Carcinogenesis vol.19 no.12 pp.2145–2149, 1998

Implications of *p53* mutation spectrum for cancer etiology in gastric cancers of various histologic types from a high-risk area of central Italy

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Examination of *p53* mutation spectra may provide clues to molecular mechanisms involved in different histologic types of gastric cancer. A total of 105 gastric cancer cases classified according to the Laurén's system were selected from a high-risk area around Florence, Italy. Exons 5–8 of the *p53* gene were examined for mutations by the polymerase chain reaction–single strand conformation polymorphism technique and DNA sequencing, using DNA from formalin-fixed paraffin-embedded tissues. Mutation frequency was similar in intestinal-type (12/28) and unclassified tumors (9/18), but was significantly lower in diffuse cancers (12/57, $P < 0.05$). A similar frequency of *p53* mutations was observed among tumor stages in both intestinal-type and unclassified cancers, but in diffuse tumors mutations tended to be associated with invasion beyond the muscularis propria. When base changes were considered, G:C→A:T transitions at CpG sites were the most common mutations for all the three tumor types with 6 of 11 (55%) in intestinal type, 8 of 12 (67%) in diffuse type, and 5 of 8 (63%) in unclassified tumors. Frequent *p53* mutations in both intestinal-type and unclassified tumors support the hypothesis that unclassified tumors represent variants of the intestinal type and suggest that unclassified tumors, like the intestinal type, may also associate with environmental exposures. The predominance of G:C→A:T transitions at CpG sites, which are associated with methyltransferase-induced DNA methylation at carbon 5 of cytosine, in all three tumor types suggests that the status of DNA methylation may be the major determinant for *p53* mutations and may be also equally important in gastric carcinogenesis regardless of histology.

Introduction

Gastric cancer has been estimated to be the second most common cancer in the world (1), even though the global incidence has declined over time (2,3). Gastric carcinoma is classified histologically by Laurén's criteria into two principal types, intestinal-type and diffuse, with a third category of 'unclassified' tumors that do not clearly fit into either of the principal types (2). The declining trend of gastric cancer worldwide has been attributed to a decrease in intestinal-type tumors, whereas the incidence of diffuse tumors has remained stable (3–6). Intestinal-type tumors are more common than diffuse tumors in high-risk areas (7) while the opposite has been reported in some low-risk areas (3). Unclassified tumors comprise ~15% of all gastric cancers (2,7,8) and are mainly poorly differentiated carcinomas (9).

Several etiologic hypotheses have been proposed for gastric cancer. Intestinal-type tumors have been associated with environmental factors, including high consumption of salt and nitrite, low intake of antioxidants, and *Helicobacter pylori* infection (7,10–13). In contrast, the diffuse cancers may have a stronger genetic component, as suggested by an earlier age at diagnosis and association with blood group A (9,14). However, a protective effect of raw vegetables and micronutrients, and an increased risk from infection with *Helicobacter pylori* have been reported for diffuse tumors in some studies (7,15). Although the etiology of unclassified tumors has not been well studied, they may represent variants of less differentiated intestinal-type tumors (9).

The *p53* gene, the most frequently mutated gene in human tumors, may serve as a reporter gene reflecting exposures to specific carcinogens (16). Among environmental exposures, genotoxic agents including nitrosating, alkylating and oxidative compounds cause various base mutations in test genes in *in vivo* and *in vitro* studies (17–21). The most common mutation type, G:C→A:T transitions at CpG sites, has been attributed to the methyltransferase-initiated methylation of cytosine at carbon 5 followed by deamination (22).

In this study, we examined the *p53* mutation spectra in intestinal-type, diffuse and unclassified gastric carcinomas from a high-risk area around Florence, Italy. Comparison of mutation spectra among tumor types may provide a clue to cancer etiology.

Materials and methods

Subjects

Patients were selected from a population-based study conducted in central Italy (10) in which gastric cancers categorized as intestinal, diffuse or unclassified type according to Laurén's system (2) were studied previously (7). Briefly, intestinal-type carcinoma exhibits atypical glandular structures characterized by cohesive cells. The diffuse tumor type features individual cells or small clusters of cells that diffusely infiltrate the layers of the gastric wall. Cases which did not fit either of these categories were defined as unclassified. In all, 28 cases with intestinal-type, 57 with diffuse, two with mixed intestinal-type/diffuse and 18 with unclassified gastric tumors (female: 41 cases; male: 64 cases; mean age: 62 years; range: 34–76 years) were retrieved from archives at the Department of Pathology, University of Florence, Italy. Tumors were located at the antrum (45 cases), the body (31 cases), the cardia (12 cases), and others (17 cases). Our case series was selected to include adequate numbers of diffuse gastric cancers and subjects with a positive family history.

The χ^2 test for heterogeneity or Fisher's exact test were used as appropriate

Abbreviations: PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

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Table I. Sequences of primers used for the amplification of *p53* exons 5 to 8

Primer	Sequence	PCR product	Gene locus
E5A	Upstream: 5′-TGCCCTGACTTTCAACTCTGT-3′ Downstream: 5′-CATGTGCTGTGACTGCTTGTA-3′	172 bp	3′-intron 4 and exon 5
E5B	Upstream: 5′-CTGTGCAGCTGTGGGTTGATT-3′ Downstream: 5′-GCAACCAGCCCTGTCGTCTCT-3′	174 bp	Exon 5 and 5′-intron 5
E6	Upstream: 5′-TTGCTCTTAGGTCTGGCCCC-3′ Downstream: 5′-CAGACCTCAGGCGGCTCATA-3′	128 bp	Exon 6
E7	Upstream: 5′-TAGGTTGGCTCTGACTGTACC-3′ Downstream: 5′-TGACCTGGAGTCTTCCAGTGT-3′	117 bp	Exon 7
E8	Upstream: 5′-AGTGGTAATCTACTGGGACGG-3′ Downstream: 5′-ACCTCGCTTAGTGCTCCCTG-3′	141 bp	Exon 8

Table II. The frequency of *p53* mutations (p53+) in tumors by depth of invasion and histologic type

	Mucosa or submucosa		Muscularis propria		Adventitia		Adjacent structures	
Histologic type	p53+ (%)	P53–	p53+ (%)	p53–	p53+ (%)	p53–	p53+ (%)	p53–
Intestinal type	1 (33)	2	3 (43)	4	8 (44)	10	NA	NA
Diffuse type	0 (0)	6	1 (9)	10	9 (21)	29	2 (100)	0
Unclassified	0 (0)	1	6 (60)	4	2 (40)	3	1 (50)	1

NA, not available.

to compare the frequency of mutations among different groups. The two mixed intestinal-type/diffuse tumors were later excluded from statistical analysis because of no detectable *p53* mutations.

DNA extraction

Serial 5 μm formalin-fixed paraffin-embedded sections were cut and neoplastic tissue microdissected as previously described (23). DNA was extracted as previously described (24) with some modification. A final 500 μg/ml concentration of proteinase K was used for digestion at 55°C for 48 h. After proteinase K digestion, DNA purification was carried out by adding one-third volume of saturated NaCl with vigorously shaking followed by centrifugation for 15 min at 14 000 r.p.m. Supernatant was transferred to a new tube and DNA was then precipitated by 3 vols cold absolute ethanol. A negative control containing only reagents and no tissue was run in parallel for each DNA extraction. Purified DNA was then quantified fluorometrically using the Hoechst 33258 dye procedure (25).

Polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP)

The primers used for PCR are shown in Table I. The procedure for PCR–SSCP was followed as previously described (23). In summary, 50 ng of genomic DNA was used in a final 50 μl PCR mixture containing 10 mM Tris–base, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 125 μM deoxyribonucleoside triphosphates, 0.2 μM primers and 1.25 U of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). PCR was performed as follows: 40 cycles of denaturation (92°C for 1 min); annealing (55, 62, 62, 60 and 60°C for 1 min for primers E5A, E5B, E6, E7 and E8, respectively); and extension (72°C for 1 min) followed by a final 7 min extension at 72°C.

Five microliters of PCR product was mixed with 0.4 μl 1 M methylmercury hydroxide (Johnson Matthey Electronics, Ward Hill, MA), 4 μl of loading buffer (15% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol) and 10.6 μl of 1× TBE buffer to yield a total volume of 20 μl. This mixture was heated to 85°C for 5 min to denature double-stranded DNA and then plunged into ice prior to SSCP analysis. Cold SSCP analysis was performed with 1.25× TBE (111 mM Tris–base, 111 mM boric acid, 2.5 mM Na_2EDTA, pH 8.4) running buffer, 20% polyacrylamide TBE gels (Novex, San Diego, CA) and a constant 300 V for 2–3 h, as described previously (26). Constant inner and outer buffer temperatures (25, 30, 16, 10 and 25°C for E5A, E5B, E6, E7 and E8, respectively) were carefully controlled throughout the SSCP analysis to maximize sensitivity. Gels were then stained with SYBR Green II (Molecular Probes, Eugene, OR) at a 1:10 000 dilution in deionized water for 20 min and photographed with the IS-1000 Imaging System (Alpha Innotech, San Leandro, CA). Putative mutations identified by PCR–SSCP were confirmed by a second independent PCR–SSCP from genomic DNA prior to DNA sequencing.

DNA sequencing

Twenty microliters of PCR product were purified by size filtration using Microcon 50 according to manufacturer's instructions (Amicon, Beverly, MA). One-third volume of purified product was used for DNA sequencing. DNA sequencing was performed by the dideoxynucleotide method using the CircumVent Thermal Cycle Sequencing Kit (New England BioLabs, Beverly, MA) and [α-^{35}S]dATP. The sequence ladder was resolved in 7.7 M urea and 6% (w/v) polyacrylamide gel using a 35×40 cm electrophoresis chamber (Gibco BRL, Gaithersburg, MD). After electrophoresis, the gel was soaked in 10% acetic acid for 30–60 min and then dried in the Bio-Rad (Hercules, CA) gel dryer before exposure to X-ray film for 3–5 days at room temperature. Base changes were confirmed by repeatedly sequencing the same or complementary DNA strand.

Results

Mutations in the *p53* sequence were detected in 12 of 28 (43%) intestinal-type, 12 of 57 (21%) diffuse and 9 of 18 (50%) unclassified gastric cancers. Mutation frequency was significantly lower in diffuse tumors ($P < 0.05$). Unclassified tumors appear to have *p53* mutation frequency similar to intestinal-type cancers. When depth of invasion was considered, two specific patterns were observed (Table II). For diffuse cancers, *p53* mutations in advanced tumors (invading deeply into adventitia and adjacent structures) tended to be more frequent (11/40) than in tumors involving only the mucosa, submucosa or muscularis propria (1/17) ($P = 0.08$, Fisher's exact test). In contrast, intestinal-type and unclassified tumors did not show any trend related to depth of invasion.

The precise location and type of *p53* mutations in relation to tumor histology are summarized in Table III. Missense mutations predominated in each histologic type. Other mutations including nonsense, intron and silent mutations were observed, but only in unclassified tumors of our series. Mutations at two 'hotspot' codons 175 and 273, were observed

in intestinal-type (5/12) and diffuse (8/12) tumors but not in unclassified cancers (0/9).

The mutation spectra of the *p53* gene for each histologic type are summarized in Table IV. G:C→A:T transitions at CpG sites were most commonly noted for all tumor types. These accounted for 6 of 11 (55%), 8 of 12 (67%), and 5 of 8 (63%) base substitution mutations for intestinal-type, diffuse and unclassified tumors, respectively. Other point mutations (G:C→A:T transitions at non-CpG sites, G:C→T:A transversions, A:T→T:A transversions, A:T→G:C transitions, A:T→C:G transversions and G:C→C:G transversions) occurred sporadically in all three types of gastric cancer. Base changes other than G:C→A:T transitions at CpG sites showed little variability among tumor types, being observed in 5 of 11 (46%) intestinal-type, 4 of 12 (33%) diffuse and 3 of 8 (38%) unclassified tumors.

Table III. *p53* Mutations in gastric cancer by histologic type

Case no.	Base change	Amino acid	Codon	Exon	Mutation type	Age
Intestinal type						
6	CGC→CAC	Arg→His	175	E5	Missense	73
9	CGC→CAC	Arg→His	175	E5	Missense	72
13	CCG→CTG	Pro→Leu	152	E5	Missense	52
34	ACA→CCA	Thr→Gln	284	E8	Missense	65
36	ATC→AAC	Ile→Asn	232	E7	Missense	59
37	TAC→T		234	E7	Deletion	68
61	CGT→TGT	Arg→Cys	273	E8	Missense	63
63	CGC→CAC	Arg→His	175	E5	Missense	74
66	AGT→AGA	Ser→Arg	215	E6	Missense	65
70	GTG→GAG	Val→Glu	197	E6	Missense	69
75	CGC→CAC	Arg→His	175	E5	Missense	74
78	TCC→TTC	Ser→Phe	127	E5	Missense	62
Diffuse type						
2	CGC→CAC	Arg→His	175	E5	Missense	71
3	CGT→GGT	Arg→Gly	273	E8	Missense	73
17	GGC→GTC	Gly→Val	153	E5	Missense	67
39	CGT→CAT	Arg→His	273	E8	Missense	45
48	CGT→TGT	Arg→Cys	273	E8	Missense	65
68	CGC→CAC	Arg→His	175	E5	Missense	75
71	CGC→CAC	Arg→His	175	E5	Missense	65
73	ATG→AAG	Met→Lys	133	E5	Missense	49
79	CGT→CAT	Arg→His	202	E6	Missense	52
80	CGT→CAT	Arg→His	273	E8	Missense	64
91	CGC→CAC	Arg→His	175	E5	Missense	75
105	GTC→CTC	Val→Leu	157	E5	Missense	44
Unclassified						
11	ATG→GTG	Met→Val	246	E7	Missense	65
16	CGG→CAG	Arg→Gln	248	E7	Missense	50
23	TAC→TAA	Tyr→Stop	126	E5	Nonsense	65
25	GGC→GGT	Gly→Gly	244	E7	Silent	73
30	CAA→CA		136	E5	Deletion	62
40	CGG→TGG	Arg→Trp	282	E8	Missense	64
56	CGG→CAG	Arg→Gln	248	E7	Missense	68
84	CGA→TGA	Arg→Stop	196	E6	Nonsense	66
87	G→C	3rd base from 5′-end			Intron 5	67

Discussion

The mutation spectrum of *p53* or any other gene in unclassified gastric cancers has not been examined previously. In this study, frequent *p53* mutations in unclassified tumors, similar to those observed in tumors of intestinal type, is striking and suggests that environmental exposures are a key component of carcinogenesis in this tumor type. Previously, our group also demonstrated that gastric cancers of any histologic type are significantly associated with family history (7), especially families with more than two first-degree relatives affected, suggesting a close interaction of genetic and environmental factors in intestinal-type and unclassified tumors.

The pattern of *p53* mutations in both intestinal-type and unclassified cancers, independent of depth of invasion, provides further evidence that the *p53* gene is a common target for carcinogen(s) leading to these tumor types. Unclassified tumors had a higher proportion of nonsense, intronic and silent mutations, but since they probably comprise more than one histologic type, mutation heterogeneity was expected. On the basis of morphologic evaluation, Correa *et al.* (9) have proposed that the unclassified tumors represent poorly differentiated variants of intestinal-type tumors. The *p53* findings support Correa's hypothesis.

Low frequency of *p53* mutations in diffuse gastric cancers and the association of these mutations with invasion beyond the muscularis propria are consistent with a report from other Italian series (27). It is not clear whether mutations contribute to the invasive potential of the neoplasm, or whether mutant clones are selected later during tumor progression. In any event, our results suggest that the *p53* mutation frequency in diffuse cancers tends to be stable across populations at varying risk of gastric cancer.

Mutations at the 'hotspot' codons 175 and 273 were frequently detected in diffuse tumors (67% of detected mutations) and to a lesser extent in intestinal tumors (42%), but were not seen in unclassified tumors. Mutations at these codons have previously been documented for cancers of the lung, colon, breast and thyroid, but not for gastric cancer (16). Mutations at specific codons may be an indication of specific exposures or genomic susceptibility.

Regardless of differences in the frequency of *p53* mutations and the target codon affected, it is noteworthy that G:C→A:T transitions at CpG sites, which are frequently methylated (28), were the most common mutations for all types of gastric cancer. The mutations have been also frequently detected in intestinal-type and diffuse gastric cancers from other geographic areas (27,29–32). In a previous study, we observed an

Table IV. Mutation spectra of the *p53* gene in gastric cancers by histologic type

Histologic type	Deletion	G:C→A:T		G:C→T:A	A:T→T:A	A:T→G:C	A:T→C:G	G:C→C:G
		CpG	Non-CpG					
Intestinal type	1	6	1	0	3	0	1	0
Diffuse type	0	8	0	1	1	0	0	2
Unclassified	1	5	0	1	0	1	0	1
Total	2	19	1	2	4	1	1	3

association, of borderline statistical significance, of G:C→A:T transitions at CpG sites with *Helicobacter pylori* infection but not with smoking or dietary intake of nitrite (33). This suggests that endogenous agents generated from inflammatory reactions in response to infection may contribute to the mutations at CpG sites. *N*-nitroso compounds, which may also induce G→A mutations at CpG sites through alkylation reaction (34), do not appear responsible for the high frequency of these mutations in gastric cancers because of lack of association with tobacco smoke and dietary intake of nitrite. Rather, the mechanism for these mutations may involve deamination of 5-methylcytosine to generate thymine, which cannot be repaired by DNA-uracil glycosidase and results in G:C→A:T transitions (35). Deamination of 5-methylcytosine can be initiated by nitrosating agents (e.g. N_2O_3, N_2O_4) (17,18), methyltransferase when *S*-adenosylmethionine is deficient (36) or other unknown mechanisms. Epidemiological evidence of etiologic difference among tumor types of gastric cancers may reflect these distinct deamination processes.

Point mutations other than G:C→A:T transitions at CpG sites might have resulted from either deamination, alkylation or oxidation of DNA bases (17–21). Our previous observation of association of non-CpG mutations with tobacco smoking and high dietary intake of nitrite and animal fat supports the involvement of multiple mechanisms resulting in these mutations. Most damaged DNA bases are readily repaired by host repair enzymes (19,37), so that defective repair systems may predispose to these kinds of mutations. A similar frequency of mutations other than G:C→A:T transitions at CpG sites among the tumor types suggests that repair defects contribute to *p53* mutations in all forms of gastric cancer.

In conclusion, frequent *p53* mutations in both intestinal-type and unclassified tumors support the hypothesis that unclassified tumors represent variants of the intestinal type. Prominence of G:C→A:T transitions at CpG sites in all three tumor types suggests that the status of DNA methylation is a common determinant in gastric carcinogenesis regardless of histology.

Acknowledgements

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US Government. This project was funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-56000. This work was partially supported by Regione Toscana, Firenze, Italy.

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Received on April 21, 1998; revised on August 21, 1998; accepted on August 26, 1998