KRAS Mutations Predict Progression of Preneoplastic Gastric Lesions[1]

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Abstract

Eight hundred sixty-three subjects with atrophic gastritis were recruited to participate in an ongoing chemoprevention trial in Nariño, Colombia. The participants were randomly assigned to intervention therapies, which included treatment to eradicate *Helicobacter pylori* infection followed by daily dietary supplementation with antioxidant micronutrients in a 2 × 2 × 2 factorial design. A series of biopsies of gastric mucosa were obtained according to a specified protocol from designated locations in the stomach for each participant at baseline (before intervention therapy) and at year three. A systematic sample of 160 participants was selected from each of the eight treatment combinations. DNA was isolated from each of these biopsies ($n = 320$), and the first exon of *KRAS* was amplified using PCR. Mutations in the *KRAS* gene were detected using denaturing gradient gel electrophoresis and confirmed by sequence analysis. Of all baseline biopsies, 14.4% (23 of 160) contained *KRAS* mutations. Among those participants with atrophic gastritis without metaplasia, 19.4% (6 of 25) contained *KRAS* mutations, indicating that mutation of this important gene is likely an early event in the etiology of gastric carcinoma. An important association was found between the presence of *KRAS* mutations in baseline biopsies and the progression of preneoplastic lesions. Only 14.6% (20 of 137) of participants without baseline *KRAS* mutations progressed from atrophic gastritis to intestinal metaplasia or from small intestinal metaplasia to colonic metaplasia; however, 39.1% (9 of 23) with baseline *KRAS* mutations progressed to a more advanced lesion after 3 years [univariate odds ratio (OR), 3.76 ($P = 0.05$); multivariate OR adjusted for treatment, 3.74 ($P = 0.04$)]. In addition, the specificity of the *KRAS* mutation predicted progression. For those participants with G→T transversions at position 1 of codon 12 (GGT→TGT), 19.4% (5 of 17) progressed (univariate OR, 2.4); however, 60.0% (3 of 5) of participants with G→A transitions at position 1 of codon 12 (GGT→AGT) progressed (univariate OR, 8.7; $P = 0.004$ using χ^2 test).

Introduction

Gastric cancer is one of the most frequent cancers in the world (1). The 5-year survival rate in the United States is only 21% for all histological stages (2). According to the Lauren classification (3), there are two major types of gastric carcinomas: intestinal and diffuse. The most frequent gastric malignancy is the intestinal type, which is often preceded by sequential steps of precancerous changes (Fig. 1), including atrophic gastritis, intestinal metaplasia (type I, complete, or small intestinal metaplasia; and type III, incomplete, or colonic metaplasia), and dysplasia (reviewed in Ref. 4). These progressive stages, which usually proceed over decades, have been defined as a sequence of histopathological events that confer an increasing risk of malignant transformation. The conversion of normal epithelial cells to cancer cells requires the accumulation of multiple genetic abnormalities. Interestingly, the two types of gastric carcinoma have some common genetic components but differ in some important aberrations. Abnormal expression and amplification of the *MET* gene, inactivation of the *p53* tumor suppressor gene, abnormal transcription of CD44, and loss of telomeres are common events in both types (5–7). Reduction or loss of cadherins and catenins and *KSAM* gene amplification are unique to the diffuse type of gastric cancer (8). *KRAS* mutations, *ERBB2* gene amplification, LOH[3] and mutations of the *APC* gene, LOH of *BCL2* gene, and LOH of the *DCC* locus are preferentially associated with the intestinal type (8). However, because few studies have been done on preneoplastic lesions, the significance of genetic events in gastric carcinogenesis remains unclear.

Mutations of *KRAS* are detected in many types of human malignancies and are associated with the development and progression of human cancer (9). The *KRAS* gene encodes a M_r 21,000 membrane-associated protein ($p21^{RAS}$) with intrinsic GTPase activity involved in cellular signal transduction. Point mutations of *KRAS* at specific codons lead to activated oncoprotein (GTP-RAS) with reduced GTPase activity (9). *KRAS* codons 12, 13, and 61 are the most frequently detected mutation "hot spots" in human cancers. The frequency of mutated *KRAS* varies greatly among different tumor types. *KRAS* mutations are found in ~10% of intestinal type gastric carcinomas but are rarely detected in the diffuse type (8). Very few studies have been done on premalignant gastric mucosa, and the significance

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[3] The abbreviations used are: LOH, loss of heterozygosity; DGGE, denaturing gradient gel electrophoresis; OR, odds ratio.

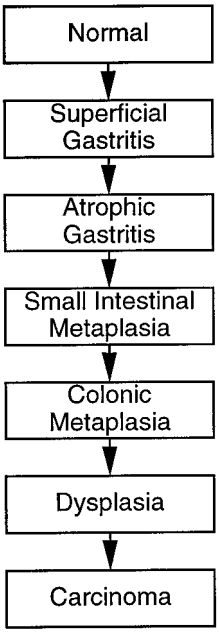

Fig. 1. A schematic representation of the preneoplastic progression of stomach.

of *KRAS* activation in the carcinogenesis of gastric cancer is not clear.

Helicobacter pylori infection has been recognized as a risk factor for gastric cancer (5, 10). The bacterium can colonize the gastric mucosa of the host for decades and is associated with both gastric ulcers and gastric cancer (10). It has been hypothesized that as a result of chronic infection with concomitant chronic inflammation, inducible nitric oxide synthase and the sustained production of reactive nitrogen species eventually lead to cancer (11). Activation of proto-oncogenes, inactivation of tumor suppressor genes, and loss of function of DNA repair genes are likely mechanisms for tumorigenic transformation. Eradication of *H. pylori* infection and dietary supplementation with vitamin antioxidants reduce inducible nitric oxide synthase induction and the formation of reactive oxygen and nitrogen species (11).

In this study, a systematic sample of 160 participants from a Colombian population with a high risk for developing gastric cancer participated in an ongoing clinical trial of a 2^3 factorial design, which tested the effect of anti-*H. pylori* therapy as well as dietary supplementation with ascorbic acid and/or β-carotene. Using DNA extracted from paraffin-embedded gastric biopsies obtained before and after intervention therapy, *KRAS* mutations were detected with PCR-DGGE. We determined whether the presence of baseline *KRAS* mutations in biopsies predicted the frequency of progression of premalignant lesions.

Materials and Methods

Study Population. Eight hundred sixty-three individuals with chronic multifocal atrophic gastritis were recruited from the towns of Pasto and Tuquerres of Nariño in the southern Colombian Andes. In this region, the incidence of gastric cancer ranks as one of the highest in the world (150 per 100,000; Refs. 12–15). The estimated *H. pylori* prevalence is 93% among asymptomatic adults (16). The volunteers were agricultural or blue-collar workers of Spanish-Indian ("mestizo") extraction.

Table 1 $2 \times 2 \times 2$ factorial study design

Antibacterial treatment[a]		No antibacterial treatment	
AA + BC[b]	AA + P	AA + BC	AA + P
BC + P	P + P	BC + P	P + P

[a] Triple therapy included amoxicillin, metronidazole, and bismuth subsalicylate.
[b] AA, ascorbic acid; BC, β-carotene; P, placebo.

The demographic characteristics of the volunteer population have been described previously (11). Endoscopic evaluations of individuals from the community who volunteered to participate in the study were performed in the Hospital Departmental (Pasto, Colombia) after obtaining informed consent approved by the local Human Subjects Committee and the Louisiana State University Medical Center Institutional Review Board. Infection with *H. pylori* was detected by the Steiner modification of the Warthin-Starry staining method (17) using baseline biopsies.

Study Design. Volunteer individuals were randomized into treatment groups using a 2^3 factorial design as illustrated in Table 1. A systematic sample of 160 participants was selected from each of the eight treatment combinations for this study. The anti-*Helicobacter* treatment consisted of a 2-week course of amoxicillin (500 mg three times per day), metronidazole (400 mg three times per day), and bismuth subsalicylate (262 mg four times per day). Bismuth subsalicylate (262 mg once per day) was continued until 2 weeks before the second endoscopy. Ascorbic acid (1-g tablet, twice per day) and/or β-carotene (30-mg capsule, once per day) or matched placebos for these two drugs (provided by Hoffman-La Roche, Inc.) were given throughout the study. Compliance was assessed by quarterly pill counts as well as by measurement of serum antioxidant levels at the time of second endoscopy. Compliance was consistently >90% as measured by pill count. Biopsies from 160 individuals (20 from each group; Table 1) were used to detect *KRAS* mutations in a double-blinded study.

Biopsies. Gastric biopsies were obtained through endoscope at the start of the trial (baseline) and then again after 3 years (follow-up). Two biopsies for this study were taken from the lesser curvature around the incisura angularis, where the most advanced lesions are usually found. Fresh biopsies were fixed immediately in 90% alcohol, dehydrated, and embedded in paraffin within 24 h. At embedding, tissues were carefully oriented. Five 4-μm sections of embedded biopsies were sliced for DNA isolation.

DNA Isolation and Amplification by PCR. DNA was isolated from specimens using Puregene DNA isolation kits (Gentra Systems, Inc., Minneapolis, MN) as described previously (18). A 152-bp region of exon one of human *KRAS* was amplified using the following primers and conditions as described previously (18): 5′-ATG ACT GAA TAT AAA CTT GTG-3′ and 5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GCC TCT ATT GTT GGA TCA TAT TC-3′. The amplified products were separated by electrophoresis in 3% 3:1 (Nuseive:Seakem; FMC BioProducts, Rockland, ME) agarose gels containing 0.1 μg/ml ethidium bromide in Tris borate-EDTA buffer. Amplified bands were excised for sequencing as described below.

Mutation Screening by DGGE. DGGE was used to screen for mutations in DNA. The apparatus used was the DGene System (Bio-Rad, Hercules, CA). The PCR products were electrophoresed at 60 V through a 10% polyacrylamide gel with a linear increasing gradient from 25 to 40% denaturant [100%

Table 2 Baseline *KRAS* mutations and clinicopathological findings

	Baseline *KRAS* mutation		Total
	Absent	Present	
Age groups			
<60	110 (86.6%)	17 (13.4%)	127
>60	26 (81.3%)	6 (18.8%)	32
Total	136 (85.5%)	23 (14.5%)	159
Sex			
Male	66 (88.0%)	9 (12.0%)	75
Female	71 (83.5%)	14 (16.5%)	85
Total	137 (85.6%)	23 (14.4%)	160
Metaplasia at baseline			
Absent	25 (80.6%)	6 (19.4%)	31
Present	112 (86.8%)	17 (13.2%)	129
Total	137 (85.6%)	23 (14.4%)	160
Lymphocytes in stroma			
Mild + moderate	118 (86.7%)	18 (13.3%)	136
Severe	19 (79.2%)	5 (20.8%)	24
Total	137 (85.6%)	23 (14.4%)	160

(v/v) denaturant: 7 M urea (Bio-Rad), 40% formamide (Fisher Scientific, Pittsburgh, PA)] in 1× Tris-acetate EDTA buffer, as described previously (18). After electrophoresis, the gel was stained with SYBR-GREEN I (Molecular Probes, Inc., Eugene, OR) and examined by UV light transillumination.

DNA Sequencing. The PCR products were isolated from agarose using QIAquick Gel Extraction kit (Qiagen, Inc., Chatsworth, CA). The isolated DNA was sequenced using [^{33}P]dideoxynucleotide triphosphates and THERMOSequenase (Amersham Life Sciences, Inc., Cleveland, OH).

Statistical Analysis. Cross-tabulation of the *KRAS* mutation levels and clinicopathological parameters were evaluated using the Pearson χ^2 test. ORs are reported as measures of association. Multivariate analysis was carried out to adjust for treatment status using logistic regression. All statistical analyses were accomplished using software from SPSS, Inc. (Chicago, IL).

Results

Detection of *KRAS* Mutations in Biopsies. Specimens were obtained from 75 male and 85 female volunteers whose mean age was 54 years (Table 2). Biopsies from baseline and follow-up were examined for mutations in *KRAS* using DGGE (Fig. 2). Those samples that were positive for mutations were sequenced to confirm the presence of mutations and to determine the type of mutation. The overall frequency of *KRAS* mutations in the biopsies was 12.5% (40 of 320, 160 baseline biopsies and 160 follow-up biopsies; Table 3). The frequency of *KRAS* mutations in baseline biopsies was 14.4% (23 of 160). All mutations detected were in the first base of codon 12. The mutations detected were either G→T transversions or G→A transitions, which change the encoding wild-type glycine (GGT) to either cysteine (TGT) or serine (AGT), respectively. Among the 40 mutations detected in the 320 biopsies, 33 (82.5%) were GGT→TGT (Gly→Cys), 2 (5%) were GGT→AGT (Gly→Ser), and 5 (12.5%) were mixed GGT→A/TGT (Gly→Cys/Ser).

***KRAS* Mutation and Progression of Preneoplastic Lesions.** Baseline mutations of *KRAS* occurred more often in individuals who did not have metaplasia at baseline (19.4%) as compared with those with metaplasia (13.2%; Table 2). For those individuals who had mutations in *KRAS* at baseline, 39.1% progressed to a more advanced premalignant lesion as compared with 14.6% who did not have baseline *KRAS* mutations. Therefore, those with baseline *KRAS* mutations were 3.8 times more likely to progress from either atrophy to metaplasia or from complete metaplasia (type I) to incomplete metaplasia (type III; $P = 0.05$; Table 4). When the OR was adjusted for intervention therapy, the estimate was unchanged and statistically significant (OR, 3.74; $P = 0.04$; Table 4). The presence of baseline *KRAS* mutations was a significant predictor of progression. For those individuals who had *KRAS* mutations in follow-up biopsies but did not have them at baseline, there was a trend toward a risk of progression as compared with individuals who never contained *KRAS* mutations. However, the odds of progression were significantly increased for those who had mutations only at baseline (OR, 3.47; $P = 0.02$) or for those who had mutations at baseline and at follow-up (OR, 12.4; $P = 0.04$). As seen in Table 4, this trend toward a risk of progression was significant after univariate analysis ($P = 0.014$) and after multivariate analysis adjusting for treatment ($P = 0.03$). The overall goal of this study was to determine whether antibiotic treatment and/or micronutrient supplementation of volunteer subjects would effect the subsequent progression of premalignant lesions. There was no significant independent effect of *H. pylori* infection status or micronutrient treatment with *KRAS* mutation status.

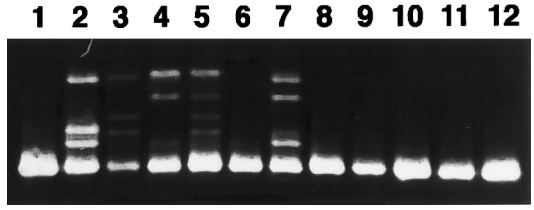

Fig. 2. DGGE demonstrates *KRAS* mutations in some gastric biopsies. Normal human DNA was used as a negative control (*Lane 1*). DNA from the lung adenocarcinoma cell line Calu-1 with a known mutation in codon 12 of *KRAS* (GGT→AGT) was used as a positive control (*Lane 2*). *Lanes 3–12*, biopsy samples. The samples in *Lanes 3*, *4*, *5*, and *7* were found to be positive for mutation. These samples were subsequently found to contain mutations GGT→A/TGT, GGT→TGT, GGT→A/TGT, and GGT→TGT, respectively, upon sequencing.

***KRAS* Mutation Type and Progression.** We have demonstrated previously that specific *KRAS* mutations are prognostic indicators of survival in lung cancer (18). To determine whether specific mutations of *KRAS* predict progression of preneoplastic lesions to a more advanced stage, the specific mutations were associated with progression. As seen in Table 5, those individuals with G→A transitions (Gly→Ser) were more likely to progress from atrophy to intestinal metaplasia than those individuals who lacked this mutation (OR, 8.7; $P = 0.004$).

Discussion

Many studies have shown that the frequency of *KRAS* mutation is relatively low for gastric cancer (9%) as compared with pancreatic (90%) and colorectal (50%) cancers (19–21). However, the recent study by Gulbis *et al.* indicated that high levels of $p21^{RAS}$ are present in preneoplastic gastric tissues as well as in tumors (22). We found a higher frequency of *KRAS* mutation in earlier stages of premalignant lesions (12.5%) as compared with that reported for tumor (9%; Ref. 8).

Interestingly, we also observed that *KRAS* mutations were more frequent in atrophic gastritis as compared with intestinal metaplasia. There are two possible explanations as to why early

Table 3 Global histology, mutation status, and treatment assignment

ID	Sex	Age (baseline)	Global histology[a]		Mutation status		Treatment assignment[b]		
			Baseline	Follow-up	Baseline	Follow-up	AB	BC	AA
1	F	66	IM-Mix	IM-COL	TGT GGC	TGT GGC	−	−	+
2	F	35	IM-SI	IM-Mix	AGT GGC	TGT GGC	+	+	+
3	F	42	IM-SI	IM-SI	A/TGT GGC	TGT GGC	+	+	+
4	F	51	IM-SI	IM-Mix		TGT GGC	−	−	+
5	M	53	MAG	MAG		TGT GGC	−	−	+
6	F	41	MAG	MAG		TGT GGC	−	+	−
7	F	59	IM-Mix	IM-COL		TGT GGC	−	+	−
8	F	62	IM-Mix	IM-SI		TGT GGC	+	+	+
9	F	30	MAG	MAG		TGT GGC	+	+	+
10	F	53	IM-Mix	IM-Mix		TGT GGC	−	−	+
11	F	56	IM-SI	IM-COL		TGT GGC	−	−	+
12	F	41	IM-Mix	IM-SI		TGT GGC	−	−	+
13	M	53	MAG	MAG		TGT GGC	−	+	−
14	M	42	MAG	MAG		TGT GGC	−	−	+
15	M	49	IM-SI	MAG		TGT GGC	−	−	+
16	M	50	MAG	MAG		TGT GGC	−	+	−
17	M	55	IM-SI	IM-SI		A/TGT GGC	+	+	+
18	F	62	IM-Mix	IM-SI	TGT GGC		−	−	+
19	M	54	IM-SI	IM-SI	TGT GGC		−	−	+
20	F	55	MAG	MAG	TGT GGC		−	−	−
21	F	66	MAG	MAG	TGT GGC		−	+	−
22	F	39	MAG	MAG	TGT GGC		−	+	−
23	F	42	MAG	MAG	TGT GGC		−	−	−
24	F	58	IM-SI	MAG	TGT GGC		+	+	+
25	F	65	IM-SI	MAG	TGT GGC		−	+	−
26	F	52	IM-COL	IM-COL	TGT GGC		−	+	−
27	M	59	IM-Mix	IM-COL	TGT GGC		−	−	+
28	M	55	IM-SI	IM-Mix	TGT GGC		+	+	+
29	F	59	IM-Mix	IM-SI	TGT GGC		−	−	+
30	M	57	IM-COL	IM-Mix	TGT GGC		−	−	−
31	F	46	IM-Mix	IM-COL	TGT GGC		−	−	+
32	M	58	IM-SI	IM-SI	TGT GGC		−	+	−
33	F	55	IM-Mix	IM-COL	TGT GGC		−	+	−
34	M	67	IM-Mix	IM-SI	AGT GGC		−	+	−
35	M	49	IM-Mix	DYS	A/TGT GGC		+	+	+
36	M	58	MAG	IM-Mix	A/TGT GGC		−	−	+
37	M	64	IM-SI	IM-Mix	A/TGT GGC		−	−	−

[a] MAG, atrophic gastritis; IM-SI, small intestinal metaplasia; IM-Mix, metaplasia of mixed type; IM-COL, colonic metaplasia; DYS, dysplasia.
[b] AB, antibiotic therapy; BC, β-carotene; AA, ascorbic acid.

Table 4 *KRAS* mutations and progression of preneoplastic lesions

Mutation	Number that progressed[a]	Univariate OR (*P*)	Multivariate OR (*P*)[b]
Baseline mutation		3.76 (0.05)	3.74 (0.04)
Absent	14.6% (20/137)		
Present	39.1% (9/23)		
Change of mutation status		(0.038, *P* for trend = 0.014)	(0.039, *P* for trend = 0.03)
(−) baseline, (−) follow-up	13.8% (17/123)	1.0	1.0
(−) baseline, (+) follow-up	21.4% (3/14)	1.7 (0.45)	1.6 (0.45)
(+) baseline, (−) follow-up	35.0% (7/20)	3.4 (0.02)	3.3 (0.03)
(+) baseline, (+) follow-up	66.0% (2/3)	12.4 (0.04)	12.7 (0.04)

[a] Progression includes the following situations: atrophy→intestinal metaplasia (IM); within IM: small intestinal metaplasia (SIM)→colonic intestinal metaplasia (CIM).
[b] Adjusted for treatment.

preneoplastic lesions appear to have a higher frequency of *KRAS* mutations as compared with later preneoplastic lesions or to tumors: (*a*) mutations of *KRAS* may be involved in the formation of early hyperplastic (preneoplastic) cells. Mutations of *KRAS* may impart a slight growth advantage to cells that contain the mutation, causing a field of cells in which subsequent mutations occur. In this model, mutations of *KRAS* are not necessary for neoplastic growth but do not harm the cells that contain the mutations; (*b*) the presence of mutant $p21^{RAS}$ may impart a negative growth advantage on tumor cells but not on preneoplastic cells. Other studies have shown that *KRAS* mutations are associated with gastric tumor progression and a poor prognosis (23). Therefore, it seems most likely that mutations of *KRAS* impart a growth advantage to cells that contain the mutation. This is further supported by our data that *KRAS* mutations are associated with progression of preneoplastic lesions.

Table 5 Specific *KRAS* mutations associated with progression to intestinal metaplasia

Mutation type	No change or regression	Progression[a]	Univariate OR ($P = 0.004$)[b]
None	85.3% (116/136)	14.7% (20/136)	1.0
TGT	70.6% (12/17)	19.4% (5/17)	2.4
AGT and A/TGT	40.0% (2/5)	60.0% (3/5)	8.7
Total	82.3% (130/158)	17.7% (28/158)	

[a] Progression from atrophy to intestinal metaplasia.
[b] Using χ^2 test.

Our data also indicate that individuals with *KRAS* mutations in their baseline premalignant stomach biopsies were more than three times as likely to progress to a higher premalignant stage than those who lacked baseline mutations. Furthermore, those who lost their mutations or who never had mutations were less likely to progress as compared with those that gained mutations or did not lose their mutations. It is not surprising that those individuals that had mutations in *KRAS* at both baseline and follow-up were more than 12 times as likely to progress to higher preneoplastic stages than were individuals who lacked mutations at baseline and at follow-up. However, there are some interesting caveats to consider for those individuals who had a change in mutation status between baseline and follow-up. There are two possible scenarios for those individuals who were negative for *KRAS* mutations at baseline and subsequently developed detectable mutations at follow-up: (*a*) the stomach did not have mutations, and mutations developed in the stomach after the initial biopsies were taken; or (*b*) *KRAS* mutations were present in a small subset of the cells and were below the detection limit for DGGE. This is an interesting caveat because the presence of detectable *KRAS* mutations at baseline is such a strong predictor of future progression, thus making the stage at which *KRAS* mutations develop an important biomarker for progression. Although it is impossible for one to differentiate these two scenarios, both seem likely events within a large study population. Therefore, it can be unambiguously stated that the presence of detectable *KRAS* mutations at baseline is a strong predictor of future progression.

All of the mutations detected were observed in codon 12. In the literature, ~90% of the *KRAS* mutations in pancreatic carcinomas were reported to be in codon 12 (24); this was true for ~70% of colorectal cancers (25) and ~90% of gastric cancers (8). The glycine at position 12 is crucial for the GTP-binding affinity of $p21^{RAS}$. A mutation at codon 12 alone is sufficient for oncogenic activation (9). In colorectal tumors, G→T transversions at codon 12 were associated with malignant transformation, and G→A transitions were associated with metastasis. In our study, 82.5% involved G→T transversions, 5% contained G→A transitions, and 12.5% contained both, indicating that these mutations may be important for the progression of gastric mucosal cells to a more advanced premalignant stage.

It seems likely from these data that the presence of *KRAS* mutations in early preneoplastic lesions will be a significant negative prognostic indicator for the development of stomach cancer. This information may be an important diagnostic tool for the physician managing patients with atrophic gastritis.

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