Oncogene Mutations in Colorectal Polyps Identified in the Norwegian Colorectal Cancer Prevention (NORCCAP) Screening Study

Jon A. Lorentzen[1,2], Krzysztof Grzyb[1], Paula M. De Angelis[1], Geir Hoff[2–4], Tor J. Eide[1,2] and Per Arne Andresen[1]

[1]Department of Pathology, Oslo University Hospital, Oslo, Norway. [2]University of Oslo, Oslo, Norway. [3]Department of Medicine, Telemark Hospital, Skien, Norway. [4]Cancer Registry of Norway, Oslo, Norway.

ABSTRACT: Data are limited on oncogene mutation frequencies in polyps from principally asymptomatic participants of population-based colorectal cancer screening studies. In this study, DNA from 204 polyps, 5 mm or larger, were collected from 176 participants of the NORCCAP screening study and analyzed for mutations in *KRAS*, *BRAF*, and *PIK3CA* including the rarely studied *KRAS* exons 3 and 4 mutations. *KRAS* mutations were identified in 23.0% of the lesions and were significantly associated with tubulovillous adenomas and large size. A significantly higher frequency of *KRAS* mutations in females was associated with mutations in codon 12. The *KRAS* exon 3 and 4 mutations constituted 23.4% of the *KRAS* positive lesions, which is a larger proportion compared to previous observations in colorectal cancer. *BRAF* mutations were identified in 11.3% and were associated with serrated polyps. None of the individuals were diagnosed with de novo or recurrent colorectal cancer during the follow-up time (median 11.2 years). Revealing differences in mutation-spectra according to gender and stages in tumorigenesis might be important for optimal use of oncogenes as therapeutic targets and biomarkers.

KEYWORDS: colonic polyps, oncogenes, colorectal cancer screening

CITATION: Lorentzen et al. Oncogene Mutations in Colorectal Polyps Identified in the Norwegian Colorectal Cancer Prevention (NORCCAP) Screening Study. *Clinical Medicine Insights: Pathology* 2016:9 19–28 doi: 10.4137/CPath.S40143.

TYPE: Original Research

RECEIVED: May 13, 2016. **RESUBMITTED:** July 14, 2016. **ACCEPTED FOR PUBLICATION:** July 16, 2016.

ACADEMIC EDITOR: Dama Laxminarayana, Editor in Chief

PEER REVIEW: Six peer reviewers contributed to the peer review report. Reviewers' reports totaled 1515 words, excluding any confidential comments to the academic editor.

FUNDING: This project has been made possible by the Norwegian Extra Foundation for Health and Rehabilitation (grant number: 2013/FOM5627) and the Oslo University Hospital. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

CORRESPONDENCE: Jon.Andreas.Lorentzen@rr-research.no

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide with an estimated incidence of more than 1.3 million cases and close to 700,000 deaths annually.[1] The high prevalence and benefit of early detection and prophylactic removal of potential precursor lesions justify general CRC screening.[2] CRC develops from adenomas through a sequence of genetic events often initiated by the mutational inactivation of *APC* followed by oncogene mutations in *KRAS* and increasing genomic instability throughout the later stages of tumor development.[3] A gradual increase in *KRAS* mutation frequency during the evolution from early to advanced adenoma supports a direct role of *KRAS* in colorectal tumorigenesis.[4–6] A similar scenario has been suggested for CRC development from serrated polyps where the *BRAF* oncogene is commonly mutated.[7]

The activation of oncogenes is of clinical relevance both as potential targets for treatment and as markers for predicting treatment response.[8] Activated KRAS and BRAF are critical drivers in mitogen-activated protein kinase (MAPK) signaling, while PIK3CA activates PI3K/AKT signaling, both pathways are crucial in cell proliferation, differentiation, and migration.[9–11] Assessments of *KRAS* mutations in clinical studies typically focus on the most common mutations in codons 12 and 13 (exon 2) frequently occurring in advanced adenomas and CRCs.[5,12] Mutations in *KRAS* exons 3 and 4 have been reported in CRC, although at low frequencies of about 1.5% and 2%, respectively.[13–15] The frequency of *KRAS* exon 3 and 4 mutations in colorectal adenomas is mainly unknown. The valine to glutamine substitution in codon 600 (V600E) is the predominant *BRAF* mutation being significantly associated with microvesicular hyperplastic polyps (MVHPs) and sessile serrated polyps (SSP).[7,16] The V600E mutation is present in around 20% of unselected cases of metastatic CRC.[17] In traditional serrated adenomas (TSAs), both *BRAF* and *KRAS* mutations are common.[18] The mutation frequency of *PIK3CA* is low in adenomas, but increases significantly in CRC.[19] In general, *KRAS* and *BRAF* mutations are considered to be mutually exclusive, but both can appear together with mutated *PIK3CA*.[19]

Colorectal polyps are common in the normal population and for a majority of cases, the affected individuals remain oblivious to their presence.[20] During CRC screening studies, polyps are routinely classified histopathologically, but only limited data exist on their oncogene mutation profiles.

Our literature search revealed only one study in which a *KRAS* mutation frequency of 23% was reported in adenomas from average risk individuals, aged 50–84 years, and with positive fecal occult blood tests.[21] In order to establish a representative impression of oncogene mutations in sporadic colorectal polyps, samples were collected from the prospectively designed NORCCAP study, a unique population-based CRC screening study carried out in two counties of Norway in the period 1999–2001.[22] Flexible sigmoidoscopy (FS) was the primary screening tool followed by colonoscopy if any polyp sized ≥10 mm or a bioptically verified neoplasia of any size was identified. A total of 204 lesions measured to be ≥5 mm in diameter during endoscopy were collected from 176 individuals and subjected to oncogene analyses. Lesions <5 mm were excluded from the study because smaller lesions rarely develop into CRC without transitional stages of increasing growth and dysplasia.[23] Frequencies of mutations in *KRAS, BRAF,* and *PIK3CA,* including the mutations in rarely assessed *KRAS* exons 3 and 4 were examined. Clinical data were examined for associations between oncogene status and histopathological data, as well as for de novo or CRC relapse and survival during the follow-up time (median 11.2 years).[2]

Materials and Methods

Histological terminology. Adenomas include tubular, tubulovillous, and villous adenomas. Serrated polyps include hyperplastic polyps, sessile serrated polyps (also known as sessile serrated adenomas), and traditional serrated adenomas. Polyps include any kind of adenomas or serrated polyps.

Samples. The design and the results from the Norwegian Colorectal Cancer Prevention (NORCCAP) screening study have been published elsewhere.[2,22,24] In brief, eligible participants aged 50–64 years in two Norwegian counties were randomized directly from the Norwegian Population Register to receive screening with FS for comparison with a control group receiving no screening. Out of 12,960 screened participants, 6201 (47.8%) were identified with adenomas, serrated polyps, or adenocarcinomas and 1991 (15.4%) had lesions ≥5 mm. In accordance with the protocol, caps of 1–3 mm were sectioned laterally from opposite sides of polyps ≥5 mm in diameter and separately frozen in liquid nitrogen, while residual middle sections were fixed in formalin and embedded in paraffin (FFPE) for histological evaluation.[24] Because this procedure turned out to be resource-intensive, only a subset of the lesions was processed according to the original protocol. No other criteria than size were utilized for the selection of a series of 216 frozen lesions from 187 individuals for oncogene analysis. At the hospital of Telemark, 159 samples were successively frozen from January 1999 to December 2000 and in October 2001 and 57 were likewise collected at the Oslo University Hospital from October 1999 to November 2000. Twelve samples were excluded from analysis due to insufficient clinical data or poor DNA quality, leaving a total of 204 samples from 176 individuals, of which 128 (72.7%) were males and 48 (27.3%) females. Among the 176 individuals, 21 (11.9%) had more than one (range 2–4) lesion ≥5 mm of which all were included in this study. Five of the 204 lesions fulfilling the inclusion criteria were adenocarcinomas (all pT2N0MX). The mean age at the time of screening was similar for males and females (58.3 and 58.4 years, respectively). Polyps originally classified as hyperplastic were reclassified (by KG) for subdivision into hyperplastic polyps, SSP, and TSA according to the revised WHO guidelines of 2010.[25] Grading of dysplasia was changed accordingly from slight/moderate to low grade and from severe/carcinoma-in-situ to high-grade dysplasia. Proximal location corresponds to cecum, ascending and transverse colon, while distal colon contains the segments from left splenic flexure to rectum. Polyps were further classified into large (≥10 mm in diameter) and small size (5–9 mm). The research presented herein was approved by the Ethics committee of South-East Norway.

DNA analysis. DNA was extracted using a QIAsymphony SP instrument (Qiagen) according to the manufacturer's protocol (QIAsymphony SP Protocol Sheet: Tissue_HC_200_V7_DSP, 2012). The samples were screened for mutations in *KRAS* (NM_004985.3; exons 2, 3, and 4), *BRAF* (NM_004333.3; exon 15), and *PIK3CA* (NM_006218.2; exons 9 and 20) using high resolution melting (HRM) analysis on a LightCycler 480 (Roche Diagnostics) to distinguish aberrant sequences from normal sequences. All samples with aberrations were subjected to Sanger sequencing on a 3130 Genetic Analyzer (Applied Biosystems Inc.). The sequence analyses were performed in forward and reverse directions except for *KRAS* exon 4 where two different forward primers were used for verification of results due to suboptimal results with the exon 4 reverse primer. The sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific) and the sequencing products were purified using the BigDye XTerminator® Purification Kit (ThermoFisher Scientific). All HRM and sequencing reactions were performed in duplicate for result validation. Sequencing results were analyzed with the software SeqScanner 2 version 2.0 (Applied Biosystems Inc.) and Mutation Surveyor version 4.0 (SoftGenetics®). Primers used for HRM analysis and sequencing are listed in Supplementary Table 1. Mutations verified by sequencing were checked against the catalogue of somatic mutations in cancer (COSMIC) database.[26]

Statistical analysis. *P* values were calculated using the univariate chi-square test or mid-*P* exact test (if one or more expected values were less than 5) on the count data and with the Mann–Whitney *U*-test on the continuous variables. *P* values < 0.05 were defined as statistically significant. The statistical calculations were performed using IBM SPSS Statistics software version 21 (SPSS Inc.) and OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 3.03a).

Results

Histopathological and oncogene data are listed in Table 1. According to data from the Norwegian Cancer Registry, none

Table 1. Histopathological data and oncogene status in relation to gender in 176 individuals participating in the NORCCAP study.

VARIABLE	ALL (%) N = 176	MALES (%) N = 128	FEMALES (%) N = 48	*P*-VALUE
Number of lesions ≥ 5 mm				
Multiple	21 (11.9)	16 (12.5)	5 (10.4)	0.70
Single	155 (88.1)	112 (87.5)	43 (89.6)	0.70
Histology				
Tubular adenomas*	121 (68.8)	87 (68.0)	33 (68.8)	0.92
Tubulovillous adenomas†	31 (17.6)	22 (17.2)	9 (18.8)	0.81
Serrated polyps	16 (9.1)	13 (10.2)	3 (6.3)	0.45
Tubular adenomas and serrated polyps	3 (1.7)	3 (2.3)	0 (0.0)	0.38
Adenocarcinomas‡	5 (2.8)	2 (1.6)	3 (6.3)	0.15
Oncogene mutations				
Any oncogene	63 (35.8)	41 (32.0)	22 (45.8)	0.09
KRAS	45 (25.6)	25 (19.5)	20 (41.7)	0.003
BRAF	17 (9.7)	15 (11.7)	2 (4.2)	0.13
PIK3CA§	2 (1.1)	1 (0.8)	1 (2.1)	0.55

Notes: *Including one individual with a nonspecified adenoma. †Including five individuals with both tubular and tubulovillous adenomas. ‡Including one individual with a synchronous serrated polyp. §One *PIK3CA*-positive (H1047R) case concomitant with a *KRAS* codon 146 (A146T) mutation.

of the analyzed individuals relapsed from previous adenocarcinomas or developed de novo malignant tumors of the colon during the follow-up time (median 11.2 years). Independent of histology, 35.8% of the individuals had at least one polyp with an oncogene mutation. *KRAS* mutations predominated (25.6%) followed by *BRAF* (9.7%) and *PIK3CA* (1.1%). There was a significant difference in *KRAS* mutation frequency, between males (19.5%) and females (41.7%; $P = 0.003$; Fig. 1). Electrophoretograms illustrating mutations in *KRAS*, *BRAF*, and *PIK3CA* are shown in Supplementary Figure 1. No significant associations were found between mutational status, polyp multiplicity, and age.

Associations between oncogenes and histopathological features of the individual lesions are summarized in Table 2 and visualized in Figure 2. The overall *KRAS*, *BRAF*, and *PIK3CA* mutation frequencies were 23.0%, 11.3%, and 1.0%, respectively. *KRAS* mutations were significantly associated with tubulovillous histology ($P < 0.001$) and large size (average: 13.5 mm; $P = 0.002$). The tubulovillous adenomas were associated with high-grade dysplasia ($P < 0.001$, data not shown) and had a *KRAS* mutation frequency of 50.0% when based on exon 2 alone and 65.6% when exons 3 and 4 were included (both $P < 0.001$). Polyps with mutations in exons 3 and 4 were associated with large size (average: 15.3,

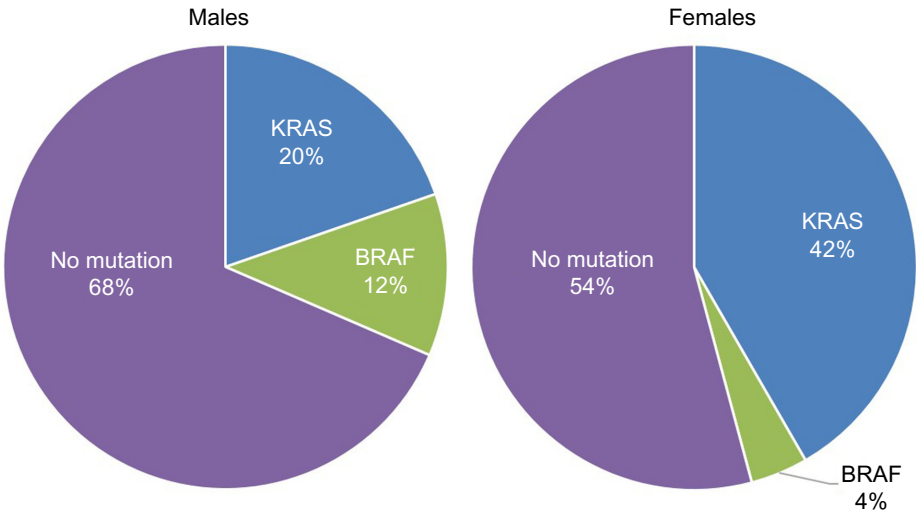

Figure 1. The percentage distribution of the presence of *KRAS*- and *BRAF*-positive lesions among males and females. *PIK3CA* mutations are not included due to the low number of mutations.

Table 2. Oncogene mutation frequencies distributed according to histology, location, dysplasia, and size in 204 colorectal lesions from 176 individuals.

VARIABLE	NUMBER (%)	AVERAGE mm SIZE	*KRAS* ALL	*KRAS* EXON 2*	*KRAS* EXON 3/4	*BRAF* EXON 15	*PIK3CA* EXON 9/20
All lesions	204 (100)	11.3	47 (23.0)	36 (17.7)	11 (5.4)	23 (11.3)	2 (1.0)
Adenomas	174 (85.3)	11.3	45 (25.9) ***P* = 0.021**	34 (19.5)	11 (6.3)	1 (0.6)	2 (1.4)
Tubular†	142 (69.6)	10.5	24 (16.9)	18 (12.7)	6 (4.2)	1 (0.7)	1 (0.7)
Tubulovillous	32 (15.7)	14.8 ***P* = 0.001**	21 (65.6) ***P* < 0.001**	16 (50.0) ***P* < 0.001**	5 (15.6) ***P* = 0.019**	0	1‡ (3.2)
Serrated polyps	25 (12.3)	8.9	1§ (4.0)	1§ (4.0)	0	22 (88.0) ***P* < 0.001**	0
Adenocarcinomas	5 (2.9)	21.2	1 (20)	1 (20)	0	0	0
Location¶							
Proximal	21 (10.3)	12.5	4 (19.0)	4 (19.0)	0	2 (9.5)	1 (4.8)
Distal	181 (88.7)	11.1	42 (23.2)	31 (17.1)	11 (6.1)	20 (11.1)	1§ (0.6)
Dysplasia**							
Low grade	152 (74.5)	10.7	35 (23.0)	26 (17.1)	9 (5.9)	6 (4.0)	2 (1.3)
High grade	28 (13.7)	13.7 ***P* = 0.005**	10 (35.7)	8 (28.6)	2 (7.1)	0	0
Average mm size	–	–	13.5 ***P* = 0.002**	12.9 ***P* = 0.02**	15.3 ***P* = 0.047**	8.4 ***P* = 0.005**	5

Notes: The samples are evaluated individually and independent of origin. *P* values for statistically significant positive associations are highlighted. *KRAS* mutations include codons 12 and 13 in exon 2 and codons 61, 117, and 146 in exons 3 and 4. *Including two samples with both codon 12/13 and a codon 61 *KRAS* mutation. †Including one case of an unspecified adenoma. ‡Combined with a *KRAS* (A146T) mutation. §MVHP with cytological dysplasia. ¶Two samples had unknown location. **19 of the serrated polyps had no dysplasia and the adenocarcinomas were not graded according to dysplasia.

$P = 0.047$) and tubulovillous histology ($P = 0.019$). Histological re-evaluation of the serrated polyps resulted in a predominance of sessile ($n = 13$) and unspecified ($n = 8$) serrated polyps with *BRAF* mutation frequencies of 84.6% and 100.0%, respectively (both $P < 0.001$). The two cases of TSA and one case of MVHP also tested *BRAF* mutation-positive, whereas another case of MVHP with cytological dysplasia tested *KRAS* mutation-positive.

KRAS mutation frequencies in adenomas according to size (5–9 mm and ≥10 mm) and gender are listed in Table 3. Lesions originating from females were significantly associated with a high *KRAS* mutation frequency, especially among large tubular adenomas. The high mutation frequency was mainly attributed to mutations in codon 12 ($P < 0.001$), as remaining *KRAS* mutations were more evenly distributed among males and females ($P = 0.987$, not shown). None of the females had more than one *KRAS*-positive lesion excluding multiple *KRAS*-positive polyps in a single individual as cause of the higher frequency. In contrast to the high mutation frequencies for *BRAF* observed in serrated polyps independent of size, the *KRAS* frequency increased significantly from 9.0% to 24.7% between 5–9 mm and ≥10 mm tubular adenomas, respectively.

When *KRAS* mutations were examined in more detail (Supplementary Table 2), the c.35G>A (G12D) and c.35G>T (G12V) mutations predominated and the *KRAS* codon 13 mutations identified were exclusively c.38G>A (G13D). Mutations identified in *KRAS* exon 3 resulted in Q61H and were caused by either a c.183A>C or a c.182A>T transversion. The mutations c.351A>T, c.436G>A, and c.436G>C in exon 4 result in K117N, A146T, and A146P, respectively. *BRAF* mutations were dominated by the common c.1799T>A transversion resulting in V600E. The only non-V600E mutation was a c.1781A>G transition resulting in D594G and was observed in a 10 mm tubular adenoma. One apparently tandem *KRAS* mutation (c.34_35GG>CA; Fig. 3) observed in a tubulovillous adenoma could result in either a mono-allelic G12H change or in G12D and G12R if caused by bi-allelic c.35G>A and c.34G>C mutations, respectively, or representing different clonal cell populations with mono-allelic mutations. Three other cases of multiple *KRAS* mutations in one polyp were represented by one case of two codon 12 (c.35G>A + c.35G>C) mutations (Supplementary Fig. 2) and two cases involving Q61H combined with a codon 12 or a codon 13 mutation, respectively. Heterozygosity and intratumor heterogeneity are options for all three cases.

Discussion

Polyps precede a majority of colorectal cancers, and activation of oncogenes is essential for tumor progression. Few studies have addressed the prevalence of oncogene mutations in colorectal lesions from a population-based CRC screening study. The NORCCAP study revealed that approximately 15% of the screened individuals between the ages of 50 and 64 had

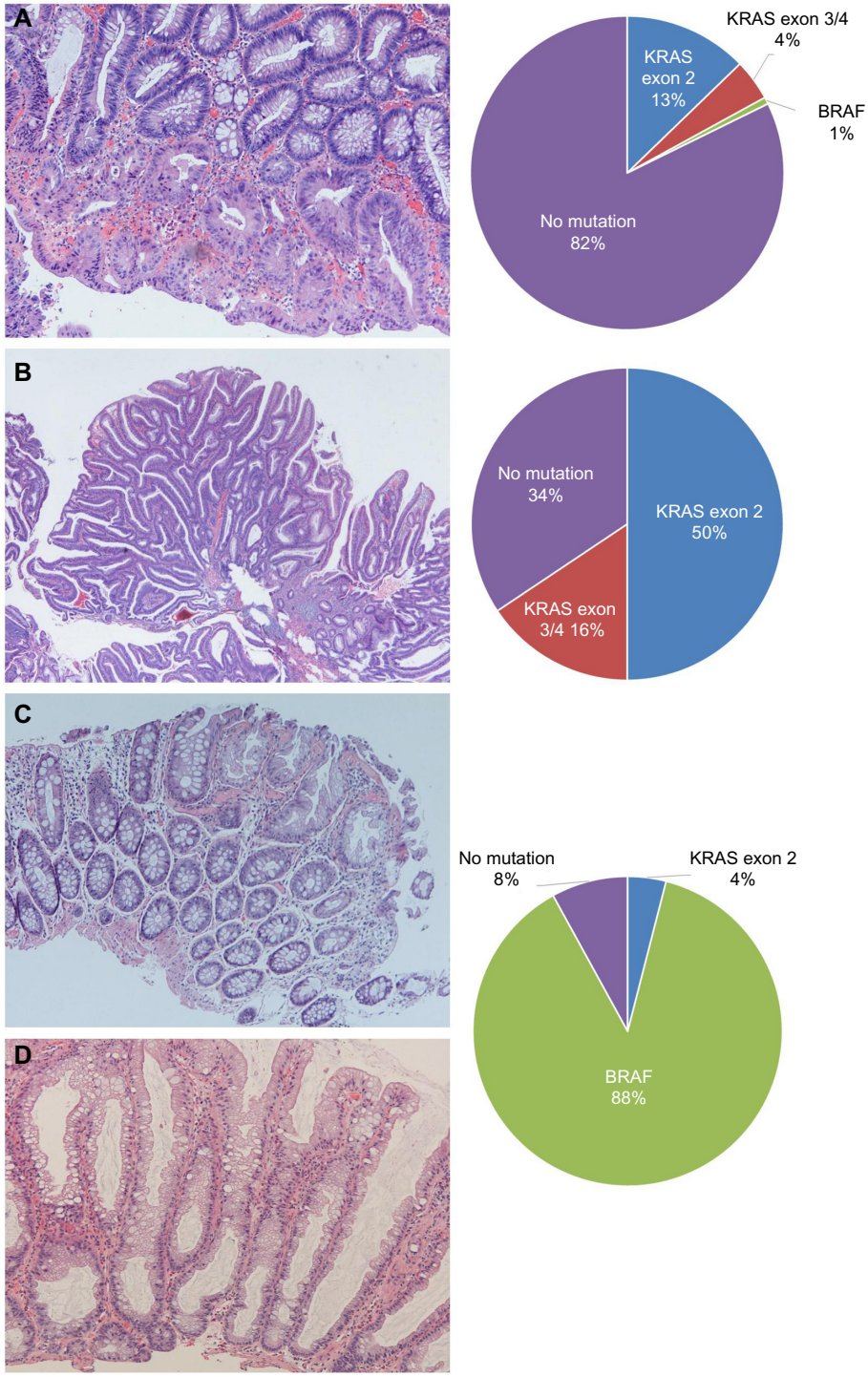

Figure 2. Pictures of the different polyp types represented in this study together with the mutation spectra of *KRAS* and *BRAF* mutations within each type.
Notes: A: A tubular adenoma with focal high-grade dysplasia (100 × magnification). **B**: A tubulovillous adenoma (20 × magnification). **C**: A microvesicular hyperplastic polyp (MVHP) (100 × magnification). **D**: A sessile serrated polyp. The column to the right shows pie charts of the *KRAS* and *BRAF* mutation frequencies in tubular adenomas (top), tubulovillous adenomas (middle), and serrated polyps (bottom). *PIK3CA* mutations are not included due to the low number of mutations.

lesions of 5 mm or larger. Among the 204 lesions collected from 176 individuals, 35% tested positive for oncogene mutations. As expected, the most frequent mutations were detected in *KRAS* and *BRAF* being strongly associated with adenomas and serrated polyps, respectively. Unexpectedly, a significantly higher frequency of *KRAS* codon 12 mutations was found in adenomas from females and a noteworthy increase in the *KRAS* mutation frequency was seen when exon 3 and 4 mutations were included. Oncogene heterogeneity and variation in its functional consequences necessitate a more precise

Table 3. *KRAS* mutation frequency in all lesions distributed according to gender and adenomas distributed according to size category (5–9 mm and ≥10 mm) and gender.

		KRAS MUTATIONS %					*KRAS* CODON 12 MUTATIONS %			
	N	TOTAL	*P*-VALUE*	MALES	FEMALES	*P*-VALUE†	TOTAL	MALES	FEMALES	*P*-VALUE†
All lesions	204	23.0 47/204		18.0 27/150	37.0 20/54	0.004	13.7 28/204	8.7 13/150	27.8 15/54	<0.001
All adenomas	172	26.2 45/172		21.8 27/124	37.5 18/48	0.035	15.1 26/172	10.5 13/124	27.1 13/48	0.006
5–9 mm	77	18.2 14/77	0.03	19.6 11/56	14.3 3/21	0.62	11.7 9/77	10.7 6/56	14.3 3/21	0.66
≥10 mm	95	32.6 31/95		23.5 16/68	55.6 15/27	0.003	17.9 17/95	10.3 7/68	37.0 10/27	0.004
Tubular adenoma	140	17.1 24/140		12.9 13/101	28.2 11/39	0.03	9.3 13/140	5.0 5/101	20.5 8/39	0.01
5–9 mm	67	9.0 6/67	0.014	10.4 5/48	5.3 1/19	0.51	3.0 2/67	2.1 1/48	5.3 1/19	0.57
≥10 mm	73	24.7 18/73		15.1 8/53	50.0 10/20	0.004	15.1 11/73	7.5 4/53	35.0 7/20	0.008
Tubulovillous adenoma	32	65.6 21/32		60.9 14/23	77.8 7/9	0.41	40.6 13/32	34.8 8/23	55.6 5/9	0.32
5–9 mm	10	80.0 8/10	0.28	75.0 6/8	100 2/2	0.62	70.0 7/10	62.5 5/8	100 2/2	0.47
≥10 mm	22	59.1 13/22		53.3 8/15	71.4 5/7	0.47	27.3 6/22	20.0 3/15	42.9 3/7	0.32

Notes: Two adenomas were not included due to missing size information. *5–9 mm vs. ≥10 mm. †Males vs. females.

specification of mutations relative to tumor stage and histology for a better understanding of polyp diversity and development. Oncogenes in premalignant lesions should probably be considered in the context of its tumor-suppressive capabilities and not only as an unrestrained proliferative force.

Oncogenes and histology. Screening for mutations with HRM analysis prior to sequencing is a sensitive and cost-effective method.[27] However, false negative results might have occurred in cases of low or no representation of oncogene-positive cells in the analyzed tissue due to tumor cell heterogeneity. When based on *KRAS* mutations in exon 2 alone versus exons 2, 3, and 4 combined, the overall mutation frequencies in adenomas from screened individuals were 19.5% and 25.9%, respectively. These are frequencies at the lower end of the wide range (12%–68%) reported in adenomas from previous clinical studies, commonly limited to the analysis of exon 2.[4–6,28–30] Differences in design and inclusion criteria affect the composition of adenoma stages and contribute to the highly variable frequencies found in various studies.[4–6,29,30] One extensive study revealed that the *KRAS* mutation frequency increased gradually from less than 10% in small adenomas with low-grade dysplasia and no villous components, to close to 60% in adenomas with high-grade dysplasia and villousness.[5] Thus, the overall low frequency as observed in the present study is in accordance with the large proportion of early-stage adenomas expected to be found in asymptomatic individuals. However, an increasing frequency according to adenoma stage is still observed as adenomas measuring 5–9 mm and ≥10 mm had mutation frequencies of 18.2% and 32.6%, respectively, while the more advanced tubulovillous adenomas reached frequencies higher than 65%. Serrated polyps were strongly associated with *BRAF* V600E as expected from observations in clinical studies.[16,31] The high *BRAF* V600E mutation rate in serrated polyps independent of size and differentiation indicates a direct role at an earlier stage than for *KRAS* in adenomas. Interestingly, the only lesion that tested positive for a non-V600E *BRAF* mutation was a 10 mm tubular adenoma with a c.1781A>G transition predicting a D594G substitution at the protein level. This mutation has a minimal impact on MAPK signaling in vitro and has been associated with good response to EGFR-directed therapy and a favorable prognosis in metastatic CRC patients.[14,32] Several individuals with multiple polyps had both serrated and adenomatous polyps associated with *BRAF* and *KRAS* mutation, respectively. Multiple serrated polyps have been associated with an increased risk of synchronous advanced neoplasia.[33] As expected, the prevalence of *PIK3CA* mutations was low (~1%) and in accordance with other and more comprehensive studies of colorectal polyps.[19]

KRAS mutation spectrum. The vast majority of *KRAS* mutations in CRC are identified in codons 12 and 13 of exon 2. Consequently, these have become the cornerstone of *KRAS* oncogene analyses.[5,13] When analyzing adenomas from screened individuals, the addition of exon 3 and 4 mutations to the commonly identified exon 2 mutations increased the frequencies with around 30%: from 12.9% to 16.9% and 50.0% to 65.6% in tubular and tubulovillous adenomas, respectively. The prevalence of exon 3 and 4 mutations in

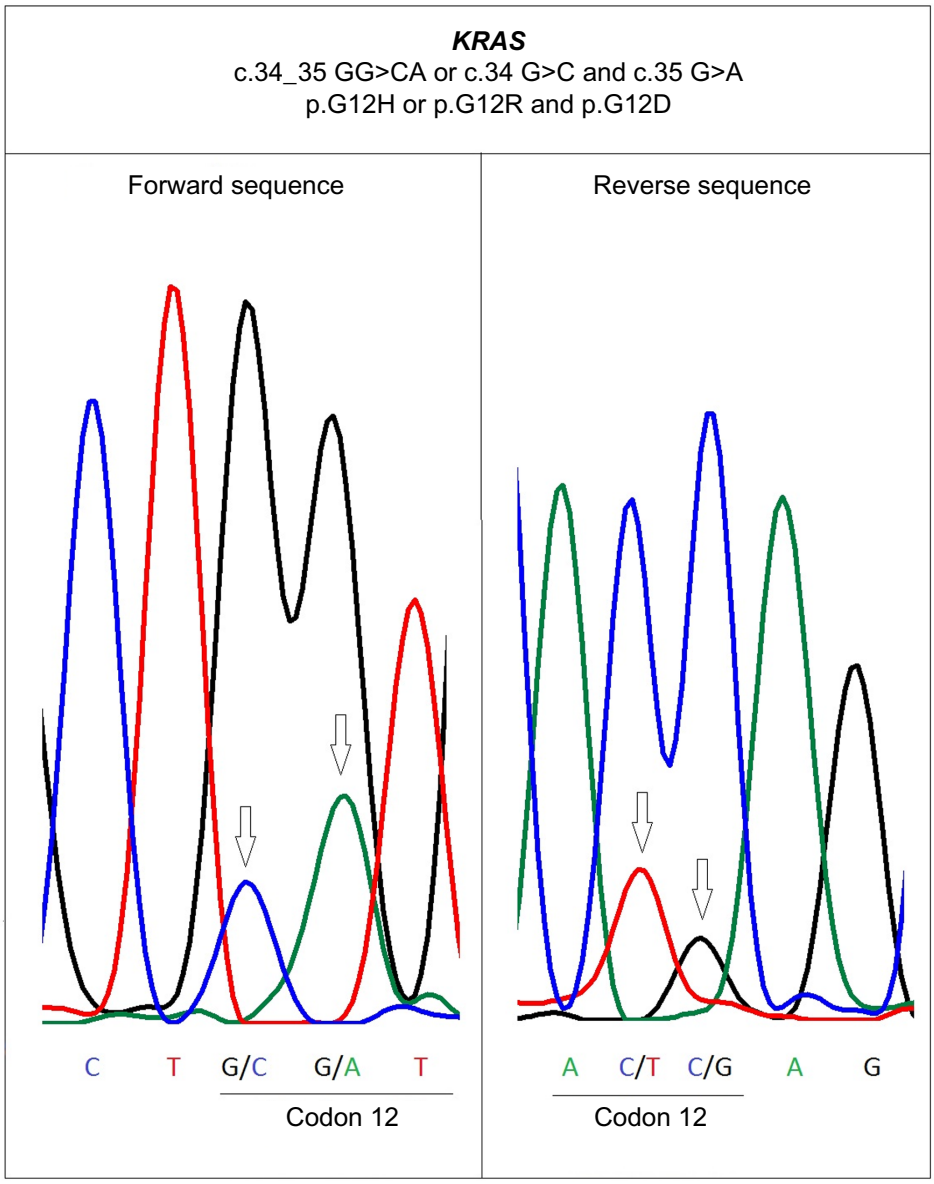

Figure 3. Electrophoretogram of the sequenced DNA from a tubulovillous adenoma in the forward and the reverse directions showing a mutation in two nucleotides (position 34 G>C and 35 G>A) in codon 12 of *KRAS*.

adenomas is unclear due to limited data from previous studies. However, when comparing with studies analyzing CRC, a comparatively higher frequency of exon 3 and 4 mutations are observed in adenomas (Fig. 4).[13–15] Important differences in oncogenic and transformative potentials have been demonstrated for the various *KRAS* mutations. In CRC, the majority of codon 12 mutations are represented by G12D (45%) followed by G12V (30%).[12–14,34] These mutations were equally distributed in adenomas from the screened individuals (35%), while others have reported G12V to dominate.[5] Thus, the seven times higher proliferation activity demonstrated for G12V when compared to G12D is not necessarily reflected in a higher frequency in CRC.[35] However, G12V has been associated with a poorer prognosis than G12D in patients with Dukes C tumors.[12] Whether this is explained by regulatory differences between various mutants is not clear, but G12V and G12D have been shown to interact with different pathways in mouse models.[36] Two adenomas had two mutations detected in the same codon and may represent bi-allelic mutations or reflect clonal heterogeneity. Such rare cases have previously been reported in CRC and similar observations in adenomas indicate that tumor heterogeneity can occur at an early stage if different mutations represent multiple clones.[37]

Gender-specific variation. The lower representation of females in the present study reflects the lower incidence of polyps normally detected in females.[22,38] The discrepancy may explain the reduced CRC-preventive effect of screening in the female population as experienced in the NORCCAP study.[2] A lower incidence probably results from a combination of a detection biases and gender-specific biological conditions.

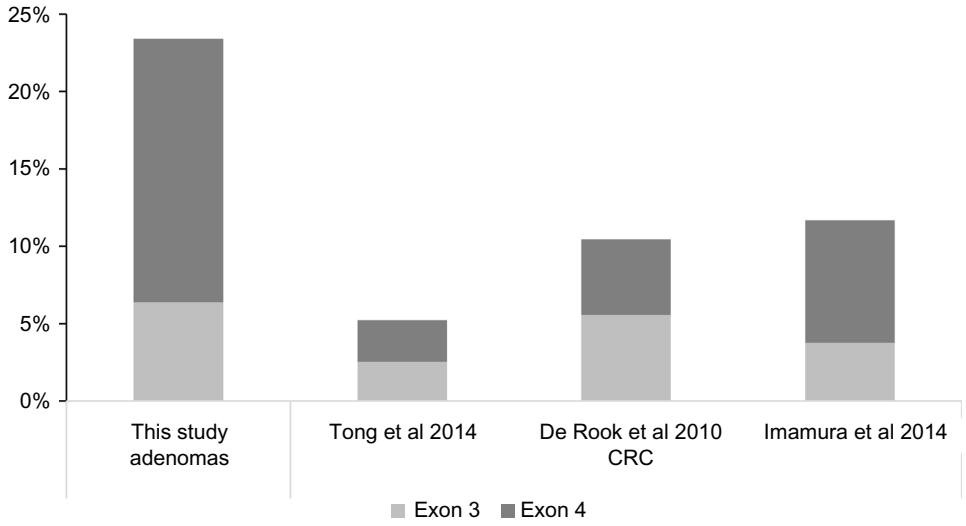

Figure 4. The percentage contribution of exons 3 and 4 to the total *KRAS* mutations in 45 *KRAS* positive adenomas from the present study and *KRAS*-positive CRCs from three publications.[13–15]

Polyps located in the proximal part of the colon, which are more common in women, would be missed if results from FS did not meet the criteria for a full colonoscopy.[22,39] Also, flat and sessile polyps are more common in the proximal colon and consequently easily overlooked during full colonoscopy. Biologically, the increased levels of natural estrogens during pregnancy and the use of synthetic estrogens in contraceptives and hormone-replacement therapies, result in the lower production of bile acids, thereby reducing the level of oxidative stress and DNA damage.[40,41] Estrogens thus appear to offer some protection against polyp development. The disparity in polyp occurrence has raised discussions as to whether women should attend screening at an older age than men.[42] However, the significantly higher *KRAS* mutation frequency in females argues against postponed screening due to the association between *KRAS* mutations and advanced adenomas.[5] In this study, both tubular and tubulovillous adenomas were more frequently *KRAS* mutated in females than in males with a particularly large difference in larger (≥10 mm) tubular adenomas (50.0% vs. 15.1%, respectively; Table 3). The steroid adrenal steroid dihydroepiandrosterone (DHEA) has been hypothesized to have a protective effect toward mutationally activated KRAS through post-translational interaction with the RAS protein.[39] However, the levels of DHEA decrease with age and especially in women after menopause, possibly contributing to a selection of *KRAS* mutation positive polyps in females.[39] This complies with observations of higher *KRAS* mutation frequencies in CRC from female than in male patients.[13,43] Some studies, however, find the higher *KRAS* frequency in females only relevant in younger individuals or limited to CRC located in the distal colon or rectum.[39,44–46] These factors are consistent with our data as both young age in terms of CRC risk and dominance of distally located polyps are present. Why the elevated *KRAS* frequencies in females are attributed to codon 12 mutations exclusively is interesting and should be studied further for the disclosure of potential gender-specific differences in colorectal tumorigenesis.

CRC Tumorigenesis

The gradient of increasing *KRAS* mutation frequency throughout adenoma development is broken during the transition to adenocarcinoma as the frequencies in advanced adenomas in general are higher than the 30–45% frequency reported in CRC.[5,6,13–15,45] This indicates that several *KRAS* positive adenomas never progress to CRC. This is probably explained by the potential of oncogenes to induce premature senescence and as such undergo cell cycle arrest.[47] However, even essentially tumor suppressive, secretion of proteins from senescent cells may contribute to neoplastic growth in neighboring cells.[48] This fits well with observed cases of *KRAS*-positive adenomas containing mutation-negative carcinomas.[49] Our observations that 5–9 mm tubulovillous adenomas had a higher mutation frequency (80.0%) than those ≥10 mm (59.1%) support the role of activated KRAS in restraining rather than promoting polyp growth (Table 3). A higher *KRAS* mutation frequency in smaller compared to larger tubulovillous adenomas has been reported by others.[5] Furthermore, the differences between somatic *KRAS* genotypes in adenomas and CRC suggest that certain *KRAS* mutations are more likely to promote the transition to CRC than others. Whether these differences also mean that certain *KRAS* mutations are more likely to induce or maintain replicative senescence than others remain to be seen. Elevated levels of GTP-bound (activated) KRAS are common for the mutations identified in this study, but this does not exclude differences in their prognostic and predictive properties.[50] While codon 12 and 13 mutations are known to restrict GAP-mediated GTP hydrolysis of the RAS protein through steric hindrance, codon 61 mutations disrupt a hydrogen bond between RAS and GAP, and codon 117 and 146 are located in a region predicted to interact with the guanine

base of GDP/GTP.[50,51] The G13D variant has been found to act more like the *KRAS* wild type in terms of response to EGFR-targeted therapy.[52] Exon 3 and 4 mutations have been associated with resistance to EGFR-targeted therapy, but have also been linked to a better prognosis when compared with *KRAS* codon 12–13 mutations in CRC.[51] In this study, mutations in *KRAS* exons 3 and 4 were associated with advanced adenomas (tubulovillous histology and large size), indicating a potent promotion of early tumorigenesis. However, the lower mutation frequencies observed in CRC studies suggest that the influence might decline at later stages of tumor development. The prevalence of *PIK3CA* mutations in CRC is much higher (10%–30%) than in polyps (~1%), indicating a more prominent role for *PIK3CA* mutations at malignant stages.[19] Assessment of *NRAS* was not included in this study because of the low frequency (<5%) observed for mutations in this gene in CRC.[14] However, with its homology to *KRAS* and similar significance as predictive marker in EGFR-directed therapy, *NRAS* should be included in future studies as little is known about its prevalence and role in colorectal polyps. Overexpression of constitutively active mutants of RAS oncogenes, including NRAS, results in classical senescence-like response in human melanocytes.[53] Consequently, all oncogenes should be of interest for processes including cell cycle arrest like oncogene-induced senescence.

Protocols based on Sanger sequencing remain the most available methods for in-house oncogene analysis in most diagnostic and research laboratories. Empirically, these protocols should be adequate for comparison with other studies. The major limitation of this study, though, was the small number of individuals accessible for analysis leaving little room for establishing meaningful associations between age, polyp multiplicity, prognosis, and oncogene genotype. Potential roles of oncogene induced senescence in CRC tumorigenesis remain to be determined. Colorectal polyps are common and relatively few make the transition to a malignant state. Little is known about the factors involved in the transitions and whether these can be directly linked to the proliferative effect of oncogenes or to the senescence-associated secretory phenotype induced by oncogenes. In summary, this study shows oncogene heterogeneity and mutation frequencies as expected in polyps from an average risk population. It was, however, unexpected that mutations in *KRAS* exons 3 and 4 constituted a large proportion of the total *KRAS* mutation load compared to previous studies on CRC and that mutations in exon 3 and 4 were significantly associated with advanced adenomas. The associations between *KRAS* codon 12 mutations and polyps in females deserve closer investigations for a better understanding of gender-specific tumorigenesis. Even when studied exhaustively, gaps remain in the knowledge of oncogene diversity and the relationship to histological subtypes, tumorigenic stage and gender. Their predictive and prognostic significance are likely to vary depending on the context they are acting within.

Acknowledgments

The authors wish to thank Lisa Yuen Løvold for her technical assistance and Magnus Løberg for providing data from the NORCCAP database.

Author Contributions

Conceived and designed the experiments: JAL, TJE, PAA. Analyzed the data: JAL, PAA. Wrote the first draft of the manuscript: JAL, PAA. Contributed to the writing of the manuscript: JAL, PMD, GH, TJE, PAA. Agree with manuscript results and conclusions: JAL, KG, PMD, GH, TJE, PAA. Jointly developed the structure and arguments for the paper: JAL, KG, PMD, GH, TJE, PAA. Made critical revisions and approved final version: JAL, KG, PMD, GH, TJE, PAA. All authors reviewed and approved of the final manuscript.

Supplementary Material

Supplementary Table 1. Sequences of the primers used for high resolution melting (HRM) and sequencing analysis.

Supplementary Table 2. A detailed overview of the oncogene mutations identified.

Supplementary Figure 1. Electrophoretograms showing mutation in *KRAS, BRAF* and *PIK3CA*.

Supplementary Figure 2. Electrophoretograms showing two mutations at position 35 (G>A and G>C) in codon 12 of *KRAS*.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;136(5):E359–86.
2. Holme Ø, Løberg M, Kalager M, et al. Effect of flexible sigmoidoscopy screening on colorectal cancer incidence and mortality. *JAMA*. 2014;312(6):606.
3. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61(5):759–67.
4. Yadamsuren E, Nagy S, Pajor L, Lacza A, Bogner B. Characteristics of advanced- and non advanced sporadic polypoid colorectal adenomas: correlation to KRAS mutations. *Pathol Oncol Res*. 2012;18(4):1077–84.
5. Maltzman T, Knoll K, Martinez ME, et al. Ki-ras proto-oncogene mutations in sporadic colorectal adenomas: relationship to histologic and clinical characteristics. *Gastroenterology*. 2001;121(2):302–9.
6. Ishii T, Notohara K, Umapathy A, et al. Tubular adenomas with minor villous changes show molecular features characteristic of tubulovillous adenomas. *Am J Surg Pathol*. 2011;35(2):212–20.
7. Sweetser S, Smyrk TC, Sinicrope FA. Serrated colon polyps as precursors to colorectal cancer. *Clin Gastroenterol Hepatol*. 2013;11(7):760–7.
8. Therkildsen C, Bergmann TK, Henrichsen-Schnack T, Ladelund S, Nilbert M. The predictive value of KRAS, NRAS, BRAF, PIK3CA and PTEN for anti-EGFR treatment in metastatic colorectal cancer: a systematic review and meta-analysis. *Acta Oncol*. 2014;53(7):852–64.
9. Kranenburg O. The KRAS oncogene: past, present, and future. *Biochim Biophys Acta*. 2005;1756(2):81–2.
10. Govender D, Chetty R. Gene of the month: BRAF. *J Clin Pathol*. 2012; 65(11):986–8.
11. Lai K, Killingsworth MC, Lee CS. Gene of the month: PIK3CA. *J Clin Pathol*. 2015;68(4):253–7.
12. Andreyev HJ, Norman AR, Cunningham D, et al. Kirsten ras mutations in patients with colorectal cancer: the "RASCAL II" study. *Br J Cancer*. 2001;85(5):692–6.
13. Tong JHM, Lung RWM, Sin FMC, et al. Characterization of rare transforming KRAS mutations in sporadic colorectal cancer. *Cancer Biol Ther*. 2014;15(6):768–76.
14. De Roock W, Claes B, Bernasconi D, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol*. 2010;11(8):753–62.

15. Imamura Y, Lochhead P, Yamauchi M, et al. Analyses of clinicopathological, molecular, and prognostic associations of KRAS codon 61 and codon 146 mutations in colorectal cancer: cohort study and literature review. *Mol Cancer*. 2014;13(1):135.
16. Carr NJ, Mahajan H, Tan KL, Hawkins NJ, Ward RL. Serrated and non-serrated polyps of the colorectum: their prevalence in an unselected case series and correlation of BRAF mutation analysis with the diagnosis of sessile serrated adenoma. *J Clin Pathol*. 2009;62(6):516–8.
17. Sorbye H, Dragomir A, Sundström M, et al. High BRAF mutation frequency and marked survival differences in subgroups according to KRAS/BRAF mutation status and tumor tissue availability in a prospective population-based metastatic colorectal cancer cohort. *PLoS One*. 2015;10(6):e0131046.
18. Kim M-J, Lee E-J, Suh J-P, et al. Traditional serrated adenoma of the colorectum: clinicopathologic implications and endoscopic findings of the precursor lesions. *Am J Clin Pathol*. 2013;140(6):898–911.
19. Whitehall VLJ, Rickman C, Bond CE, et al. Oncogenic PIK3CA mutations in colorectal cancers and polyps. *Int J Cancer*. 2012;131(4):813–20.
20. Eide TJ. Risk of colorectal cancer in adenoma-bearing individuals within a defined population. *Int J Cancer*. 1986;38(2):173–6.
21. Morris RG, Curtis LJ, Romanowski P, et al. Ki-ras mutations in adenomas: a characteristic of cancer-bearing colorectal mucosa. *J Pathol*. 1996;180(4): 357–63.
22. Gondal G, Grotmol T, Hofstad B, Bretthauer M, Eide TJ, Hoff G. The Norwegian colorectal cancer prevention (NORCCAP) screening study: baseline findings and implementations for clinical work-up in age groups 50–64 years. *Scand J Gastroenterol*. 2003;38(6):635–42.
23. Church JM. Clinical significance of small colorectal polyps. *Dis Colon Rectum*. 2004;47(4):481–5.
24. Bretthauer M, Gondal G, Larsen IK, et al. Design, organization and management of a controlled population screening study for detection of colorectal neoplasia: attendance rates in the NORCCAP study (Norwegian colorectal cancer prevention). *Scand J Gastroenterol*. 2002;37(5):568–73.
25. Bosman FT, World Health Organization, International Agency for Research on Cancer. *WHO Classification of Tumours of the Digestive System*. 4th ed. Lyon: IARC Press; 2010.
26. Forbes SA, Beare D, Gunasekaran P, et al. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res*. 2014;43(D1):D805–11.
27. Ihle MA, Fassunke J, König K, et al. Comparison of high resolution melting analysis, pyrosequencing, next generation sequencing and immunohistochemistry to conventional Sanger sequencing for the detection of p.V600E and non-p.V600E BRAF mutations. *BMC Cancer*. 2014;14(1):13.
28. Rashid A, Zahurak M, Goodman SN, Hamilton SR. Genetic epidemiology of mutated K-ras proto-oncogene, altered suppressor genes, and microsatellite instability in colorectal adenomas. *Gut*. 1999;44(6):826–33.
29. Kobayashi M, Watanabe H, Ajioka Y, Honma T, Asakura H. Effect of K-ras mutation on morphogenesis of colorectal adenomas and early cancers: relationship to distribution of proliferating cells. *Hum Pathol*. 1996;27(10):1042–9.
30. McLellan EA, Owen RA, Stepniewska KA, Sheffield JP, Lemoine NR. High frequency of K-ras mutations in sporadic colorectal adenomas. *Gut*. 1993;34(3):392–6.
31. Jass JR, Baker K, Zlobec I, et al. Advanced colorectal polyps with the molecular and morphological features of serrated polyps and adenomas: concept of a "fusion" pathway to colorectal cancer. *Histopathology*. 2006;49(2):121–31.
32. Cremolini C, Di Bartolomeo M, Amatu A, et al. BRAF codons 594 and 596 mutations identify a new molecular subtype of metastatic colorectal cancer at favorable prognosis. *Ann Oncol*. 2015;26(10):2092–7.
33. Ng SC, Ching JYL, Chan VCW, et al. Association between serrated polyps and the risk of synchronous advanced colorectal neoplasia in average-risk individuals. *Aliment Pharmacol Ther*. 2015;41(1):108–15.
34. Samowitz WS, Curtin K, Schaffer D, Robertson M, Leppert M, Slattery ML. Relationship of Ki-ras mutations in colon cancers to tumor location, stage, and survival: a population-based study. *Cancer Epidemiol Biomarkers Prev*. 2000;9(11):1193–7.
35. Al-Mulla F, Going JJ, Sowden ET, Winter A, Pickford IR, Birnie GD. Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. *J Pathol*. 1998;185(2):130–8.
36. Cespedes MV, Sancho FJ, Guerrero S, et al. K-ras Asp12 mutant neither interacts with Raf, nor signals through Erk and is less tumorigenic than K-ras Val12. *Carcinogenesis*. 2006;27(11):2190–200.
37. Improta G, Zupa A, Possidente L, et al. Coexistence of two different mutations in codon 12 of the Kras gene in colorectal cancer: report of a case supporting the concept of tumoral heterogeneity. *Oncol Lett*. 2013;5(5):1741–3.
38. Elmunzer BJ, Hayward RA, Schoenfeld PS, Saini SD, Deshpande A, Waljee AK. Effect of flexible sigmoidoscopy-based screening on incidence and mortality of colorectal cancer: a systematic review and meta-analysis of randomized controlled trials. *PLoS Med*. 2012;9(12):e1001352.
39. Breivik J, Lothe RA, Meling GI, Rognum TO, Børresen-Dale AL, Gaudernack G. Different genetic pathways to proximal and distal colorectal cancer influenced by sex-related factors. *Int J Cancer*. 1997;74(6):664–9.
40. Lin Y-C, Chiu H-M, Lee Y-C, Shun C-T, Wang H-P, Wu M-S. Hyperplastic polyps identified during screening endoscopy: reevaluated by histological examinations and genetic alterations. *J Formos Med Assoc*. 2014;113(7):417–21.
41. Gerhardsson de Verdier M, London S. Reproductive factors, exogenous female hormones, and colorectal cancer by subsite. *Cancer Causes Control*. 1992;3(4): 355–60.
42. Chacko L, Macaron C, Burke CA. Colorectal cancer screening and prevention in women. *Dig Dis Sci*. 2015;60(3):698–710.
43. Chow L, Lin P-C, Chang JS, et al. Differences in the frequencies of K-ras c12-13 genotypes by gender and pathologic phenotypes in colorectal tumors measured using the allele discrimination method. *Environ Mol Mutagen*. 2012;53(1):22–31.
44. Brink M, de Goeij AF, Weijenberg MP, et al. K-ras oncogene mutations in sporadic colorectal cancer in The Netherlands Cohort Study. *Carcinogenesis*. 2003;24(4):703–10.
45. Rosty C, Young JP, Walsh MD, et al. Colorectal carcinomas with KRAS mutation are associated with distinctive morphological and molecular features. *Mod Pathol*. 2013;26(6):825–34.
46. Jönsson M, Ekstrand A, Edekling T, et al. Experiences from treatment-predictive KRAS testing; high mutation frequency in rectal cancers from females and concurrent mutations in the same tumor. *BMC Clin Pathol*. 2009;9(1):8.
47. Di Micco R, Fumagalli M, Cicalese A, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature*. 2006;444(7119):638–42.
48. Rodier F, Campisi J. Four faces of cellular senescence. *J Cell Biol*. 2011; 192(4):547–56.
49. Ohnishi T, Tomita N, Monden T, et al. A detailed analysis of the role of K-ras gene mutation in the progression of colorectal adenoma. *Br J Cancer*. 1997;75(3):341–7.
50. Jancík S, Drábek J, Radzioch D, Hajdúch M. Clinical relevance of KRAS in human cancers. *J Biomed Biotechnol*. 2010;2010:150960.
51. Janakiraman M, Vakiani E, Zeng Z, et al. Genomic and biological characterization of exon 4 KRAS mutations in human cancer. *Cancer Res*. 2010;70(14): 5901–11.
52. De Roock W, Jonker DJ, Di Nicolantonio F, et al. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA*. 2010;304(16):1812–20.
53. Denoyelle C, Abou-Rjaily G, Bezrookove V, et al. Anti-oncogenic role of the endoplasmic reticulum differentially activated by mutations in the MAPK pathway. *Nat Cell Biol*. 2006;8(10):1053–63.