAS mutation was inversely associated with PC-CRCs [32]. Comparably, FIT-interval CRCs were less often APC mutated in the present study (40% in FIT-interval CRCs vs 68% in SD-CRCs, $P = 0.035$). However, although KRAS was less often mutated in FIT-interval CRCs, the difference was not significant (30% in FIT-interval CRCs vs 37% in SD-CRCs, $P = 0.705$). In the previously mentioned study on PC-CRCs, BRAF-mutation was present in 28% of PC-CRCs vs 19% of prevalent CRCs (not significant) [32]. These percentages are comparable to our findings on FIT-interval CRCs (BRAF mutated in 30% of FIT-interval CRCs vs 12% of SD-CRCs, not significant). Previously, it has been shown that PC-CRCs were more likely CIMP positive and MSI than sporadic CRCs [33,34]. A separate study in a large PC-CRC cohort in the Netherlands also shows PC-CRCs to be more likely CIMP positive, MSI and BRAF mutated than prevalent CRCs [35]. This suggests that the molecular patterns observed in the interval cancers suggest that these cancers arose *via* non-polypoid precursors and/or serrated precursors. This may reflect that interval cancers, both FIT interval CRCs and PC-CRCs, indeed have a different biology compared to prevalent CRCs but at the same time pose technical challenges because of their morphology (difficult to be detected during colonoscopy) as well their lack of bleeding (difficult to be detected by FIT).

Some CNAs are associated to progression of adenoma to carcinoma chromosomal instability canonical pathway, and these are labeled as cancer associated events [19]. As of yet, no CNAs have been well characterized in the CRCs originating from SSLs. A study of serrated polyps, with data on a set of 38 serrated polyps (12 traditional serrated adenomas and 26 SSLs) found gains at chromosome 7, 13 and 15q [36]. The present study did not show any differences at these specific regions. However, FIT-interval CRCs had less frequent gains at chromosome 8q, and more frequent gains at chromosome 20p than SD-CRCs. As 8q is one of the genomic regions associated with the canonical adenoma-to-carcinoma progression, this finding could mean that to a certain extent, FIT-interval CRCs follow a different progression pathway.

As stated above, FIT-interval CRCs identified in multiple screening rounds represent a case-mix of cancers originated from, not only the difficult to detect NP-CRNs and sessile serrated polyps, but also classic adenomas. Precursor lesions could simply be missed by FIT just because of the low sensitivity of this test to detect advanced adenomas and not representing a different biology as reason for missing the lesion. Yet, while molecular differences were observed between FIT-interval CRCs and SD-CRCs, not all of these were statistically significant. Still these findings provide an indication that FIT-interval CRCs are a heterogeneous mixture of phenotypes and underlying molecular biology, including CRCs from flat serrated lesions, from flat adenomas, and others, that for whatever reason shed blood in a way that levels are, at least intermittently, below the limit of detection of the FIT test. In view of this, there is a clinical need to improve in screening tests for CRC early detection.

Multi-target molecular stool DNA (mt-sDNA) testing has recently been recognized as a valid CRC screening option by the American Cancer Society [37], and its test characteristics seem especially favorable for the detection of serrated lesions. In a large trial, mt-sDNA testing showed a higher detection rate of larger serrated sessile polyps than FIT (sensitivity of 42.4% for mt-sDNA-test and 5.1% for FIT, for serrated polyps > 1 cm) [10]. The combined sensitivity for advanced precancerous lesions was also higher for mt-sDNA testing than for FIT (42.4% vs 23.8%). However, although the sensitivities are better compared to FIT, the mt-sDNA test shows lower specificity, compared to FIT, which is very

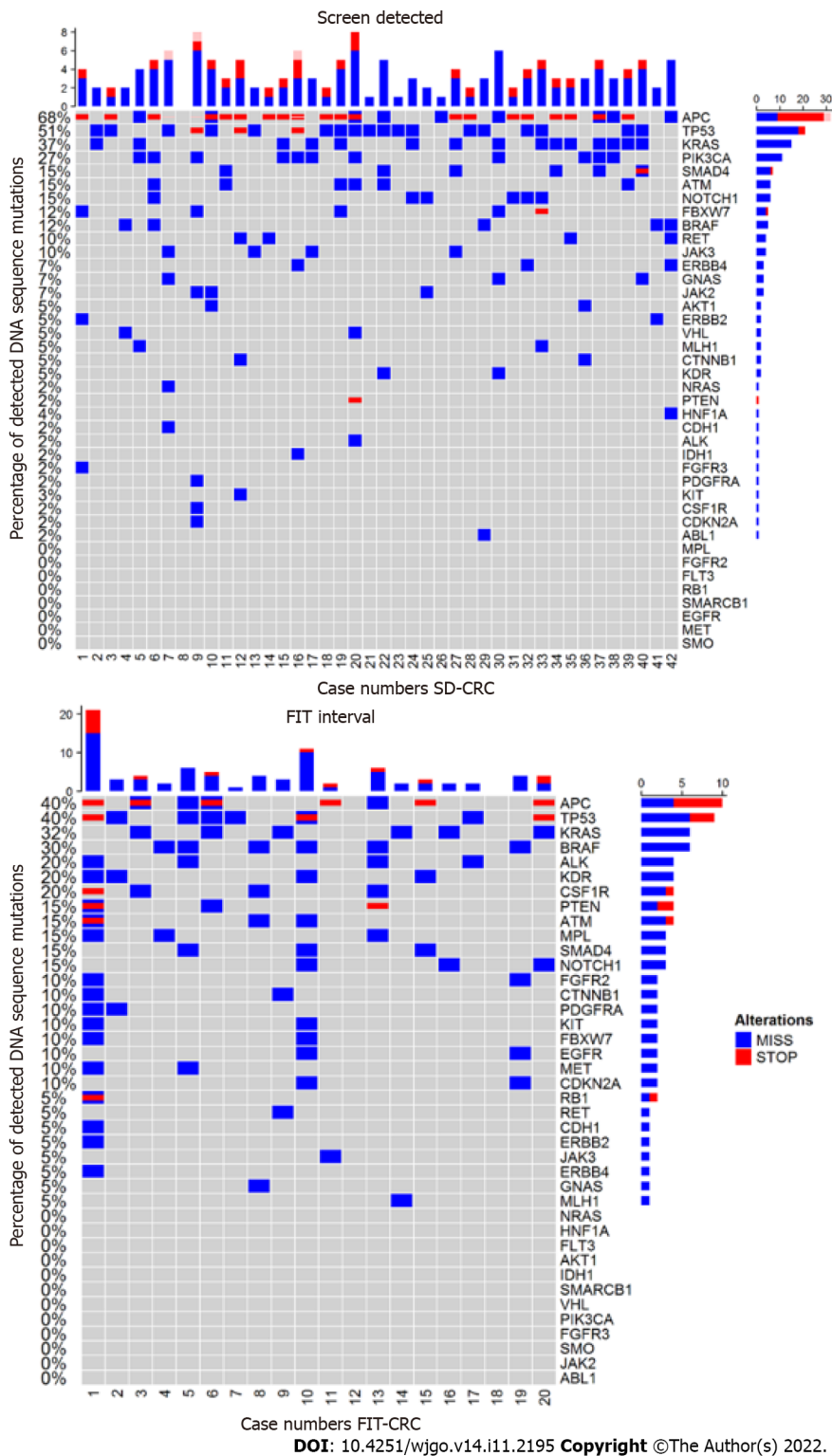


Figure 1 DNA sequence mutation analysis. A and B: Overview of mutations detected in screen-detected colorectal cancer (CRC) (A) and in fecal immunochemical test-CRC (B). SD-CRC: Screen-detected colorectal cancer; FIT-CRC: Fecal immunochemical test- colorectal cancer.

important in programmatic screening. Recently, a panel of protein markers detected in stool showed also a higher sensitivity for advanced adenomas without losing in specificity, in comparison to FIT. This protein-based approach would have the potential to improve effectivity of FIT screening without major impact for program logistics or cost effectivity[38]. Implementation of a more accurate test could have the potential to detect a substantial number of CRCs or precursor lesions that would otherwise result in FIT-interval CRCs.

Strengths of our study include that FIT-interval CRCs were identified over multiple rounds in a biennial FIT-based screening cohort, and tissue specimens of each tumor could be obtained. We were able to compare FIT-interval CRCs to a control group of SD-CRCs within the same screening

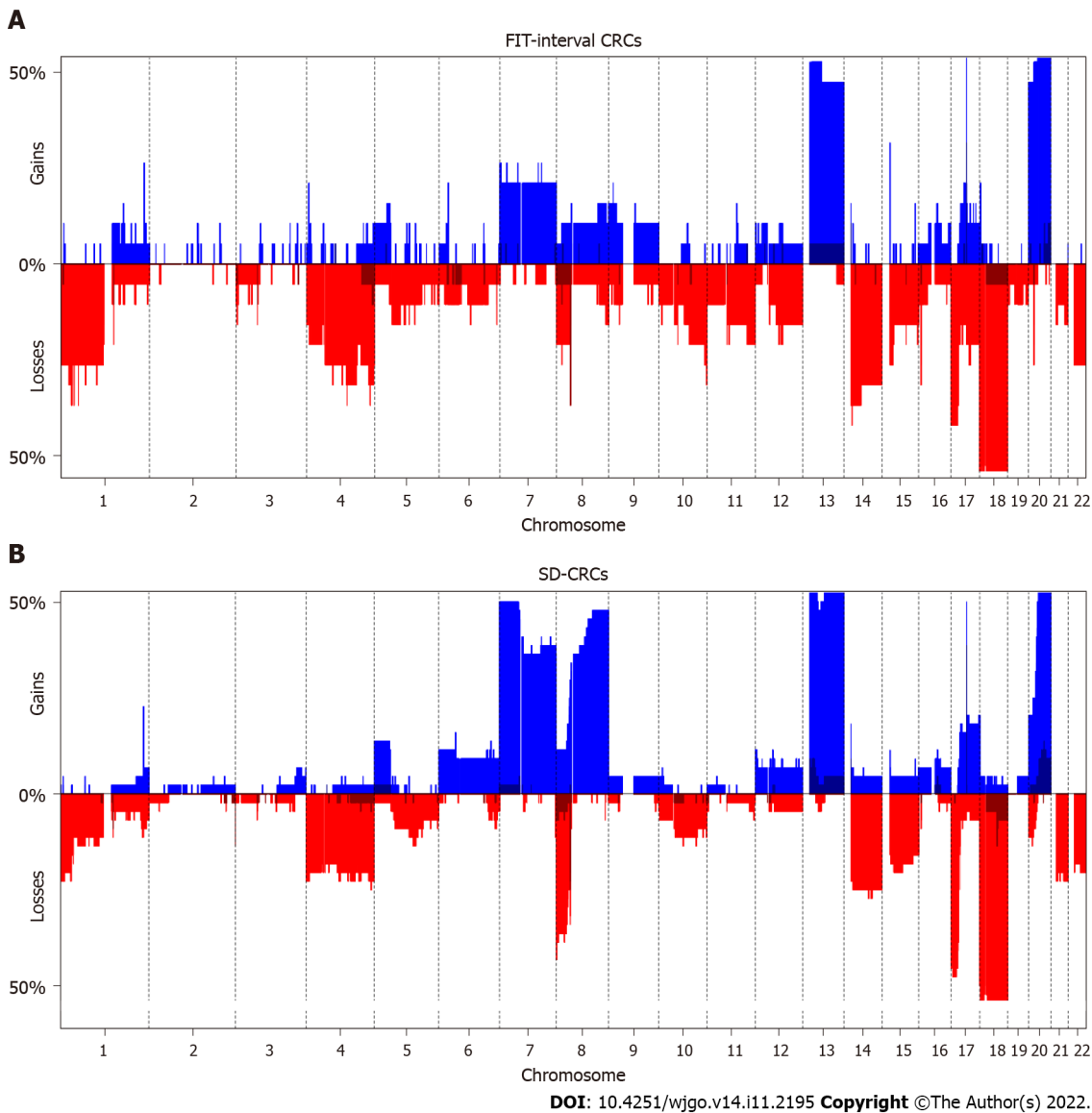


Figure 2 DNA copy number alterations in fecal immunochemical test-interval and screen-detected colorectal cancers, scale at 50%. A: Fecal immunochemical test-interval colorectal cancers (CRCs); **B:** Screen-detected CRCs. SD-CRC: Screen-detected colorectal cancer; FIT-CRC: Fecal immunochemical test- colorectal cancer.

population, region and time-span. However, an important limitation is the sample size. A total of 27 FIT-interval CRCs is still a limited number, reflecting the rarity of this entity, with inherent consequences for the statistical power of the study. To address this issue, a larger control group was composed through random selection in a 1:2 ratio. Due to budgetary and logistical constraints, we were not able to enlarge the control group. Also, we were not informed about the family history for cancer among the persons included in the study. Although rare, some of the FIT-interval CRCs might be related to familial CRC. So, although the findings of our study are suggestive for a difference in molecular make-up, further studies are needed to support our findings. We did not report tumor size and morphology of all CRCs, as this is not easy to determine in cancers (as compared to adenomas or SSLs). We were, therefore, not able to correlate size or morphology to the molecular analysis.

CONCLUSION

In conclusion, the present study provides evidence that SD-CRCs and FIT interval CRCs differ in the distribution of molecular tumor profiles. These findings can provide guidance on strategies for further improving stool-based CRC screening strategies. Future research should focus on the role of incorporating biomarkers in screening for identifying more CRCs during screening, thereby reducing the number of screening interval CRCs.

ARTICLE HIGHLIGHTS

Research background

Fecal immunochemical test (FIT) is effective in reducing colorectal cancer (CRC) but FIT testing is not perfect. FIT interval cancers, *i.e.* CRCs diagnosed after a negative FIT but before the next FIT invitation, still occur. FIT sensitivity for CRC is approximately 75%-85% and sensitivity drops to low detection rates for adenomas and even more so for sessile serrated lesions (SSLs).

Research motivation

In order to lower the number of missed lesions, we need to understand which lesions are more often missed by FIT in order to improve the screening strategy. Previous studies have shown that FIT sensitivity for SSLs is low, but a correlation between the occurrence of FIT interval cancers and the serrated pathway has not been established.

Research objectives

Our aim was to generate more insight in the molecular features of FIT-interval CRCs, as this could help in developing a more optimal screening strategy with the aim to reduce the incidence of FIT interval cancers.

Research methods

We compared the molecular make up of screen-detected CRCs (SD-CRCs) and FIT-interval CRCs, detected in a Dutch pilot-program of FIT-based screening. Molecular analyses included microsatellite instability (MSI), CpG island methylator phenotype (CIMP), DNA sequence mutations and copy number alterations.

Research results

FIT-interval CRCs were more often CIMP- and MSI-positive as compared to SD-CRCs. They also harbored more often *BRAF* and *PTEN* mutations as compared to SD-CRCs.

Research conclusions

The serrated pathway-associated molecular features seem to be more common in FIT-interval CRCs as compared to screen detected CRCs. This might indicate that proximal serrated lesions are overrepresented among FIT interval CRCs. Further research needs to be performed. Adding molecular markers of the serrated-pathway to the FIT needs to be further explored.

Research perspectives

These findings can provide guidance on strategies to further improve stool-based CRC screening with incorporating biomarkers for SSLs, thereby reducing the number of screening interval CRCs.

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FOOTNOTES

Author contributions: van der Vlugt M, Carvalho B, Meijer GA and Dekker E contributed to the study concept and design, acquisition of data, analysis and interpretation of data; van der Vlugt M and Carvalho B contributed to drafting of the manuscript and statistical analysis; Fliers J contributed to the study concept and design; Fliers J, Montazeri N, Rausch C, Grobbee EJ, van Engeland M, Spaander MCW, Meijer GA and Dekker E contributed to critical revision of the manuscript for important intellectual content; Montazeri N and Rausch C contributed to study design and statistical analysis; Grobbee EJ, van Engeland M and Spaander MCW contributed to the study concept and acquisition of data.

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Institutional review board statement: Ethics approval for performing FIT-based screening including linkage to the Netherlands Cancer Registry was provided by the Dutch National Health Council (WBO 2642467, 2832758, 3049078)

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Clinical trial registration statement: This study is registered at the Dutch Trial Registry. Registration number: NTR5874 (<http://www.trialregister.nl>).

Informed consent statement: All screenees participating in FIT screening filled in an informed consent form.

Conflict-of-interest statement: Meijer GA has research collaborations with Exact Sciences, Sysmex and Sentinel for other studies regarding early detection of colorectal cancer. The companies provide materials, equipment or (sample) analyses. Meijer GA is CSO and shareholder of CRCbioscreen BV. Carvalho B has several patents pending. Dekker E received a research grant from FujiFilm. She has received honorarium for consultancy from FujiFilm, Olympus, Tillots, GI Supply, CPP-FAP and PAION, and speakers' fees from Olympus, Roche, GI Supply and Norgine. Spaander MC received research support of Sysmex and Sentinel. All other coauthors don't have conflicts of interest.

Data sharing statement: Raw mutation data (concerning the mutation analysis and DNA copy number analysis) have been deposited in the European Genome-Phenome Archive with the study ID: EGAS00001004683.

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