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Short Communication

Low somatic *K-ras* mutation frequency in colorectal cancer diagnosed under the age of 45 years

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ABSTRACT

Somatic mutation of *K-ras* is known to be a common event in colorectal cancer tumourigenesis however its association with age at onset has not been widely explored. In this study, we have analyzed tumours from a population-based study of colorectal cancer diagnosed before the age of 45 years, in which cases had been previously screened for germ-line mismatch repair gene mutations and for microsatellite instability. We used a micro-dissection and sequencing approach to search for somatic *K-ras* mutations in codons 12, 13 and 61 in 101 early-onset colorectal cancers. Six (6%) somatic *K-ras* mutations were detected; five in codon 12 (4 G > T transitions and 1 G > A) and one in codon 13 (G > A transition). All codon 12 mutations were identified in microsatellite stable tumours and the codon 13 mutation was identified in a MSI-high tumour. Four cases with *K-ras* mutations had no reported family history of colorectal cancer and two had some family history of colorectal cancer. None were known to carry a germ-line mutation in *hMSH2*, *hMLH1*, *hMSH6* or *hPMS2*.

The role of somatic *K-ras* mutations in early-onset colorectal cancer carcinogenesis appears to be minor, in contrast to its significant role in colorectal cancer of later age of onset.

1. Introduction

A large number of studies have investigated the frequency, clinical features and outcomes of colorectal cancers (CRC) that have acquired *K-ras* mutations during tumourigenesis [see Refs. [1,2] for reviews]. Reports of *K-ras* somatic mutation frequency in colorectal cancer range between ~30% and 50%: (Ref. [3] (27%), Ref. [4] (32%), Ref. [5] (34%), Ref. [6] (37%), Ref. [7] (38%), Ref. [8] (39%), Ref. [9] (50%), Ref. [10] (50%)). Larger studies have found some evidence for *K-ras*

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mutations having an impact on disease site, progression and outcome.[3,5,7] These reports are consistently from studies of colorectal cancer unselected for age at diagnosis (therefore few with early age at onset). There have been no specific reports on the frequency of somatic *K-ras* mutations in early-onset disease.

In a population-based study of early-onset colorectal cancer (diagnosed under the age of 45 years in Victoria, Australia) we have previously reported the prevalence of mismatch repair (MMR) gene germ-line mutations to be 17% and that only a small proportion (3%) has *hMLH1* silencing via a mechanism other than germ-line mutation.[11] Previous studies have linked *K-ras* mutations with hypermethylation of gene promoters, a phenomenon associated with increasing age at onset in colorectal cancers. Therefore, we sought to examine other molecular pathways involved in the tumourigenesis of these early-onset colorectal cancers specifically addressing the contribution of mutation in the *K-ras* proto-oncogene. To address this question we applied micro-dissection and sequencing to 101 colorectal cancers from our population-based study and screened the tumour-enriched DNA for *K-ras* codon 12, 13 and 61 somatic mutations.

2. Patients and methods

2.1. The Victorian Colorectal Cancer Family Study

The Victorian Colorectal Cancer Family Study is a population-based, case–control study of early-onset CRC that was conducted in Melbourne, Victoria during 1993–1997. Approval for the study was obtained from the ethics committees of The University of Melbourne and The Cancer Council of Victoria. All subjects provided written informed consent for participation in the study.

Eligible cases comprised adult men and women living in the Melbourne metropolitan area who were under the age of 45 years when diagnosed with histologically confirmed, first primary, adenocarcinoma of the colon or rectum (ICD-9 153 and 154, respectively) during the period 1 July 1992 to 30 September 1996 identified through the Victorian Cancer Registry as described previously.[11–13] Cases participated by completing a risk factor questionnaire and family pedigree (first- and second-degree relatives of the case patient) during an in-person interview, by giving a small blood sample and providing consent to obtain the invasive tumour samples from the diagnostic laboratories. Self-reported ancestry comprised: Anglo-Saxon (75%), European (21%), Asian (4%). Relatives of the case patients were also interviewed and attempts were made to verify all reports of cancer using death certificates, medical records, and cancer registries.

A total of 131 cases participated in the study and we were able to collect tumour specimens for 118 of these cases (90%). Of the 13 cases that we were unable to collect, 6 were due to lack of participant consent to obtain pathology material and seven due to the diagnosing centre not being able to provide the material. In addition, lack of suitable material resulted in only 101 tumours being screened for mutations in *K-ras*. The average age at diagnosis for these cases was 39 years (range 24–44 years). Previous germ-line *MMR* gene mutation screening of the cases had identified 9 *hMLH1*, 4 *hMSH2*, 4 *hMSH6* and 1 *hPMS2* mutation carriers.[11]

2.2. K-ras mutation detection

Sections of 101 invasive colorectal cancers (5 μm) were stained in 0.1% v/v methyl green for 30–60 s. DNAs from tumour enriched tissue and histologically normal tissue were obtained by micro-dissection of cells from the methyl green stained slides using a 21 gauge needle followed by proteinase K digestion as described previously.[14]

Invasive tumour samples were screened for *K-ras* mutations in codons 12, 13 and 61, utilizing the polymerase chain reaction with micro-dissected tumour DNA (3 μl) as the template. Exon 1 (containing codons 12 and 13) was amplified using primers (forward) 5′ CTTATGTGTGACATGTTCT and (reverse) 5′ AGAATGGTCCTGCACCAGTA and Exon 2 (containing codon 61) using primers (forward) 5′ TTCCTACAGGAAGCAAGTAG and (reverse) 5′ CACAAAGAAAGCCCTCCCCA in a 12 μl PCR reaction containing 2.5 mM $MgCl_2$, 1× PCR buffer II (ABI), 0.2 μM each primer, 0.1 μM dNTPs and 0.5 U of AmpliTaq Gold. Exon 1 was amplified in a PCR program involving a 10 min hold at 94 °C followed by 40 cycles of 94 °C, 53 °C and 74 °C and finally a 7 min hold at 74 °C. Exon 2 was amplified using a 94 °C, for 10 min hold followed by 20 cycles of 94 °C, 64 °C (decreasing 0.5 °C every cycle) and 72 °C followed by 20 cycles of 94 °C, 54 °C, 72 °C and a final hold at 72 °C for 5 min. PCR products were visualised via agarose gel electrophoresis and ethidium bromide staining.

PCR product (5 μl) was cleaned using 1 μl of exonuclease I (10 U/μl), and 1 μl of shrimp alkaline phosphatase (2 U/μl), and heated to 37 °C for 15 min followed by 80 °C for 15 min. Sequencing was performed using either DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Buckinghamshire, England) or Big Dye Sequencing Kit (applied Biosystems). Reactions were purified via Qiagen DyeEx Columns (Qiagen) or Amersham Clean-up trays (Amersham Biosystems) and analysed on an ABI Prism 3100 or 377 Genetic Analyser.

Any sequences showing a change from the wild-type *K-ras* sequence were reamplified and resequenced for verification. If the same result was obtained, the variant was considered to reflect a somatic mutation within the tumour rich micro-dissected cells. If the repeat analysis did not confirm the first analysis the analysis was repeated a third time. *K-ras* sequence variants that could not be confirmed in a second or third attempt were regarded to be artifacts of the PCR.

2.3. Microsatellite instability testing and immunohistochemistry

DNA for microsatellite instability testing was prepared from micro-dissected tissues and MSI was determined by using the 5 marker panel[15] where the degree of instability in each tumour was scored as stable (MSS), low (MSI-L) and high (MSI-H) when 0, 1, and 2–5 markers were identified as unstable, respectively. The proportion of tumours in this study found to be MSI-high, MSI-low and MS stable by this method was 29%, 12% and 59%, respectively. The expression of MLH1, MSH2, MSH6 and PMS2 in the tumour specimens were

assessed by immunohistochemistry as described and reported previously.[11]

3. Results

3.1. K-ras somatic mutation status

A total of six somatic mutations were identified in the 101 tumours screened for *K-ras* mutations (6%). All mutations were G > T or G > A transitions and were in exon 1, five in codon 12 (four G > T transitions, G12V and one G > A, G12A) and one in codon 13 (a G > A transition, G13D). All codon 12 mutations were identified in individuals with MS stable tumours, three had no family history and two had some family history of colorectal cancer but not sufficient for the family to meet the Amsterdam criteria for HNPCC.[16]

The codon 13 somatic mutation was identified in an MSI-high tumour from an individual with no family history of colorectal cancer. The tumour from this case had been previously reported to express MSH6 and MSH2 but lack the expression of MLH1 and PMS2 (Table 1).[11]

K-ras mutations were not identified in tumours of cases with a family history of cancer that met the Amsterdam criteria for HNPCC. None of the *K-ras* mutation carrying tumours were identified in cases known to carry a germ-line mutation in *hMLH1*, *hMSH2*, *hMSH6* or *hPMS2* although the tumour carrying the G13D showed some evidence of MMR dysfunction. Four *K-ras* positive tumours were identified in the 65 tumours arising in the colon (6%) and two of the *K-ras* positives tumours were identified in the 36 tumours arising in the rectum (6%).

3.2. Clinical characteristics of K-ras mutation carriers

The age of diagnosis, histological characteristics, MSI status, MMR protein expression, family cancer history, site of resection and survival of the 6 participants of the study that were found to carry somatic *K-ras* mutations in their colorectal tumours were examined and summarized in Table 1.

4. Discussion

We observed that 6% of colorectal cancers diagnosed before the age of 45 years were associated with somatic gain-of-function *K-ras* mutations in codon 12, 13 or 61 less than one-quarter the prevalence observed in previous studies of later-onset disease. A large number of studies have investigated the frequency and clinical features of colorectal cancers that acquire *K-ras* mutations,[1,2] and reports of *K-ras* mutation frequency range between ~30% and 50% (Ref. [4] (32%), Ref. [9] (50%), Ref. [10] (50%)).

Studies of incident colorectal cancer patients have found the frequency of *K-ras* somatic mutations to range from 27% to 39%.[3,5–8] However, none of these studies were population-based and early onset which may explain some of the difference in prevalence compared to that of our study. It is possible that a more extensive screen for *K-ras* mutations in our cases outside codons 12, 13 and 61 may have yielded more mutations but it is unlikely that the number detected outside these codons would have been very substantial, given previous reports.

The ages of participants in previous studies were considerably older than in our study (mean 39 years, range 24–44 years). In Brink and colleagues, the age of the patients at baseline was between 55 and 69 years.[6] Smith and colleagues,

Table 1 – Summary of K-ras mutation carriers

Age at onset (years)	Family history[a]	MSI[b]	MMR IHC[a,c]	*K-ras* mutation[d]		Histological type	Survival
40	None	High	MLH1 neg PMS2 neg	133G > A (C13)	Right Colon	Poorly differentiated	Alive and well after 7 years follow-up
41	Some	Stable	–	130G > T (C12)	Left colon	Moderately differentiated	Alive and well after 6 years follow-up
42	Some	Stable	–	130G > A (C12)	Sigmoid colon	Poorly differentiated	Liver metastasis at diagnosis Developed lung and brain metastasis Died 3 years post-diagnosis
41	None	Stable	–	130G > T (C12)	Rectum	Well differentiated	Liver metastasis at diagnosis Developed bone metastasis Died 1.5 years post-diagnosis
38	None	Stable	–	129G > T (C12)	Rectum	Well differentiated	Developed second cancer after 6 years follow-up Alive and well after 7 years follow-up
42	None	Stable	–	130G > T (C12)	Right colon	Moderately differentiated	Alive and well after 7 years follow-up

a As reported in Southey and colleagues.[11] None, no other cancer reported in family, some, some other cancer reported in family but insufficient to meet the Amsterdam criteria for HNPCC.[16]
b As described in Boland and colleagues.[15]
c MLH1 neg, lack of MLH1 protein expression, PMS2 neg, lack of PMS2 protein expression as determined via immunohistochemistry.
d *K-ras* mutation expressed as nucleotide number, nucleotide change (codon number).

Andreyev and colleagues and Andreyev and colleagues, report the age of their study participants as 45–80 years (median age 67 years), 17–95 years (median 67 years) for males and 19–103 years (median 69 years) for females and 17–95 years (median 68 years) for males and 19–103 years (median 68 years) female, respectively.[3,5,7]

Our study did not identify any association between *K-ras* mutation status and Duke's stage, histological characteristics (including differentiation), site of resection, survival, MSI status, MMR gene mutation status or family history. However, our study of 101 colorectal cancers (only 6 *K-ras* mutations) did not have the power to detect anything but very strong associations. Larger studies involving many thousands of colorectal cancers (of all ages of onset) have found that *K-ras* mutations are associated with an increased risk of relapse and death and that tumours with a specific mutation at codon 12 (gly-val) have reduced failure-free survival ($P = 0.004$, HR 1.3) and reduced overall survival ($P = 0.008$, HR 1.29).[7,5] Further work in larger studies of early-onset colorectal cancer will be needed to determine if the codon 12 (gly-val) mutation has a similar impact on early-onset disease.

Smith and colleagues reported that *K-ras* mutations were slightly more common in the rectal tumours in their study (colon tumours 13/65 (20%) versus rectal tumours 16/41 (39%)).[3] We found *K-ras* mutations in 2 of the 36 rectal cancers tested (6%) to be no different to the rate of *K-ras* mutations observed in colon cancers tested 4/65 (6%).

While some studies have suggested that the pattern of *K-ras* oncogene mutations in colorectal cancers may be ethnicity dependent,[17] our series of cases is almost exclusively Caucasian, and therefore no different from the majority of previous studies.

Previous published work has presented evidence that the frequency and nature of oncogenic activation of *K-ras* differs between colorectal cancers that have arisen via different genetic and epigenetic mechanisms. Although the numbers of *K-ras* mutations in our study are very small, their character is consistent with the observations of Oliveira and colleagues, who reported that G12V, resulting from a G > T nucleotide substitution, was observed more frequently in MSS sporadic than in HNPCC or sporadic MSI-high colorectal cancer cases.[18] Also, Andreyev and colleagues reported that G12V mutations made up 24% of all *K-ras* positive "sporadic" colorectal cancers.[5] Four of the six *K-ras* mutations (66%) identified in our study of early-onset colorectal cancer were G > T nucleotide substitutions (all G12V) and all were in cases that were MSS (three had no family history and one had some family history of colorectal cancer but not sufficient for the family to meet the Amsterdam criteria for HNPCC).[16] Furthermore, the frequency of G > A transitions has been reported to be higher in MMR deficient than in MSS tumours, perhaps due to these alterations being more difficult to repair than other alterations even in the presence of intact MMR cellular machinery.[18,19] Fujiwara and colleagues have also reported that G13D (resulting from a G > A transition) was the most common type of *K-ras* mutations in HNPCC.[20] We identified two G > A transitions resulting in one case with G12A and another with G13D. The G13D was identified in a case with no family history of colorectal cancer but the tumour had some evidence for lack of MMR function (MSI-high and lack of MLH1 and PMS2 expression) but, despite extensive mutations screening in *hMLH1*, *hMSH2*, *hMSH6* and *hPMS2*,[11] was not known to carry a germ-line MMR gene mutation. The second case identified with a G > A transition (G12A) had some family history of colorectal cancer but the tumour had no indication of MMR dysfunction (MSS and expressed MLH1, MSH2, MSH6 and PMS2).

Therefore the role of *K-ras* mutations in early-onset (diagnosed before the age of 45 years) colorectal cancer carcinogenesis appears to be minor when compared to its role in colorectal cancer of later age of onset. We postulate that the explanation for this may be partly due to the acquisition of *K-ras* mutations being dependent on exposure (perhaps prolonged exposure) to environmental carcinogens. Colorectal cancers arising at an early age would therefore have had less opportunity to have acquired a somatic *K-ras* mutation. We have observed a similar phenomenon for MLH1 protein expression in early-onset colorectal cancer. Lack of MHL1 expression (associated with *hMLH1* promoter methylation) is observed frequently (50%) in studies of all or later onset colorectal cancer.[21] However, in our population-based study of early-onset disease we only identified 3% of the cases lacked expression of MHL1 (via immunohistochemistry) and lacked the identification of a germ-line MMR gene mutation (suggesting promoter methylation) despite extensive screening.[11] We therefore suggest that *K-ras* somatic mutations and *hMHL1* epigenetic silencing are a feature of later onset but not early-onset disease.

Conflict of interest statement

None declared.

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