Risk Factors for Ki-ras Protooncogene Mutation in Sporadic Colorectal Adenomas

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ABSTRACT
The Ki-ras protooncogene frequently is mutated in colorectal adenocarcinomas, but the etiology of this molecular event is uncertain. We investigated the association between variables known or suspected to be related to risk for colorectal cancer and the occurrence of Ki-ras mutations in colorectal adenomas. This study was conducted among 678 male and female participants, 40–80 years of age, enrolled in a phase III trial through the effects of a wheat bran fiber supplement on adenoma recurrence. Exposure information on the risk factors of interest was assessed through self-administered questionnaires. Mutations in codons 12 and 13 of the Ki-ras protooncogene were analyzed in baseline adenomas 0.5 cm or larger by PCR amplification followed by direct sequencing. Eighteen percent (120 of 678) of the participants had one or more adenoma(s) with Ki-ras mutations. A higher risk of Ki-ras mutations was associated with increasing age and a lower intake of total folate. The odds ratio (OR) for Ki-ras mutations for individuals >72 years of age was 1.98 [95% confidence interval (CI) = 1.19–3.27; P for trend = 0.008] compared with those less than 65 years of age. Compared with individuals in the lower tertile of total folate, those in the upper tertile had an ~50% lower risk of having Ki-ras mutation-positive adenomas (OR = 0.52; 95% CI = 0.30–0.88; P for trend = 0.02). There was a suggestion of a stronger inverse association of total folate with G → T transitions (OR = 0.41; 95% CI = 0.20–0.87) than G → A transitions (OR = 0.61; 95% CI = 0.31–1.21), although the CIs for the associations overlap. The results of these analyses suggest that the protective effect of folate in colon cancer observed in published studies may be mediated through folate’s effect on Ki-ras mutations.

INTRODUCTION
There is extensive clinical and histopathological evidence indicating that the development of colorectal cancer is a multistep process. Mutations in the Kirsten ras (Ki-ras) gene are widely accepted to be early molecular events in the progression from colorectal adenoma to carcinoma (1, 2). These events are thought to follow the initiation of the neoplastic process by APC gene mutations (3). Thus, Ki-ras mutations may be involved in the progression of adenomas from one of small size and low malignant potential to one that is larger and more clinically important (1).

The prevalence of ras mutations is estimated to be ~50% for colorectal carcinomas and 50% for adenomas greater than 1 cm in size; however, these mutations are seen only in ~10% of adenomas 1 cm or smaller in diameter (1). Ki-ras mutations also appear to be more common in tumors with an increased degree of dysplasia (4). The vast majority of ras mutations in colorectal tumors are present at codons 12 and 13 (88%) of the Ki-ras gene, whereas mutations of the N-ras or H-ras genes are infrequent (5). It has also been shown that Ki-ras mutations in colorectal carcinomas are predictive of a poor prognosis in some studies (6–8).

Despite the frequency of Ki-ras mutations in colorectal neoplasms, data on their etiology are sparse. Undoubtedly, understanding the role of environmental influences on the nature and rate of mutations in colorectal neoplasms, such as mutations in Ki-ras, is crucial. If mutational events play an important role in the colorectal carcinogenesis sequence, one can hypothesize that modification of these events by life-style or other factors would be a useful prevention strategy. The purpose of this study was to investigate the relationship of risk factors known to be associated with colorectal neoplasia as they relate to Ki-ras mutations in colorectal polyps.

MATERIALS AND METHODS
Study Population. The analysis was conducted among participants in the WBF study, whose details have been described previously (9). Briefly, the WBF study is a double-blind, phase III trial of high versus low fiber, designed to measure the effects of WBF supplementation for 3 years on adenoma recurrence. Men and women 40–80 years of age who had removal of one or more colorectal adenoma(s) 3 mm or larger at colonoscopy within 3 months prior to study entry were recruited from the Phoenix metropolitan area. Questionnaire data and biological specimens, including polyp tissue, were collected at baseline and throughout the trial. The study was approved by the University of Arizona Human Subjects Committee and local hospital committees.

Questionnaire Information. Self-administered questionnaires were used to obtain data on family history of colorectal cancer in first-degree relatives, history of polyps prior to the baseline adenoma, aspirin use, cigarette smoking, physical activity (defined as participation in aerobic exercise for at least 20 min and involving some heavy breathing), and postmenopausal hormone use. Height and weight were measured during the baseline visit. Weight in kilograms was divided by the square of height in meters to calculate BMI. We ascertained dietary intake from a 113-item food frequency questionnaire that inquired about diet during the prior year. Vitamin and mineral supplement information was also obtained through this questionnaire. We examined dietary and total (dietary plus supplemental) intake of calcium and folate.

Ki-ras Mutational Analysis. Histological slides and paraffin tissue blocks from all polyps removed during the qualifying colonoscopy were requested from the community pathology laboratory. Retrieved specimens were processed through the project laboratory. Baseline adenomas 0.5 cm or larger in size resected from participants who were randomized into the WBF trial were selected to undergo mutational analyses for Ki-ras. A total of 955 tissue samples were identified among 723 participants and prepared for mutational analysis. We were unable to sequence 115 (12%) of the prepared samples because of insufficient tissue or lack of adenoma tissue in the slide(s) provided. Thus, 840 adenoma specimens from 678 participants were analyzed and included in this analysis.

DNA Extraction and Amplification. Sections (5 μm) were cut from formalin-fixed paraffin-embedded polyps. One slide for each polyp was H&E stained so that normal tissue could be microdissected away from the polyp. The tissue was scraped from the slide and transferred to a microcentrifuge tube for DNA extraction with a QiAamp Tissue Kit (Qiagen) according to the manufacturer’s directions. DNA quality and quantity were assessed using agarose gel electrophoresis and PicoGreen staining, respectively. DNA was diluted to 100 ng/μl in Tris-EDTA buffer prior to the PCR amplification step. PCR reactions were done in a final volume of 50 μl containing 25 μl of 1× PCR buffer (Perkin Elmer), 200 μM dNTPs, 1.5 mM MgCl2, 100 μM of each primer, 0.5 μM of Taq DNA polymerase (Perkin Elmer), and 2 μl of 10× concentrated DNA template. The primers used were designed from the sequence of human Ki-ras (GenBank accession number M17575) and were 5’-CAGGTTAAGGATATTTGCGAG-3’ and 5’-TTCCCAGCTTCTTGGATTG-3’. The expected product is 90 bp, and amplification was done as follows: 1 cycle of denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, followed by 1 cycle of extension at 72°C for 10 min. Amplified DNA products were electrophoresed on a 3% agarose gel stained with ethidium bromide and visualized under ultraviolet light.
DNA extraction. Thirty-five μl of DNA extraction buffer (0.5 mM Tris, 20 mM EDTA, 10 mM NaCl, 2 mg/ml protease K, 5% Tween 20, pH 9) were added to the tissue sample and incubated in a heat block overnight at 56°C. Ten μl of 3 mM Tris, 0.2 mM EDTA (pH 7.5) containing 5% Chelex (Bio-Rad, Hercules, CA) were then added, and the tubes were heated to 100°C for 10 min. The samples were then centrifuged at 12,000 × g for 2 min to pellet undigested tissue and chelate. For each set of samples extracted, negative control tubes containing no tissue were extracted simultaneously and tested for ability to amplify a product. Only samples from which the corresponding negative control did not amplify a product were used. One microliter of the supernatant was used in a PCR to amplify the region of exon 1 of Ki-ras containing codons 12 and 13. The PCR reaction was as follows: 94°C for 2 min, 37 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, and 72°C for 5 min. The PCR reaction buffer (Promega, Madison, WI) contained 1.5 mM MgCl2, 0.2 mM dNTPs, 0.25 μM Ki-ras sense primer (5'-GAGAATTCAT-GAATTCCTCTATTGTTGGATCATATTC-3'), 0.25 μM Ki-ras antisense primer (5'-ATCGAATTCCCTATTGTTGGATCATATTC-3'), and 1 U of Taq polymerase (Promega). An additional no-template control containing only water was run for every PCR reaction. The 118-bp PCR product was then visualized on a 2% agarose gel. DNA samples that failed to PCR with 1 μl were repeated using 5 μl of the extracted DNA.

Ki-ras Sequencing. Mutations at codons 12 and 13 of Ki-ras were detected by PCR cycle sequencing using the Ki-ras PCR product in the SequiTherm EXCEL II DNA Sequencing Kit (Epipcentre Technologies, Madison, WI). One to four μl of the PCR product were used directly in the sequencing reaction depending on the size of the PCR product on the gel. A primer downstream of codons 12 and 13 (5'-ATCGAATTCAT-GAATTCCTCTATTGTTGGATCATATTC-3') was end labeled and used in the sequencing reaction according to the protocol provided by the manufacturer. The sequencing reaction products were run on a 6% denatured polyacrylamide gel. The gel was dried and exposed to film overnight. Mutations in the first and second nucleotide positions of codon 12 and the second nucleotide position of codon 13 were identified using the sequence visible on the film (Fig. 1). A Ki-ras mutant sample from the negative control did not amplify a product were used. One microliter of the extracted DNA.

Results

Compared with the total population of randomized WBF participants, those selected for Ki-ras analysis, as well as those with Ki-ras mutational data, were slightly older, and this difference was statistically significant (P < 0.05; Table 1). Although there were additional minor differences in the distribution of demographic characteristics, dietary and nondietary factors among the three groups, none was statistically significant.

One hundred twenty four (18%) of the 678 study participants had one or more adenoma(s) positive for Ki-ras mutations. Eighty-five percent of these mutations were at codon 12; the remaining 15% were at codon 13. Among participants with multiple adenomas, the concordance rate of mutational status was 73%. Table 2 presents associations between demographic and nondietary risk factors and Ki-ras mutations. Age was positively associated with Ki-ras status; compared with individuals <65 years of age, those 72 years or older had an almost 2-fold increase in risk of their adenoma(s) having a Ki-ras mutation (OR = 1.98; 95% CI = 1.19–3.27). Ki-ras mutations did not vary by gender. Although no important association was shown for race and Ki-ras mutations, given that our study population was predominantly white (96%), we were unable to adequately explore this association. No important associations were noted for family history of colorectal cancer or aspirin use. There was a weak, nonsignificant association for history of polyps prior to baseline and Ki-ras mutations. Slightly positive but nonsignificant associations were shown for current smokers versus never-smokers and for individuals in the upper versus the lower tertile of BMI. Although we did not have complete data for physical activity, there was a suggestion of a protective effect for participants who reported participating in aerobic exercise three or more times per week, compared with those who seldom or never participated in these (OR = 0.66; 95% CI = 0.40–1.08). Female participants who reported using hormone replacement therapy in the previous 10 years had a significantly lower risk of having Ki-ras positive adenomas compared with the nonusers (OR = 0.59; 95% CI = 0.25–1.38). Because polyp size has been shown to be a strong predictor of Ki-ras mutations, we repeated these analyses with an indicator variable for size (1 cm = 1 cm; < 5 cm) included in the logistic models. The results were not appreciably different from those presented in Table 2 (data not shown).

Table 3 presents the multivariate ORs for selected dietary factors and Ki-ras mutations. The only significant association was observed for intake of total folate (dietary plus supplemental) and Ki-ras mutational status: a slight, inverse association was seen for dietary folate. In these analyses, individuals in the upper tertile of total folate had an ~50% lower risk of having Ki-ras positive adenomas (OR = 0.52; 95% CI = 0.30–0.88; P for trend = 0.02). The upper tertile of total folate intake mainly comprised supplemental sources because 93% of individuals in this category were supplement users. Therefore, we further adjusted our analyses for supplement use. In this model, there were no appreciable changes in the overall association of total folate and Ki-ras mutations, although the CIs widened (OR for the upper versus the lower tertile of total folate intake = 0.57; 95% CI = 0.24–1.36). We also analyzed dietary folate after excluding users of supplemental folate. This analysis greatly reduced the sample size given that 37% of the participants reported supplement use. In this model, compared with individuals in the lower tertile of dietary folate, the ORs for the middle and upper tertiles were 0.83 and 0.89, respectively (P for trend = 0.74). When we included the size indicator variable as noted previously, the results were not changed appreciably. When we conducted stratified analyses by polyph size, the corresponding ORs (95% CIs) for the terciles were 1.00, 0.77 (0.31–1.87), and 0.39 (0.13–1.17) for small polyps (< 1 cm) and 1.00, 0.89 (0.50–
1.60), and 0.54 (0.29–1.00) for large polyps (≥1 cm). Given the overlap in the CIs, there do not appear to be major differences between the two groups.

We next investigated the relationship between specific mutation type in Ki-ras and intake of total folate (Fig. 2). Analyses stratified by Ki-ras codon suggested a stronger effect of total folate on codon 13 than codon 12 mutations. Analyses by specific mutation suggested that although total folate was inversely related to Ki-ras mutations involving G→A transitions (OR = 0.61; 95% CI = 0.31–1.21), its effect may have the greatest impact on mutations with a G→T transversion (OR = 0.41; 95% CI = 0.20–0.87). Given the low prevalence of G→C transversions, we are unable to fully investigate this type of mutation as it relates to intake of total folate.

DISCUSSION

To our knowledge, this is the first report of host and lifestyle risk factors as they relate to Ki-ras mutations in colorectal adenomas. Contrary to previous reports comprising smaller samples of participants with adenomas (11) or carcinomas (12, 13), our analyses indicate that age is a strong predictor of these mutations. There was a suggestion of a protective effect for participation in aerobic exercise. Likewise, use of hormone replacement therapy was suggested to be protective among women. However, given the relatively small sample size and low prevalence of this mutation, we lack the statistical power to make firm conclusions regarding these two variables.

Among the dietary factors, the only variable shown to be significantly associated with having an adenoma with a Ki-ras mutation was intake of total folate. Of particular interest, our results suggest that a high folate intake is most protective for mutations involving G→T transversions, which have been shown to be related to poorer prognosis and a higher risk of recurrence in colorectal carcinomas (6, 8). Reduced dietary intake of folate has been shown to be associated with an increased risk of colon cancer (14–17). Furthermore, low dietary or erythrocyte folate levels have also been associated with an increased risk of Ki-ras mutations in colorectal adenomas.
risk for colorectal adenomas (18–21). In the most recent and comprehensive of these reports (17), a 33% reduction in risk of colon cancer was shown for women with a total folate intake $>$400 µg/day compared with those with intakes of 200 µg/day or less. The results of our study show only a slight, nonsignificant reduction in risk from dietary sources of folate. This result is supported by published data where the reduction in colon cancer risk was stronger for total folate (15, 17, 22) or supplemental folate alone (14, 16, 17) than with dietary folate. This may reflect the higher levels of folate contributed by the supplement. Conversely, this effect may be due to the higher bioavailability of folate from supplements than dietary sources (23, 24).

The precise mechanism by which folate deficiency might be associated with colorectal carcinogenesis is uncertain. Mutations in the Ki-ras gene may be an important component of this mechanism. Folate is an important coenzyme for DNA methylation and DNA synthesis. Folate deficiency has been associated with reduced DNA methylation, and this hypomethylation of DNA is one of the earliest events in colon carcinogenesis (25, 26). It has also been shown that folate supplementation increases the degree of DNA methylation in patients with colonic adenomas (27). Different endogenous forms of folate, 5-methyltetrahydrofolate and 5,10-methylenetetrahydrofolate are essential for DNA methylation and DNA synthesis. When availability of methyl donors is low, a G→A transition would be generated by spontaneous deamination of methylcytosine or enzymatic deamination of cytosine (28, 29).

It is also possible that folate deficiency impairs DNA repair mechanisms in the colon. It has been shown in an animal model (30), that folate deficiency can result in defective and/or impaired DNA repair. Lacking an effective repair mechanism, cells can develop genomic instability and rapidly accumulate somatic mutations or loss of short segments of alleles within oncogenes or tumor suppressor genes. Therefore, as the burden to repair DNA increases in the presence of a folate-deficient environment, this can in turn result in a higher probability of Ki-ras mutations. Folate deficiency can also modulate the number of strand breaks and hypomethylation of tumor suppressor genes, such as the p53 gene (31). Thus, it is plausible that individuals exposed to a low-folate environment are more likely to have altered DNA repair mechanisms, which can lead to mutations in the Ki-ras protooncogene.

Limitations of our study are inherent in its cross-sectional design, which restricted assessment of the temporality of the relationship between the risk factors of interest and Ki-ras mutations. In addition, although the concordance rate of Ki-ras mutations was higher than in other studies (11), a discordance rate of 27% points to the modest degree of misclassification encountered when only one adenoma per participant is analyzed. The select nature of the study population may limit the generalizability of the findings. Fewer than 1500 individuals participated in the WFB trial, of nearly 5000 who were identified as potentially eligible. Furthermore, the self-reported nature of the exposure may be of concern. However, calibration studies of nutritional variables indicate that supplemental nutrient sources, including folate, are reported with a high degree of accuracy (32, 33). In addition, the reporting of folate factor data is not dependent on Ki-ras status mutations. The observed results do not appear to be attributable to selection bias, given that the distribution of the selected variables was not significantly different between the total randomized participants and those with Ki-ras data. The strong evidence of benefit from the addition of supplemental folate is of interest. However, we cannot be certain that this is due to folate per se because it is derived from multivitamin intake. Further elucidation of this association might be achieved from results of ongoing folic acid trials.

Our findings support results of published data on the potential role of folate in colorectal carcinogenesis. If the observed association is true, they suggest that the protective effect of folate in colorectal cancer may be mediated through its effect on Ki-ras mutation rates. Future studies, particularly population studies with large sample sizes, should be able to confirm this association and more adequately explore the role of other host and environmental risk factors as they relate to Ki-ras mutation rates.

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