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

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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. 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

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 microvascular 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. 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. Flexible sigmoidoscopy (FS) was the primary screening tool followed by colonoscopy if any polyp sized ≥10 mm or a biotically 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. 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).

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. 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. 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 used 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. 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.

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
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,

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

| VARIABLE | ALL (%) | MALES (%) | FEMALES (%) | \( P \)-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.

**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)| 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            | 21 (65.6)| 16 (50.0)    | 5 (15.6)      | 0            | 1 (3.2)         |
| Serrated polyps     | 25 (12.3)  | 8.9             | 1 (4.0) | 1 (4.0)      | 0             | 22 (88.0)    | 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            | 10 (35.7)| 8 (28.6)     | 2 (7.1)       | 0            | 0               |
| Average mm size     | –          | –               | 13.5     | 12.9         | 15.3          | 8.4          | 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.

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...
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 \textit{KRAS} and \textit{BRAF} being strongly associated with adenomas and serrated polyps, respectively. Unexpectedly, a significantly higher frequency of \textit{KRAS} codon 12 mutations was found in adenomas from females and a noteworthy increase in the \textit{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
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. 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 lower end of the wide range (12%–68%) reported in adenomas according to adenoma stage is still observed as adenomas as asymptomatic individuals. However, an increasing frequency of 18.2% and 32.6%, respectively, while the more advanced tubulovillous adenomas reached frequencies higher than 65%. Serrated polyps were strongly associated with 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. 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 18.2% to 21.8% for a better understanding of polyp diversity and development.

**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.**

| N     | All lesions | 9.3 | 4–6,28–30 | 67 | 59.1 | 4–6,28–30 | 67 | 59.1 | 4–6,28–30 | 67 | 59.1 |
|-------|-------------|-----|-----------|----|------|-----------|----|------|-----------|----|------|
|       | TOTAL       | P-VALUE* | MALES | FEMALES | P-VALUE* | TOTAL | 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 | 6/66 | 14.3 | 3/21 | 0.66 |
| ≥10 mm | 95 | 32.6 | 31/95 | 23.5 | 18/68 | 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 | 5/9 | 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.
Oncogene mutations in colorectal polyps identified in the NORCCAP

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). 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%). These mutations were equally distributed in adenomas from the screened individuals (35%), while others have reported G12V to dominate. Thus, the seven times higher proliferation activity demonstrated for G12V when compared to G12D is not necessarily reflected in a higher frequency in CRC. However, G12V has been associated with a poorer prognosis than G12D in patients with Dukes C tumors. 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. 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.

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

**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.
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.42 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 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

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**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
base of GDP/GTP. The G13D variant has been found to act more like the KRAS wild type in terms of response to EGFR-targeted therapy. 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. 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.

Assessment of NRAS was not included in this study because of the low frequency (~5%) observed for mutations in this gene in CRC. 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. 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.

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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. 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.

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