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Barbara G. Schneider · Susan G. Hilsenbeck
Charles H. Hensel · Vladimir Pekkel
Coranelle H. Shelton · Héctor A. Rodríguez-Martínez
M. Esther Gutiérrez-Díaz C. · Donald R. Pulitzer
D. Craig Allred

p53 mutations in gastric and colorectal cancers in Texas Hispanics versus Anglos

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Abstract Gastric cancer is more than twice as common in Hispanics as in Anglos in Texas, while colorectal cancer is almost twice as common in Anglos as Hispanics. To test the hypothesis that mutations in the p53 tumour suppressor gene are involved in these differences, we examined 131 gastric and 138 colorectal cancers from Hispanic and Anglo patients from South Texas and Mexico using immunohistochemistry (IHC) as a screening assay for p53 mutations. The fraction of p53 positive cases was not significantly different in gastric cancers from Hispanics compared to Anglos (43% versus 61%, respectively, $p=0.13$) or in colorectal cancer (57% versus 58%, respectively, $p=1.0$), suggesting that p53 mutations are not involved in causing the different incidences of these cancers in these populations. In addition, the types of p53 mutations arising in gastric tumours from Hispanic patients were consistent with those reported in gastric tumours in other populations. Sequencing of mutations in five gastric cancers revealed two G: C to A: T transitions, two A: T to G: C transitions and one complex deletion. In contrast with findings in studies in other tumour types, neither stage nor survival was associated with p53 positive staining by IHC in either gastric or colorectal tumours in this study. Positive p53 immunostaining was associated with the diffuse histological subtype in gastric carcinoma ($p=0.05$) and high histological grade in colorectal carcinoma ($p=0.04$).

Key words Adenocarcinoma · Immunohistochemistry
Tumour suppressor gene · Ethnicity

Introduction

Gastric and colorectal cancers have dramatically different incidence rates in various parts of the world. Gastric cancer, though less frequent in the United States and some other Western countries, is estimated to be the second most common cancer worldwide (Parkin et al. 1988), and is common in Latin America. The opposite is true of colorectal cancer. Incidence data are available from five of the twelve Public Health Regions in Texas, including San Antonio, and show an approximately twofold higher rate of gastric cancer in Hispanics than in Anglos and a similarly elevated rate of colorectal cancer in Anglos compared to Hispanics (Table 1). Therefore the major ethnic groups in Texas provided an op-

Table 1 Incidence rates for five public health regions in Texas (1976–1985). The five public health regions of Texas examined include the cities of Amarillo, Lubbock, El Paso, San Antonio, Beaumont and their surrounding counties. (Texas Department of Health).

	Gastric cancer	Colorectal cancer
Hispanics		
Males	18.24[a]	25.44
Females	10.36	20.95
Anglos		
Males	8.16	43.68
Females	3.72	34.24

[a] Age adjusted incidence rates are expressed per 100,000, using the United States 1970 standard million population by age.

B. G. Schneider (✉) · V. Pekkel · C. H. Shelton · D. R. Pulitzer
D. C. Allred
Department of Pathology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, Texas 78284, USA

S. G. Hilsenbeck
Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA

C. H. Hensel
Department of Cellular and Structural Biology, University of Texas Health Science, Center at San Antonio, San Antonio, Texas, USA

H. A. Rodríguez-Martínez · M. E. Gutiérrez-Díaz C.
Hospital General de México, Universidad Nacional Autónoma, Mexico City, Mexico

portunity to initiate studies into the biology of these poorly understood epidemiological variations.

The p53 gene is frequently mutated in a wide range of tumour types including gastric (Imazeki et al. 1992; Kakeji et al. 1993; Kim et al. 1991; Matozaki et al. 1992; Seruca et al. 1992; Tamura et al. 1991; Yamada et al. 1991) and colorectal cancers (Baker et al. 1990; Bell et al. 1993; Campo et al. 1991; Cunningham et al. 1992; Kikuchi-Yanoshita et al. 1992; Nigro et al. 1989). The purposes of this study were to determine if different rates of of p53 mutations as detected by immunohistochemistry (IHC) were associated with the different incidences of gastric and colorectal cancers in South Texas Hispanic and Anglos, and also to compare p53 status by IHC with several clinical-pathological characteristics including tumour site, grade, stage, subtype, and patient survival. We also sequenced five of the p53 mutations found in gastric cancers in these populations, to see if they differed from previously reported mutations in gastric cancer.

Materials and methods

Formalin-fixed, paraffin-embedded surgical specimens of 109 sequential gastric and 112 colorectal adenocarcinomas (selected blinded to the ethnic origin of the patient) were obtained from three hospitals (Bexar County Medical Center Hospital, Santa Rosa Medical Center Hospital and the Audie Murphy Memorial Veterans Administration Hospital, all located in San Antonio, Texas). For comparison, paraffin-embedded samples from 22 gastric and 26 colorectal tumours were obtained from the Hospital General de México in Mexico City. Ethnicity and survival times of the patients, and grade, site and subtype of the tumours were determined from medical records and from tumour registries in the respective hospitals. Anglo patients in this area are predominantly of northern European descent, and Hispanic patients are those of Latin American (mainly Mexican) descent. Staging was performed by TNM criteria (Beahrs et al. 1992) using information obtained in surgical pathology reports. Colorectal tumours were classified as proximal, if they arose between the caecum and the splenic flexure, or distal, if they developed beyond the splenic flexure. Gastric cancers were classified by Laurén's classification (Laurén 1965).

The majority of p53 mutations appear to result in a conformationally altered, nonfunctional, and stabilized protein which accumulates in the nuclei of tumour cells in amounts detectable by IHC (Finlay et al. 1988; Oren 1985). This phenomenon, and the ease and economy of IHC, have resulted in its use as a relatively sensitive and specific screening assay for p53 mutations.

Deparaffinized sections (4 μm) of tumours (one section per tumour) were labelled with a mixture of antibodies against p53 (PAb1801, diluted 1:20 with 1% ovalbumin, and CM-1, diluted 1:1000; Novocastra, Newcastle upon Tyne, UK). This combination of antibodies was found in preliminary studies to be more sensitive than either antibody alone. The monoclonal antibody 1801 reacts with amino acids 1–91 in the N-terminus of human p53. (Vojtesek et al. 1992). In addition, the monoclonal antibody DO-7 (Novocastra), which reacts with amino acids 1–45 of p53 (Vojtesek et al. 1992), was applied to some tumours. Bound antibodies were detected with sequential incubation in biotinylated rabbit anti-mouse IgG, (16 μg/ml), biotinylated swine anti-rabbit IgG, (2.3 μg/ml) and streptavidin-biotin-horseradish peroxidase (HRP), diluted 1:100 (DAKO; Carpinteria, California). The substrate for the HRP reaction was diaminobenzidine (1 mg/ml), and the reaction product was enhanced with reduced osmium (0.2%). Sections were counterstained with methyl green (0.5%). Control cases included in IHC experiments were colon tumours previously shown to demonstrate positive and negative nuclear immunostaining for p53. As a reagent control, non-immune rabbit and mouse IgG solutions were substituted for the primary antibody. Immunolabelling was scored semi-quantitatively according to the following scheme: proportion of tumour cells which are positive (PS) 0=none, 1=1%, 2=2–10%, 3=11–33%, 4=34–66%, 5=67–100%; intensity (IS) 0=negative, 1=weak, 2=intermediate, 3=strong. The total score (TS) was computed by adding PS and IS. Previous work with breast cancer has found any positivity (that is to say, TS>0 versus TS=0) to be prognostic (Allred et al. 1993), and we have used this dichotomization here. In analysis of gastrointestinal tumours, we examined other cut-off points and employed TS, PS and IS as continuous variables without improving the predictive value.

While data for the Hispanic cases from Mexico were initially analysed as a separate category, their results were very similar to those of Hispanic cases from San Antonio, and only the combined results are reported here. Associations between p53 status and other categorical variables were analysed using contingency tables. Two by two tables were tested using Fisher's exact test. Larger tables were analysed using the Chi square test for association, or in the case of ordered categories, (i.e., grade or stage), the Mantel-Haenzel test for trend. Age at diagnosis was analysed using *t*-tests (2 sample) and one-way analysis of variance. Survival curves were estimated using the Kaplan-Meier product-limit method and compared using the log rank test. The analyses were performed with SAS (SAS Institute, Cary, North Carolina).

For the genetic identification of p53 mutations, frozen tissue was available from 13 of the gastric cancer specimens (12 of which were from Hispanic patients), where the tumour fraction was estimated as at least 1/3 of the nuclei. Single 10 μm sections 1 cm^2 or less in size were harvested in microcentrifuge tubes. Adjacent normal tissue from the same patient was similarly collected. The tissue was digested with 25–40 μl proteinase K (1 mg/ml, in 50 mM TRIS-HCl buffer, pH 8, with 1 mM EDTA, 0.45% NP-40 and 0.45% Tween-20) by overnight incubation at 52° C. The proteinase K was then denatured by heating at 95° C for 15 min. From these digested samples, highly conserved regions of the p53 gene (exons 5–9) were amplified by the polymerase chain reaction (PCR), using primers and amplification conditions described previously (Hensel et al. 1991). We screened the samples for mutations in these regions with single strand conformation polymorphism analysis (SSCP) (Orita et al. 1989), using primers which amplified exons 5–6, 7, and 8–9. An alternative primer pair employed to amplify exons 5 and 6 was TGACTTTCAACTCTGTCTCCT and CAGAGACCCCAGTTGCAAAC, used with an annealing temperature of 50° C and 1.5 mM magnesium chloride. Samples were analysed on non-denaturing gels (5% polyacrylamide) with and without 10% glycerol and electrophoresed at 45 W at room temperature with a cooling fan (Hensel et al. 1991). Where a mutation was identified by a band shift, the region of the gene was cloned into pCR 1000 (Invitrogen, San Diego, California) and sequenced. Alternatively, some amplified products were purified using the Magic PCR Preps DNA purification resin (Promega, Madison, Wisconsin) and sequenced with a dsDNA Cycle Sequencing System (GIBCO BRL, Gaithersburg, Maryland), according to the manufacturer's suggested protocols. Each mutation was confirmed by sequencing in both directions from independent clones which were derived from more than one PCR amplification to ascertain that the mutation was not a result of a misincorporation error (Mor et al. 1992) or a cloning artefact.

Results

Figure 1 shows typical examples of tumours positive and negative for accumulation of nuclear p53 protein by the IHC assay used in this study. For gastric cancers, the relationships between IHC results and various clinical-

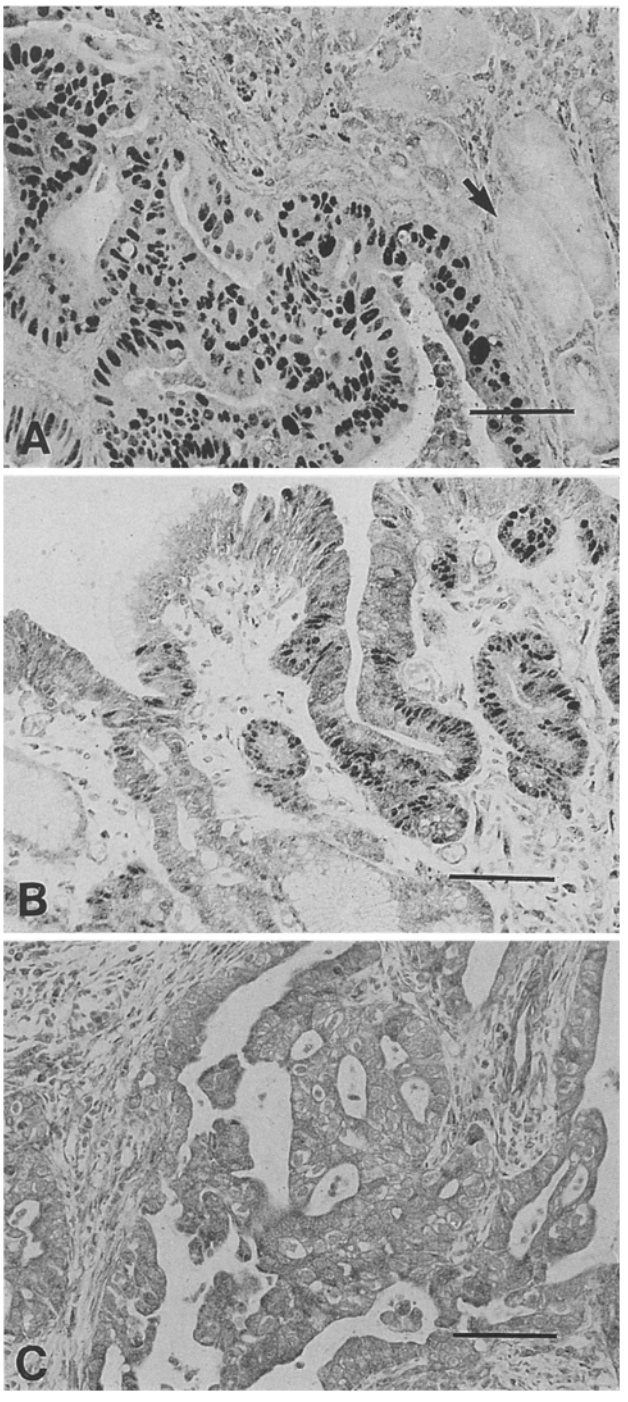

Fig. 1A–C Immunohistochemistry with anti-p53 antibodies. **A** This intestinal type gastric tumour showed dense nuclear staining with antibodies CM1/1801. The signal in colonic carcinomas had a similar appearance. Benign epithelium (*arrow*) was unlabelled. **B** A region of dysplastic gastric epithelium was p53 positive using antibodies CM1/1801. Although not frankly malignant, this tissue already showed evidence of p53 mutation. The tumour from this tissue contained a T to C missense mutation at codon 232 (Table 4, sample 1). The presence of the mutation in the dysplastic epithelium indicates that, at least in this case, the mutation in the p53 gene was an early event in the process of carcinogenesis. **C** This gastric tumour, labelled here with antibodies CM1/1801, also showed no positive labelling with antibody DO-7 (data not shown). The p53 gene from this tumour contains a mutation in exon 6 which, when translated, produces a truncated protein lacking a nuclear localization signal (Shaulsky et al. 1990) (Table 4, sample 5; Fig. 3). Scale bar, 50 μm

Table 2 Gastric tumour characteristics and p53 status ($n=131$)

	p53 negative (cases)	p53 positive (cases)	% pos.	p value
Ethnicity				
Anglo	11	17	61%	0.13[a]
Hispanic	59	44	43%	
Gender				
Female	26	13	33%	0.06[a]
Male	44	48	52%	
Grade				
Well	6	7	54%	0.34[b]
Moderate	17	16	48%	
Poor	33	23	41%	
Subtype				
Diffuse	37	20	35%	0.045[a]
Int. + Mixed	30	36	55%	
Stage				
I	4	4	50%	0.87[b]
II	30	20	67%	
IIIA	6	1	16%	
IIIA	4	6	60%	
IV	4	3	43%	

[a] Fisher's exact test
[b] Mantel-Haenzel test for trend

pathological features of tumours are shown in Table 2. A non-significant trend for a reduced rate of p53 immunostaining was noted in Hispanics compared to Anglos (43% versus 61%, respectively, $p=0.13$). A weak correlation between positive staining for p53 and the diffuse histological subtype of gastric carcinoma was observed ($p=0.05$), but no associations were detected with gender, tumour grade, or stage.

For colorectal cancers, the relationships between IHC results and various features of the tumours are shown in Table 3. The rates of positive immunostaining were almost identical in Anglos and Hispanics (58% versus 57%, respectively, $p=1.0$). Poorly differentiated tumours were more likely to be p53+ than were well-differentiated tumours ($p=0.038$). No associations were detected with stage or site of the tumour, or with gender of the patient.

As shown in Fig. 2, p53 positivity alone was not associated with differences in survival in either gastric or colorectal carcinomas ($p=0.47$ and $p=0.66$, respectively. After correcting for the highly significant correlation of stage with survival ($p<0.001$), there was no detectable association between p53 positivity and survival ($p>0.2$).

SSCP analysis of exons 5–9 in 13 frozen gastric tumours showed aberrant bands in five of these samples, suggesting the presence of a mutation in the gene. Upon

Table 3 Colorectal tumour characteristics and p53 status ($n=138$). Total numbers of cases may vary in categories where data was missing for some cases.

	p53 negative (cases)	p53 positive (cases)	% pos.	*p* value
Ethnicity				
Anglo	21	29	58%	1.0[a]
Hispanic	38	50	57%	
Gender				
Female	17	28	62%	0.47[a]
Male	42	51	56%	
Grade				
Well	16	12	43%	0.038[b]
Moderate	35	41	54%	
Poor	3	16	84%	
Site				
Distal	31	45	59%	0.47[a]
Proximal	26	28	52%	
Stage				
I	5	5	50%	0.71[b]
II	17	21	55%	
III	14	23	61%	
IV	5	12	71%	

[a] Fisher's exact test
[b] Mantel-Haenzel test for trend

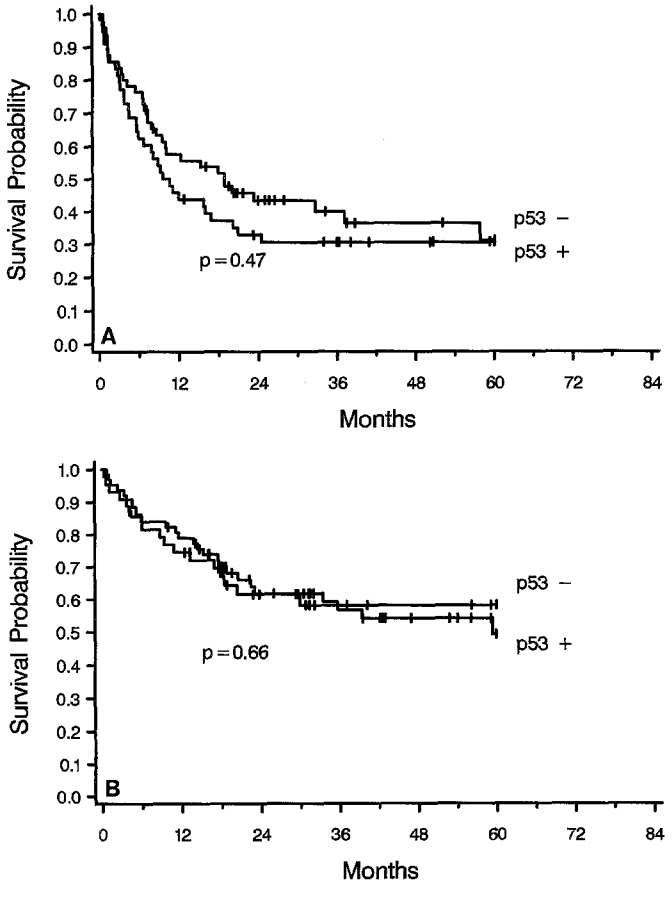

Fig. 2 Survival curves for gastric (**A**) and colorectal (**B**) cancer patients. p53 positivity by IHC did not correlate with survival in either tumour type

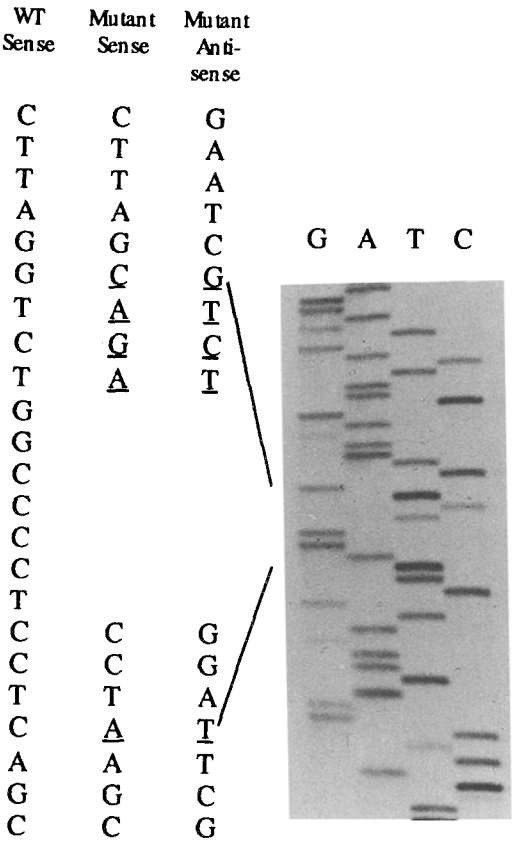

Fig. 3 Complex deletion mutation. The sequence of a cloned portion of exon 6 from the tumour represented in Fig. 1C reveals a deletion of 7 base pairs, a 4 base pair substitution and a point mutation

Table 4 p53 mutations in primary gastric tumours

Sample	Exon	Codon	Amino acid change
1	7	232, ATC>ACC	ile >thr
2	7	234, TAC>TGC	tyr >cys
3	7	248, CGG>TGG	arg >trp
4	8	282, CGG>TGG	arg >trp
5	6	190, deletion	frame shift

sequencing the amplified DNA segments, we found four of the five contained missense mutations, and one contained a complex deletion (Table 4). Two of the four missense mutations were G: C to A: T transitions, and one of these mutations occurred at a codon 248 which is frequently mutated in colorectal carcinomas (Hollstein et al. 1991). Two other missense mutations were A: T to G: C transitions. The deletion mutation (sample 5) involved the 5' end of exon 6 (Figs. 1C, 3), where 7 bp were deleted causing a frame shift, 4 bp of sequence complementary to the wild type sequence were substituted, and a C to A point mutation occurred. All of the missense mutations showed positive p53 immunostaining. The deletion mutation was negative, both with the combination of antibodies CM1 and 1801, and with DO-7. For four of the five cases (samples 1–3 and 5),

normal tissue was available from the same patient. The portion of the p53 gene mutated in the tumour was sequenced in the normal tissue from each of the four patients and found to be wild type.

Discussion

The p53 gene is distinctive in its high frequency of mutation in diverse tumour types (Hollstein et al. 1991; Nigro et al. 1989). Once the elevated frequency of p53 mutations in human tumours was appreciated, patterns in the site and type of the mutation became interesting for the insight they gave into processes of carcinogenesis (Bressac et al. 1991; Hsu et al. 1991; Somers et al. 1992). The present study asked whether different rates of p53 mutations of the missense type which are positive by IHC, were associated with the strikingly different incidences of gastric and colorectal carcinomas in Hispanic compared to Anglo populations. Although there was a non-significant trend ($p=0.13$) for a lower rate of p53 positive tumours in Hispanic gastric carcinoma, no evidence was found to suggest that p53 was playing a differential role in generating the variations in incidence rate of this tumour. It is likely that other as yet unidentified oncogenes and/or tumour suppressor genes are involved.

The relatively high incidence of gastric carcinoma in Hispanics suggests that there may indeed be unique risk factors involved in the development of this disease. For example, an association between *Helicobacter pylori* and gastric cancer has been reported in studies in other populations (Correa et al. 1990; Forman et al. 1990; Loffeld et al. 1990; Nomura et al. 1991; Parsonnet et al. 1991a, b). Asymptomatic Hispanics in a South Texas study had a rate of *H. pylori* infection approximately twice that of Anglos (Malaty et al. 1992). It is noteworthy that this ratio is similar to the ratio of gastric cancer in Hispanics versus Anglos seen in data from Texas (Table 1). In addition, mutagenic substances associated with a traditional South Texas Hispanic or Mexican diet are under investigation (Agarwal et al. 1986; Lawson and Gannett 1989; Nagabhushan and Bhide 1985; Toth et al. 1984). Identification of the putative carcinogens and their influence on various proto-oncogenes and/or tumour suppressor genes are important topics for future investigations.

Because p53+ tumours have been reported to have a worse prognosis in other tumour types, such as lung (Quinlan et al. 1992), breast (Allred et al. 1993; Isola et al. 1992; Thor et al. 1992), bladder (Sarkis et al. 1993) and prostate (Bookstein et al. 1994; Visakorpi et al. 1992), we asked whether nuclear p53 accumulation would correlate with poor survival in our patient population. We found no correlation between survival and nuclear positivity for p53 in either cancer type we studied. In previous IHC studies of gastric or colorectal tumours from other populations, some have detected an association (Martin et al. 1992; Starzynska et al. 1992; Yamaguchi et al. 1992), and some found none (Bell et al. 1993; Scott et al. 1991; Sun et al. 1992). Differences in patient populations or variation in the pathogenesis of the disease may account for the differences in results.

The relatively low rate of p53 IHC positive gastric carcinomas observed in Hispanics (43%) compared to Anglos (61%) in this study raises the possibility that gastric tumours from Hispanics may have an excess of mutations that do not result in nuclear accumulation of p53 (for example, nonsense mutations, insertions, or deletions) (Bodner et al. 1992). Our sequencing results from 5 gastric cancers argue against this, in that 4 of the 5 p53 mutations detected were missense transitions associated with positive immunostaining, and only one was a deletion associated with negative immunostaining. This is consistent with previous sequencing studies of primary gastric carcinoma showing that the majority of p53 mutations are missense mutations, while only 20% are deletions or insertions ($n=40$, including this study) (Imazeki et al. 1992; Kim et al. 1991; Matozaki et al. 1992; Renault et al. 1993; Seruca et al. 1992; Tamura et al. 1991).

Two of the 5 mutations we identified in gastric cancer were C: G to T: A transitions. The two C: G to T: A mutations occur at CpG sites, which are known to be readily mutable. The instability at these sites is attributed to the spontaneous deamination of 5-methylcytosines (Ehrlich and Wang 1981). When considered with other p53 mutations in primary gastric cancer reported in the literature (Imazeki et al. 1992; Kim et al. 1991; Matozaki et al. 1992; Renault et al. 1993; Seruca et al. 1992; Tamura et al. 1991), the C: G to T: A mutations comprise 69% of those missense mutations published (22 of 32 examples, including those of this study). This result is similar to studies of colorectal carcinoma showing that about 80% of missense mutations are C: G to T: A transitions, (Hollstein et al. 1991; Kikuchi-Yanoshita et al. 1992).

In our initial studies into the genetic basis of the relatively high and low incidence of gastric and colorectal carcinomas in Hispanics when compared with Anglos, we found that the rates of p53 mutations, as measured by IHC, were not associated with these epidemiological variations. In addition, p53 IHC status was not associated with patient survival in either gastric or colorectal carcinoma. Examination of candidate genes other than p53 for their contribution to carcinogenesis in both of these tumour types is in progress.

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