Diet, *Helicobacter pylori,* and *p53* Mutations in Gastric Cancer: A Molecular Epidemiology Study in Italy[1]

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Abstract

A series of 105 gastric cancer (GC) cases with paraffin-embedded specimens interviewed in a previous population-based case-control study conducted in a high-risk area around Florence, Italy, was examined for the presence of *p53* mutations. Overall, 33 of 105 cases had a mutation (*p53*$^+$) identified by single-strand conformational polymorphism and confirmed by sequencing (Y-H. Shiao *et al.,* submitted for publication). *p53*$^+$ cases had a more traditional dietary pattern (*i.e.,* corn meal mush, meat soup, and other homemade dishes) and reported less frequent consumption of raw vegetables (particularly lettuce and raw carrots). A positive association with a high nitrite intake and a negative association with raw vegetables and diffuse type histology persisted in a multivariate analysis. In addition, *p53*$^+$ cases tended to be located in the upper portion of the stomach and to be associated with advanced age and blood group A. No relation was found between the presence of *p53* mutations and histologically defined *Helicobacter pylori* infection, smoking history, family history of gastric cancer, education, and social class.

Of the 33 *p53*$^+$ cases, 19 had G:C→A:T transitions at CpG sites. These tumors tended to occur in females and in association with *H. pylori* infection but not other risk factors. The remaining 14 cases with a *p53* mutation had mainly transversions but also two deletions and two transitions at non-CpG sites. These tumors showed a strong positive association with a traditional dietary pattern and with the estimated intake of selected nutrients (nitrite, protein, and fat, particularly from animal sources). The findings of this case-case analysis suggest that *p53* mutations at non-CpG sites are related to exposure to alkylating compounds from diet, whereas *p53* mutations at CpG sites might be related to *H. pylori* infection.

Introduction

Despite a downward trend in incidence and mortality in nearly all countries, GC[4] was recently estimated to be the second most common cancer in the world and the second leading cause of cancer death (1). The causes of GC are not well established, although nutritional, microbial, and genetic factors have been suggested in a multifactorial and multistage process (2). Several studies have focused on alterations of *p53* in human cancers, including GC (3), and it has been suggested that a mutant allele promotes transformation by inactivating normal *p53* function in a dominant-negative fashion (4). Loss of heterozygosity on chromosome 17p13 and the occurrence of *p53* mutations have been reported by several studies of GC in different countries (5–9), including Italy (10, 11). Although there is controversy as to whether *p53* alterations occur in GC at an early or late stage, additional studies of *p53* mutations in GC may provide clues to environmental determinants similar to the specific *p53* sequence mutations reported for cancers of the liver, lung, and skin (12, 13).

We evaluated the relation of sequence mutations of *p53* to dietary and other exposures in a series of 105 GC cases identified through a case-control study carried out in a high-incidence area around Florence, Italy.

Subjects and Methods

Subjects. GC patients were selected from one of the participating centers in a population-based case-control study in several areas in Italy (14–16). All subjects of the current series were identified during 1985–1987 in Florence, Italy, a high-risk area in central Italy, at the coordinating center of the study. Overall, 382 histologically confirmed GC cases were enrolled, and surgical specimens were retrieved for approximately two-thirds of the series.

A preliminary study of *p53* mutations in GC was reported previously by our group, for 34 intestinal type cases (17). Our case series was selected to include adequate numbers of GC cases with diffuse type, as well as subjects with a positive family history.

Pathology. Formalin-fixed paraffin-embedded samples were retrieved from the archival files of the Pathology Department of Florence University. Serial 5-μm sections were cut, and neoplastic lesions were microdissected according to a previously described protocol (17). Additional sections were stained with

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[4] The abbreviations used are: GC, gastric cancer; SSCP, single-strand conformational polymorphism; TBE, Tris-borate EDTA; OR, odds ratio; CI, confidence interval.

Giemsa to detect evidence of *Helicobacter pylori* infection in nontumor gastric mucosa. The cases were classified according to the Lauren classification using all of the slides available from different blocks of GC tissue (16).

DNA Extraction, PCR-SSCP, and *p53* Analysis. Methods have been described in detail in a related report.[5] Briefly, genomic DNA extraction was performed on paraffin-embedded sections from neoplastic tissues. After proteinase K digestion, purification was carried out by adding saturated NaCl, and DNA was finally precipitated in cold ethanol. PCR-SSCP was performed with a 50-μl PCR mixture containing 10 mM Tris-base, 50 mM KCl, 1.5 mM $MgCl_2$, 125 μM deoxyribonucleoside triphosphates, 0.2 μM primers, and 1.25 units of Taq polymerase (Perkin-Elmer Corp., Norwalk, CT). PCR was performed as follows: 40 cycles of denaturation at 92°C for 1 min, annealing for 1 min at temperatures according to primer sets, and extension at 72°C for 1 min, followed by a final 7-min extension at 72°C. A 20-μl mixture (5 μl of PCR product, 0.4 μl of 1 M of methylmercury hydroxide, 1 μl of loading buffer, and 13.6 μl of 1× TBE buffer) was heated to 85°C for 5 min to denature double-stranded DNA and plunged into ice prior to SSCP analysis. "Cold SSCP" analysis was performed with 1.25× TBE running buffer, 20% polyacrylamide TBE gels, and a constant 300 V for 2–3 h. Constant inner and outer buffer temperatures were carefully controlled to obtain a maximum sensitivity. Gels were then stained with SYBR Green II (Molecular Probes, Eugene, OR) and photographed with the IS-1000 Imaging System (Alpha Innotech Corp., San Leandro, CA). Samples identified by PCR-SSCP to have putative mutations were confirmed by a second independent PCR-SSCP prior to DNA sequencing.

A dideoxy DNA-sequencing method was performed to sequence DNA using the CircumVent Thermal Cycle Sequencing Kit (New England Biolabs, Beverly, MA). Signal was revealed by incorporation of α-^{35}S-labeled dATP. The sequence ladder was resolved in a denaturing gel (7.7 M urea and 6% polyacrylamide). The gel was exposed to X-ray film for 3–5 days at room temperature. Base changes were confirmed by repeated DNA sequencing of the same or complementary strand.

Data Instruments and Statistical Analysis. The database from the previously analyzed case-control study with extensive individual information from a dietary and lifestyle questionnaire was merged with the laboratory assay results. The resulting data set was analyzed using the SAS Statistical software (SAS Institute, Inc., Cary, NC) on a mainframe computer (18). For the consumption of dietary items (single foods or complex groups), as reported in the original face-to-face interview, categorical indexes were used (quintiles or median values of weekly frequencies, specifically computed on all 105 subjects). Logarithmic transformations of nutrient intakes were carried out. Nitrite intake was calculated on the basis of available estimates of nitrite concentration in local foods (15); the highest quintile was defined (>6 mg per day).

GC cases with *p53* mutations (and two subsets, *i.e.*, CpG or non-CpG site mutations) were compared to those lacking mutations using simple cross-tabulations, nonparametric ranking statistics, and multivariate methods. Adjusted ORs were determined using logistic models, including terms for potential confounders. Parameters estimated from models with continuous nutrients were applied to average intakes of extreme quintiles to present ORs. Further analyses were carried out to identify effect modification.

Results

***p53* Mutation Analysis.** Overall, *p53* sequence mutations were detected in 33 of 105 (31.4%) subjects. Table 1 shows the distribution of GC cases with and without *p53* mutations according to selected individual characteristics. *p53* mutations tended to be more frequent among women, older subjects, those with upper gastric tumors (cardia and fundus) and those with blood group of type A. Mutations were significantly more common among GC cases with intestinal (42.9%) or unclassified (45.0%) tumors, as compared to diffuse tumors (21.2%). No association was found between the presence of *p53* mutations and various risk factors, including *H. pylori* infection (histologically defined), smoking habits, social class, education, and family history of GC. However, five of the eight cases reporting two affected first-degree relatives had a *p53* mutation.

Table 2 shows the distribution of *p53* mutations according to selected dietary characteristics. A decreasing trend with an increasing weekly frequency of consumption of raw vegetables (quintiles) was evident (χ^2 for trend $P < 0.01$). A negative association was also seen with specific raw vegetables, particularly lettuce, chicory, and carrots. On the other hand, mutations were positively associated with a high frequency of consumption of traditional soups (>3.5 times per week) and a high intake of nitrite (>6 mg per day). Among specific food items, a strong positive association was evident with polenta (a cornmeal mush). No relation was seen with the frequency of consumption of other food groups or the estimated intake of nutrients.

When terms for several variables were tested simultaneously in a multivariate model, some associations persisted after taking into account sex, family history, and age (Table 3). In particular, histological type (intestinal or unclassified), consumption of raw vegetables, and nitrite intake were all linked to the presence of *p53* mutations.

Subset Analysis. Small numbers precluded detailed analyses relating each type or site of *p53* mutations with individual characteristics. Therefore, the mutated cases were divided into two major groups that were compared to the 72 GC cases with no mutations. The "CpG" group consisted of 19 cases with a G:C→A:T transition at any CpG site. The "non-CpG" group involved 14 cases, including 10 transversions, 2 deletions, and 2 transitions at non-CpG sites.

Table 4 compares selected characteristics of the two *p53*$^+$ case groups (CpG and non-CpG) with the *p53*-negative cases and shows the results of separate multivariate analyses. Mutations at CpG sites showed a positive association with the female sex (adjusted OR, 3.2; 95% CI, 1.0–10.4) and *H. pylori* infection, although the latter did not reach statistical significance (adjusted OR, 3.3; 95% CI, 0.8–14.1).

In contrast, non-CpG mutations were associated with a traditional dietary pattern, particularly a high consumption of traditional soups, including polenta, stuffed pasta, and other homemade soups as meat broth. Because of small numbers, the CI was wide, but the lower boundary suggested a 5-fold increase in risk associated with frequent consumption of this group of dishes (adjusted OR, 40.5; 95% CI, 5.7–504). A positive association was also evident for tobacco smoking, either past or current (adjusted OR, 5.6; 95% CI, 0.8–40.8).

Multivariate analyses of non-CpG mutations identified a

[5] Y-H. Shiao, D. Palli, G. S. Buzard, N. E. Caporaso, A. Amorosi, C. Saieva, J. F. Fraumeni, Jr., L. M. Anderson, and J. M. Rice, Frequent p53 mutations at CpG sites in gastric cancer from a high-risk area of central Italy, submitted for publication.

Table 1 Distribution of GC subjects with and without *p53* mutations by sequence analysis according to selected characteristics (Florence, 1985–1987)

	p53 mutation			
	Absent	Present	Total	Significance[a]
	N (%)	*N* (%)		
Sex				NS
M	45 (70.3)	19 (29.7)	64	
F	27 (65.9)	14 (34.1)	41	
Age				NS
<65 years	40 (76.9)	12 (23.1)	52	
65+ years	32 (60.4)	21 (39.6)	53	
Family history				NS
0	54 (70.1)	23 (29.9)	77	
1	15 (75.0)	5 (25.0)	20	
2+	3 (37.5)	5 (62.5)	8	
Smoking history				NS
Never	28 (70.0)	12 (30.0)	40	
Former	17 (68.0)	8 (32.0)	25	
Current	27 (67.5)	13 (32.5)	40	
Blood group				NS
A	22 (59.5)	15 (40.5)	37	
Non-A	50 (73.5)	18 (26.5)	68	
Lauren histological type				$P < 0.05$
Intestinal	16 (57.1)	12 (42.9)	28	
Diffuse	45 (78.9)	12 (21.1)	57	
Unclassified	11 (55.0)	9 (45.0)	20	
GC site				NS
Cardia	6 (50.0)	6 (50.0)	12	
Fundus	6 (50.0)	6 (50.0)	12	
Antrum	32 (71.1)	13 (28.9)	45	
Other	28 (78.8)	8 (22.2)	36	
H. pylori[b]				NS
Yes	49 (69.0)	22 (31.0)	71	
No	23 (67.6)	11 (32.4)	34	
Total	72 (68.6)	33 (31.4)	105	

[a] NS, not significant.
[b] Giemsa staining.

Table 2 Distribution of GC subjects with and without *p53* mutations by sequence analysis according to selected dietary characteristics: frequency of consumption of raw vegetables and traditional soups and nitrite intake (Florence, 1985–1987)

	p53 mutation		
	Absent	Present	*P*
	N (%)	*N* (%)	
Raw vegetables (quintiles)			<0.01[a]
1 (low)	13 (59.1)	9 (40.9)	
2	13 (65.0)	7 (35.0)	
3	11 (52.4)	10 (47.6)	
4	16 (76.2)	5 (23.8)	
5 (high)	19 (90.5)	2 (9.5)	
Traditional soups			0.05
Low	43 (76.8)	13 (23.2)	
High	29 (59.2)	20 (40.8)	
Nitrite intake			<0.05
Low	61 (70.9)	25 (29.1)	
High (6+ mg/day)	11 (57.9)	8 (42.1)	
Total	72 (68.6)	33 (31.4)	

[a] χ^2 for trend *P*.

Table 3 Factors predicting GC *p53* mutations: ORs obtained from one logistic model, including terms for age, sex, and family history (Florence, 1985–1987)

Variable	OR	95% CI	*P*
Diffuse type	0.4	(0.1–1.0)	0.05
Raw vegetables[a]	0.2[b]	(0.1–0.9)	0.04
Nitrites[c]	3.4	(1.0–11.4)	0.05

[a] Quintiles of weekly consumption.
[b] Highest frequency of consumption (fifth quintile) *versus* lowest (first quintile).
[c] High *versus* low intake (6 mg/day).

positive association with the estimated intake of selected nutrients: nitrite, protein, and fats. The effects were more pronounced when protein and fats were derived from animal sources. Table 5 shows the parameters estimated by separate multivariate analyses and the ORs obtained by comparing the average intakes of extreme quintiles. The effect of smoking persisted and reached statistical significance in several of the nutrient-adjusted models.

In the univariate subset analyses of single food items in the group of raw vegetables, two distinct patterns were observed. The consumption of lettuce was lowest in the CpG group, whereas that of onions and leeks was lowest in the non-CpG group. On the other hand, butter tended to replace olive oil in the diet of non-CpG cases.

Two hot spots were evident, with eight mutations at codon 175 and five at codon 273 (eight females, five males), occurring mostly at CpG sites (12 of 13). These particular cases showed a positive association with increasing age (χ^2 for trend $P < 0.01$) and tended to involve tumors located in the upper portion of the stomach.

Table 4 Distribution of GC cases according to selected characteristics and presence and subtype of p53 mutations; multivariate ORs (Florence, 1985–1987)

Factor	p53 Mutations			
	Negative	Positive CpG	Positive non-CpG	Total
Sex				
Female	27 (65.9)	11 (26.8)	3 (7.3)	41
Male	45 (70.3)	8 (12.5)	11 (17.2)	64
Total cases	72	19	14	105
OR[a]	1	3.2 (1.0–10.4)	0.6 (0.1–2.5)	
H. pylori				
Yes	49 (69.0)	15 (21.1)	7 (9.9)	71
No	23 (76.6)	4 (11.8)	7 (20.6)	34
Total	72	19	14	105
OR[a]	1	3.3 (0.8–14.1)	0.4 (0.1–1.9)	
Traditional soups[b]				
Low	43 (76.8)	12 (21.4)	1 (1.8)	56
High[c]	29 (59.2)	7 (14.3)	13 (26.5)	49
Total	72	19	14	105
OR[a]	1	0.6 (0.2–2.1)	40.5 (5.7–504)	
Smoker				
Ever[d]	44 (67.7)	9 (13.8)	12 (18.5)	65
Never	28 (70.0)	10 (25.0)	2 (5.0)	40
Total	72	19	14	105
OR[a]	1	1.5 (0.4–6.4)	5.6 (0.8–40.8)	

[a] Each obtained from a separate logistic model, including terms for age, sex, family history, raw vegetable consumption, and nitrite.
[b] Including polenta, meat soup, stuffed pasta, and other homemade dishes typical of rural areas.
[c] >3.5 times per week.
[d] Former and current smokers combined.

Table 5 Multivariate analysis of selected nutrient intakes for cases with non-CpG mutations (n = 14) compared to GC cases without mutations (n = 72)

Variable	Parameter[a]	OR[b]	95% CI	P
Nitrite	2.65	16.5	(1.8–151.2)	0.013
Protein	3.36	9.8	(1.04–93.8)	0.046
Animal protein	2.93	14.3	(1.4–143.5)	0.023
Lipids	2.32	5.8	(0.9–38.1)	0.066
Animal lipids	2.27	12.0	(1.5–93.9)	0.018

[a] Each obtained from a separate logistic model, including terms for age, sex, family history, diffuse type, consumption of raw vegetables, smoking history, and the log-transformed nutrient of interest.
[b] High *versus* low intake (obtained by applying each parameter to the average values of extreme quintiles, the fifth and the first).

Discussion

The findings of our study suggest that the mechanisms leading to GC through somatic mutations of the *p53* gene may involve specific exposures identified previously to be causally related to GC. In particular, the case-control study from which our case series was derived found a strong protective effect for raw vegetables and an excessive risk associated with nitrite-rich foods (14), consistent with other reports in the literature (19).

Subset analyses, although limited by small numbers, suggested that the $p53^+$ gastric tumors involve at least two different pathways. Transitions at CpG sites have generally been considered to result from deamination of 5-methyl-cytosine by nitrosating agents commonly present in the gastric microenvironment (20). In our study, these transitions tended to occur among women, who generally have a much lower risk of GC than men. This sex difference is not easily explained by diet, suggesting the possible role of reproductive (21) or other factors.

Of particular interest is the positive, although nonsignificant, association we found between transitions at CpG sites and the presence of *H. pylori* in the gastric mucosa. *H. pylori* infection was defined on the basis of Giemsa-stained histology and may have been underestimated in our study due to the lack of serological data and the presence of severe atrophic gastritis, during which *H. pylori* tends to disappear from the stomach. We have previously reported a decrease in *H. pylori* antibody titers associated with gastric atrophy in the general population of this area (22, 23). Although the mechanism relating *H. pylori* infection to *p53* mutations is unclear, the *H. pylori*-related inflammatory process may lead to high levels of nitric oxide that can interact selectively with genomic DNA and lead to deamination of bases (24). Alternatively, in the presence of mutant *p53* cell clones, nitric oxide may induce microvascularization and thus accelerate tumor growth (25).

In contrast, the *p53* mutations identified at non-CpG sites were found to be positively associated with a traditional dietary pattern that is rich in nitrite, proteins, fat, and probably preformed *N*-nitroso-compounds. The risks primarily involved the consumption of local foods typical of rural areas where refrigeration has become available only recently. Homemade dishes were traditionally prepared for consumption over several days and stored at room temperature. Although meat broth and other soups were usually reboiled to limit bacterial proliferation, degradation of protein and reduction of nitrate to nitrite may still have occurred. A major dietary risk factor we and others have identified in Italy is polenta (14, 26), but the consumption of this staple food has steadily declined in recent decades. This dietary item can be considered a good indicator of traditional dietary habits and probable high intake of preformed *N*-nitroso compounds. The intake of preformed *N*-nitroso compounds or their formation in the gastric microenvironment in the presence of high concentrations of nitrites could result in the formation of mutations at non-CpG sites. An additional source of alkylating agents could be tobacco smoke, which was also related to this class of mutations in our data.

In addition, the group of cases with mutations at non-CpG sites also tended to use butter instead of olive oil, which was the predominant added fat in the area. In past years, the lack of domestic refrigeration for the storage of butter, dairy products, meats, and cooked foods probably led to the consumption of rancid fatty acids in the daily diet of the local population. It is noteworthy that free radical-generating compounds, such as peroxides, have been shown to cause G→T transversion mutations in cell lines (27).

Intake of dietary antioxidants, reported as protective factors in our original study (15), did not affect the overall or subset analyses of *p53* alterations. However, subset analyses of the protective effects of raw vegetables in the $p53^+$ cases revealed that lettuce consumption was lowest in the CpG group, whereas consumption of onions and leeks was lowest in the non-CpG group. This pattern suggests that different mechanisms may be involved, such as inhibition of nitrosation by lettuce and the chemopreventive effects of organosulfur and flavonols present in onions and leeks (28–30).

The relationship of mutation status with familial predisposition to GC was unremarkable in our study, except for a high prevalence of *p53* mutations in a small group of cases reporting at least two affected relatives. Such a strong family history was previously associated with a 5-fold increased GC risk (31). Other candidate genes, particularly those involved in repair mechanisms, may contribute to GC susceptibility and should be investigated in high-risk families.

Approximately one-third of *p53* mutations occurred at two hot spots, but no dietary or other risk factors (other than age) could be identified.

In summary, the availability of epidemiological data on dietary and other exposures in a series of GC cases provided an opportunity to link specific risk factors to *p53* sequence mutation status. The sample size was relatively large when compared with other studies that have evaluated *p53* mutations in GC, but too small for detailed analyses for each type or site of mutation. Moreover, our subset analyses of *p53* sequence mutations suggest that exposure to nitrosating and alkylating agents from diet and possibly tobacco smoking may be related to mutations at non-CpG sites, whereas *H. pylori* infection may be related to mutations at CpG sites in GC. Larger studies with more detailed analyses are needed to clarify the relation of *p53* sequence mutations to environmental and genetic determinants of GC.

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