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TP53 in Gastric Cancer: Mutations in the L3 Loop and LSH Motif DNA-Binding Domains of TP53 Predict Poor Outcome

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The aim of this study was to clarify whether specific p53 mutations may have biological relevance in terms of disease relapse or death in gastric carcinomas (GC). Resected specimens from a consecutive series of 62 patients with GC undergoing potentially curative surgery were prospectively studied. The mutational status of exons 5–8 of the p53 gene was investigated in 62 cases using the PCR-SSCP and sequencing. Presence of microsatellite instability (MSI) was evaluated in 56 cases by analyzing loci highly sensitive of MSI. Twenty mutations of p53 were detected in 17 of the 62 cases analyzed (27%). Ten mutations (50%) occurred in highly conserved domains. According to the p53 specific functional domains: 4/20 mutations (20%) were in the L3 loop and 3/20 (15%) in LSH motif. Eight of the 56 GC resulted MSI-H, 5 (9%) MSI-L, and 43 (77%) MSI stable (MSS). None of the 8 (14%) MSI-H GC showed p53 mutations. p53 mutations were associated with intestinal histotype. Moreover, specific mutations in functional domain (L3 and LSH), together with advanced TNM stage, node involvement, depth of invasion, diffuse histotype, proved to be significantly related to quicker relapse and to shorter overall survival. Specific mutations in p53 functional domains, rather than any mutations in this gene, may be biologically more significant in terms of patients outcome, indicating that these mutations might have biological relevance to identify subgroups of patients at higher risk of relapse or death who might benefit from a more aggressive therapeutic approach. J. Cell. Physiol. 200: 476–485, 2004.

Despite a decreasing trend in incidence and mortality in most developed countries, gastric carcinomas (GC) was still recently estimated to represent the fourth most common cancer in the world and the second leading cause of cancer death (Gonzalez et al., 2000). Moreover, this neoplasia still leads to an unfavorable prognosis, with a relative 5-year survival rate varying from 5% to 15% in the Western world. In adenocarcinomas, which represent over 95% of GCs, distinct morphological, histological, and biomolecular features have been observed, with the result that a great many classification systems for GC have been devised in the attempt to identify patient subgroups with different prognostic features.

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Lauren (1965) classified GC into two groups: (1) an intestinal, differentiated type, forming tubular or papillary structures, and (2) a diffuse undifferentiated type, where such structures are almost entirely absent (Stadtlander and Waterbor, 1999; Endoh et al., 2000). The last 20 years have seen an increase in the incidence of diffuse-type GCs (Borch et al., 2000), which generally lead to an unfavorable outcome (Karpeh et al., 2001); nevertheless, patients with identical histopathological features may follow a completely different clinical course.

From the biomolecular point of view, it has now been established that carcinogenesis also in GCs is a multistep process where a series of genetic alterations accumulate until they produce a neoplastic phenotype (Fiocca et al., 2001). At least two pathways of genomic instability have been identified in gastric carcinogenesis. The first pathway is characterized by the successive accumulation of mutations in oncogenes and tumor suppressor genes controlling epithelial cell growth and differentiation. In particular, p53 mutations have been quite frequently seen in GC (20–50% of cases), mostly presenting as puntiform mutations in exons 5–8 (Prives and Hall, 1999; Fenoglio-Preiser et al., 2003). This region is made up of about 600 bp and contains several areas which have been highly conserved during evolution, together with a great many functional domains, among which the L2 loop, which is needed for the correct folding and stabilization of the central part of the protein, the L3 loop and the L1 loop-sheet-helix motif (LSH), directly involved in interaction between the protein and the DNA (Cho et al., 1994). It has been reported that p53 alterations are often associated with greater invasiveness of tumors (Sakurai et al., 1995; Aas et al., 1996) and particular attention has recently been paid to the prognostic role played by specific alterations of the gene p53 (in conserved areas or in functional domains) in several types of human neoplasias (colorectal, lung and breast cancers) (Borresen et al., 1995; Borresen-Dale et al., 1998; Skaug et al., 2000; Russo et al., 2002).

The second pathway of genomic instability, known as microsatellite instability (MSI) pathway, is characterized by the accumulation of somatic alteration in the length of simple nucleotide repeats (microsatellite) mostly consisting in small deletions/insertions that affect short repeated DNA sequences in either coding or non-coding regions (Simpson et al., 2001). These short repeated sequences include mononucleotide repeats, that typically manifest contractions, as well as di-, tri-, and tetranucleotide repeats that may show either contractions or expansions. Since di-, tri-, and tetranucleotide repeats manifest infrequently high levels of instability, the presence of alterations at these repeats is considered as indicator of low level of instability (MSI-L), whereas contractions at mononucleotide repeats is considered the hallmark of the MSI-high (MSI-H) phenotype. Tumors presenting MSI-H have a deficit of the mismatch repair system (MMR) (Breivik and Gaudernack, 1999), present karyotype stability, and follow progression pathway(s) characterized by the accumulation of frameshift mutations at coding repetitive sequences within genes whose products play roles at different levels in the control of cell growth, apoptosis, and genome integrity (Ohmiya et al., 2001).

The aim of our study was to investigate molecular pathways underlying gastric carcinogenesis by analyzing p53 mutations and MSI status in 62 GC cases and to determine whether or not there were associations between these two variables and the traditional clinico-pathological factors, with particular emphasis on their involvement in the pathogenesis of tumoral histotype, and also to evaluate their possible predictive role with regard to the disease-free interval and survival rate of such patients.

MATERIALS AND METHODS

Clinicopathological variables

Resected specimens from a consecutive series of 62 patients with GC who had undergone potentially curative surgery were prospectively studied. All patients were operated on between January 1992 and December 1996, at the same Institute (Department of Oncology, University of Palermo, Palermo, Italy). Clinicopathological factors included: age and sex; tumor location (cardia or fundus, corpus, and antrum); tumor size ($\leq$5 cm and $>$5 cm); depth of invasion [PS($-$): invasion into the muscularis propria or into the subserosa without infiltrative growth; PS($+$): invasion into the subserosa with infiltrative growth]; TNM stage (I–IV); node status (negative and positive); histological grade (G1–G3); histotype (intestinal and diffuse).

None of them had received chemo- or radiotherapy before surgery. Curative resection included gastrectomy and extensive dissection of regional lymph nodes. The resected GC and lymph nodes were examined pathologically and staged according to the latest TNM classification. Tissue sections from all the 62 surgical specimens were examined blind by two separate pathologists (R.M.T. and M.M.). Tumors were classified according to Lauren's criteria into intestinal or diffuse (Lauren, 1965). The grade of differentation was also evaluated and ranked as G1, G2, and G3 (respectively, well, moderately, and poorly differentiated). Written informed consent was obtained from all patients included in this study.

Patients were followed-up every 3 months for the first 2 years, at six-monthly intervals for the next 2 years and annually thereafter. All reasonable attempts were made to document disease relapse (local recurrence or distant metastases) cytologically or histologically. All patients with relapse disease after surgery received a standard chemotherapeutic regimen for GC (5-fluorouracil, epirubicin, methotrexate, etoposide, doxorubicin, and cisplatin). Information on survival (disease-free survival (DFS) and overall survival (OS)) was obtained directly from clinical charts and through the Oncology Section at our Department. Clinicopathological and follow-up data of all patients have been recorded prospectively in a computerized registry database (Table 1).

Tissue-handling and DNA extraction

Multiple samples (3–10) of the primary GC tissue were taken from different representative areas (including the core and the invasive edge of the tumor) and processed within 30 min of surgical resection. The portion of primary tumor was obtained by superficial biopsy of either the tumor bulk or the edge of the malignant ulcer for more infiltrative cancer. All tissues

TABLE 1. Clinicopathologic variables of the 62 gastric cancer patients

	Patients (N)
Sex	
Male	42
Female	20
Age	
<60	18
60–69	24
>70	20
Site	
Antrum	16
Corpus	26
Cardias or fundus	20
Tumor size (cm)	
<5	28
>5	34
Tumor type	
Intestinal	50
Diffuse	12
Tumor grade	
Well differentiated (G1)	4
Mod differentiated (G2)	24
Poorly differentiated (G3)	34
Stage	
I	12
II	25
III	21
IV	4
Invasion	
PS–	37
PS+	25
Node status	
Negative	18
Positive	44

were carefully trimmed to remove as much non-neoplastic material as possible, avoiding the non-viable areas. In addition, from each patient multiple samples of normal-appearing tissue were taken in a corresponding non-tumoral area as far as possible from the tumor site, to be used as a standard reference for biomolecular analyses. Tissues were bisected and one half of each sample was fixed in 70% ethyl alcohol and paraffin-embedded for pathological examination; the remaining half of the sample pool was immediately frozen and stored at −80°C until analysis. Histopathological examination on cryostat sections stained with hemotoxylin and eosin was then performed; only those samples containing more than 80% of neoplastic cells were used for biomolecular studies. Where present, areas with a high content of non-neoplastic cells were removed from the frozen block with a scalpel. Evaluation of each biomolecular variable (p53 and MSI status) was performed independently with no knowledge of clinical data. Genomic DNA was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) with the standard protocol from primary GC and normal gastric specimens.

Detection of p53 gene mutations

Mutations within the p53 gene were detected by SSCP analysis following PCR amplification of the exons 5–8, performed as described previously (Russo et al., 1998). In every instance, negative (DNA was replaced with water) controls were amplified by PCR and included in the experiment. In all PCR assays, aerosol-resistant pipette tips were used to avoid cross-contamination. The quality and the concentration of the amplification products were verified by 1.5% agarose gel electrophoresis and ethidium bromide staining. One hundred nanogram aliquots of the amplified DNA fragments, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA), were denatured and analyzed by SSCP analysis. PCR-SSCP analysis was repeated twice for each sample to minimize the possibility of artifacts due to contamination or polymerase errors and interpretation of SSCP analysis was performed by consensus of two investigators. DNA of normal colon tissue from each patient was also amplified and run in parallel with matched tumoral DNA samples on SSCP gels, to evaluate the occurrence of germ-line mutations or polymorphisms. Individual ssDNA fragments with shifted mobilities, compared to normal control, were electroeluted from polyacrylamide gel, as described previously (Albanese et al., 1997), reamplified and sequenced. Automated sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the model 3100 DNA sequencer (Perkin-Elmer, Foster City, CA).

MSI analysis

Mutations at repetitive tracts were analyzed using a PCR-based assay. MSI status was assessed at 3 loci by analyzing markers included in the recommended reference part of markers for the detection of MSI (the mononucleotide repeats BAT25, BAT26, and the dinucleotide repeat D2S123). Tumors were classified as MSI-H when showing contraction(s) in BAT25 and/or BAT26, regardless of the status of dinucleotide loci, as MSI-L when instability was limited to dinucleotide loci, and as MSS when no instability was observed at the loci tested.

Coding repeats within genes involved in the control of cell proliferation [TGF-βRII poly$(A)_{10}$, IGFIIR poly$(G)_8$, RIZ poly$(A)_9$], transcription [TCF4 poly$(A)_9$], apoptosis [BAX poly$(G)_8$, BCL 10 poly$(A)_8$, FAS poly $(T)_7$, CASPASE5 poly$(A)_{10}$, APAF1 poly$(A)_8$] and of DNA repair [hMSH6 poly$(C)_8$, hMSH3 poly$(A)_8$, MED1 poly$(A)_{10}$, RAD50 poly$(A)_9$, BLM poly$(A)_9$] were analyzed in the MSI-H subset. PCR products were directly sequenced using the Sequenase PCR Sequencing Kit (USB), or alternatively, after insertion into a plasmid vector with the Topo TA Cloning Kit (Invitrogen, Carlsbad, CA). PCRs, electrophoretic separation, and autoradiography were as described previously (Ottini et al., 1997; Ottini et al., 1998). Paired genotypings of all cases positive for microsatellite alterations were confirmed in duplicate experiments, using independently extracted DNA samples.

Statistical analysis

Association between p53 mutations, MSI and clinicopathological variables was evaluated by means of the Chi-square test and, where appropriate, Yates' test. DFS was measured from the day of primary surgery to the date of first relapse (locoregional or metastatic) and OS from the day of surgery to the day of death specifically due to the tumor. If patients did not relapse or die, they were censored at the time of their last follow-up. Clinical and morphobiological variables were examined

by means of the Kaplan–Meier method (Kaplan and Meier, 1958); significance of differences for each prognostic factor was assessed by the logrank and Wilcoxon's tests or trend tests where appropriate. Multivariate analysis was carried out by means of Cox's proportional hazards model, using a backward procedure (Cox, 1972). *P*-values less than 0.05 were considered statistically significant.

RESULTS

General outcome

The mean follow-up of patients was of 95 months (range, 5–137 months). At the time of analysis (December, 2002), 35 patients had relapsed, (of which 1 had locoregional recurrence and 34 distant metastases), while 34 had died for tumor-related causes. Overall 5-year survival rate was of 49.3% (SE ± 6.9) for the whole series.

Mutation analysis of p53 gene

Mutation analysis of exons 5–8 of the p53 gene was performed on genomic DNA from primary GCs of 62 patients. Aberrantly migrating bands were found in 27% (17/62) of the cases (Fig. 1a). Sequence analysis of the DNA fragments with altered electrophoretic mobility made it possible to establish the exact site and nature of the genetic alteration in all tumor samples (Fig. 1b).

Overall, 20 p53 mutations were identified in 62 of those screened. The features of the p53 mutations are summarized in Table 2.

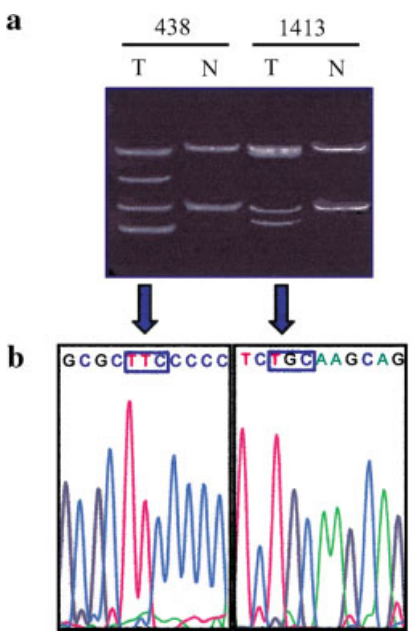

Fig. 1. SSCP analyses of exon 5 of the p53 gene amplified from gastric carcinoma and normal-appearing tissue genomic DNA of two patients (cases 483 and 1,413). In each pair of lanes, the tumor DNA is at the left (T) and the normal tissue DNA (N) is at the right (**a**). The extra bands visualized by arrows in lines 1 and 3 correspond to ssDNA molecules harboring mutations in codon 176 (TGC to TTC) and 163 (TAC to TGC), as confirmed by sequencing (**b**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Of the 20 mutations, 45% (9/20) were in exon 5, 20% (4/20) in exon 6, 20% (4/20) in exon 7, and 15% (3/20) in exon 8. Two tumor samples were found to harbor two (in two separate exons) or three (in three separate exons) different p53 mutations. Eighteen of the 20 sequenced mutations (90%) were found to be missense mutations, while silent mutations were found in two cases (all of them in codon 213 [CGA-CGG], a previously identified site of polymorphism). Moreover, transitions (75%, 15 of 20) were far more frequent than transversions (25%). No germ-line mutations were found, indicating that in every case the change was somatic. Fifty percent of the mutations (10 of 20) occurred in highly conserved domains (areas II, codons 112–141; area III, codons 171–181; area IV, codons 234–258; area V, codons 271–286). Accordingly, tumors with p53 mutations were classified into two groups: 59% of the cases (10/17 cases) with mutations in conserved areas of the p53 gene (conserved); 41% (7/17 cases) with mutations outside the conserved areas (non-conserved). In addition, by taking into account the specific functional and structural domains of p53, 30% of mutations (6/20) affected L2 loop (between codons 163 and 195), 20% (4/20) L3 loop (between codons 236 and 251), and 15% (3/20) LSH motif (between codons 273 and 286), so that the cases were classified as follows: 4/17 cases (24%) with mutations of the L3, 3/17 cases (18%) with mutations of the LSH motif, and 10/17 cases (58%) with mutations outside L3 and LSH. Since silent mutations do not determine any aminoacid change in the protein, they have been included in the wild-type group for statistical analysis.

Analysis of MSI

MSI analysis was conducted on 56 of the 62 GCs (because of the DNA exhaustion). By evaluating BAT25 and BAT26, two mononucleotide repeats considered sensitive indicators of MSI-H status, and D2S123, a dinucleotide repeat useful to discriminate MSI-L from MSS cases, 8 tumors (14%) resulted MSI-H, 5 (9%) MSI-L and 43 (77%) MSS (Table 2). The 8 MSI-H GCs were analyzed for mutations at coding repeats within genes involved in the control of cell growth (TGF-βRII, IGFIIR, RIZ, TCF4), apoptosis (BAX, BCL10, FAS, CASPASE5, APAF1), and DNA damage repair (hMSH6, hMSH3, MED1, RAD50, BLM). Mutation frequencies ranged from 0% to 50% (Table 3). Overall, the most frequently mutated target genes resulted TGFβ-RII and MSH3, in fact, frameshift mutations at the TGFβ-RII poly$(A)_{10}$ and at the hMSH3 poly$(A)_8$ were observed in 50% MSI-GCs. Mutation frequencies of 37.5% at the BAX poly$(G)_8$ and BLM poly$(A)_9$, of 25% at the hMSH6 poly$(C)_8$ and RIZ poly$(A)_9$, of 12.5% at the IGFIIR poly$(G)_8$, at the FAS poly $(T)_7$ and at the MED1 poly$(A)_{10}$ were observed in the MSI-H GCs analyzed.

Multiple target genes were simultaneously mutated in the majority of the MSI-H GCs (5/8, 62.5%) (Table 3).

Frameshift mutations in coding mononucleotide repeats consisted mostly in 1-bp deletions (19/23, 83%), 1-bp insertions, and 2-bp deletions were infrequent, respectively, accounting for 4% (1/23) and 4% (1/23) of the total number of mutations. Biallelic mutations, respectively at the TGFβ-RII poly$(A)_{10}$ and at the BAX poly$(G)_8$, were observed in only two cases (2/23, 8%) and

TABLE 2. p53 status, microsatellite instability status, and tumor histotype in the series of the 62 gastric cancer cases

Case	p53 status	Exon	Codon	Nucleotide change	Aminoacid change	Conserved area	Functional domain	MSI status	Tumor histotype
22	wt							MSI-L	IT
31	mut	5	175	cgc → ccc	arg → pro	Area III	L2	MSS	IT
46	mut	7	244	ggc → gac	gly → asp	Area IV	L3	MSI-L	IT
50	wt							MSI-H	IT
79	wt							MSI-H	IT
90	wt							MSI-L	IT
91	wt							MSI-L	IT
92	wt							n.v.	IT
128	wt							MSS	DT
138	mut	5	176	tgc → ttc	cys → phe	Area III	L2	n.v.	IT
141	wt							MSI-H	IT
226	wt							MSS	IT
236	wt							MSS	IT
279	wt							MSI-H	IT
295	wt							MSS	IT
321	wt							MSS	IT
340	wt							MSS	DT
343	wt							MSS	IT
356	wt							MSS	IT
376	wt							MSS	IT
422	wt							MSS	IT
428	mut	6	195	atc → acc	ile → thr	Out	L2	MSS	IT
431	mut	6	214	cat → cgt	his → arg	Out	Out	MSS	IT
438	mut	5	176	tgc → ttc	cys → phe	Area III	L2	MSS	IT
440	mut	7	248	cgg → cag	arg → gln	Area IV	L3	MSS	IT
447	wt							MSS	DT
474	wt							MSI-H	DT
476	wt							MSS	DT
483	mut	8	285	gag → aag	glu → lys	Area V	LSH	MSS	IT
498	wt							MSI-H	IT
500	wt							MSS	IT
533	wt							MSS	IT
561	mut	5	154	ggc → gtc	gly → val	Out	Out	MSS	IT
599	wt							MSS	IT
604	wt							n.v.	IT
621	wt							MSS	IT
654	wt							MSI-H	DT
662	wt							MSS	IT
690	wt							MSS	DT
699	wt							MSS	IT
748	wt							MSS	IT
756	mut	5	157	gtc → ggc	val → asp	Out	Out	MSS	IT
		6	213	cga → cgg	arg → arg	Out	Out	MSS	IT
		7	248	cgg → cag	arg → gln	Area IV	L3	MSS	IT
783	wt							MSS	DT
801	mut	7	249	agg → ggg	arg → gly	Area IV	L3	MSS	IT
828	mut	5	152	ccg → cgg	pro → arg	Out	Out	MSS	IT
		6	213	cga → cgg	arg → arg	Out	Out	MSS	IT
852	wt							MSS	IT
870	wt							MSS	IT
890	wt							MSI-H	IT
969	wt							MSS	DT
992	mut	8	275	tgt → tat	cys → tyr	Area V	LSH	MSS	IT
1,003	mut	5	154	ggc → atc	gly → ile	Out	Out	MSS	IT
1,037	wt							MSS	IT
1,210	wt							MSI-L	IT
1,228	wt							MSS	IT
1,241	wt							MSS	DT
1,245	mut	5	163	tac → tgc	tyr → cys	Out	L2	MSS	IT
1,303	mut	8	276	gcc → acc	ala → thr	Area V	LSH	MSS	IT
1,340	wt							MSS	DT
1,373	wt							MSS	DT
1,395	wt							n.v.	IT
1,406	wt							n.v.	IT
1,413	mut	5	163	tac → tgc	tyr → cys	Out	L2	n.v.	IT

IT, intestinal type; DT, diffuse type.

consisted in 1-bp deletions associated with 1-bp insertions. In the other cases, the occurrence of homozygous 1-bp deletions is unlikely because the wild-type allele signal was always present, with reduction in intensity within the 50% range.

Relationship between biomolecular indicators and clinical data

No association was found between p53 mutations and MSI status. Nevertheless, considering the presence of

TABLE 3. Target gene mutations in the eight gastric cancers with high microsatellite instability

Case	50	79	141	279	474	498	654	890
TGFb RII	1del/1ins	1del	—	—	—	—	1del	1del
IGFRII	—	—	—	—	—	—	—	—
RIZ	1ins	—	—	—	—	—	1del	—
TCF4	—	—	—	—	—	—	—	—
BAX	1del	—	—	—	1del	—	1del/1ins	—
BCL10	—	—	—	—	—	—	—	—
FAS	—	—	—	—	—	—	—	—
CASP5	—	—	—	—	—	—	—	—
APAF1	—	—	—	—	—	—	—	—
hMSH6	—	—	—	—	—	1del	1del	—
hMSH3	1del	1del	—	—	—	1del	—	1del
MED1	2del	—	—	—	—	—	—	—
RAD50	—	—	—	—	—	—	—	—
BLM	1del	1del	—	—	—	—	1del	—

p53 mutations and MSI status, interestingly none of the eight MSI-H GCs showed p53 mutations whereas one of the five MSI-L GCs resulted mutated.

In a comparison between these two variables and the traditional clinico-pathological variables (site, histological grade, TNM stage, lymph node metastases), there was no significant association, except for those between p53 mutations and tumoral histotype; in fact, all the 17 cases with such mutations were intestinal type ($P < 0.05$) (Table 4).

Even when the specific p53 mutations in the conserved areas or in the functional domains were taken into consideration, no other associations were observed with the traditional clinico-pathological variables examined or with MSI.

Uni- and multivariate analysis of prognostic factors

At univariate analysis, high TNM stage, node status positive, depth of invasion (PS+), diffuse histotype, and p53 mutations affecting the L3 loop and LSH motif proved to be significantly related to quicker relapse (Table 5), whereas these same factors, made exception for the diffuse histotype, were significantly related to shorter overall survival (Table 6).

Figure 2 shows the probability of DFS (Fig. 2a) and OS (Fig. 2b) according to p53 mutations in specific structural domains. The significant variables at univariate analysis were entered in a Cox's proportional hazards model with backward elimination. The major significant predictors for both disease relapse and death were high TNM stage (Tables 5 and 6).

DISCUSSION

In the last few years, several new classification systems have been formulated for GC according to their histopathological and, more recently, to biomolecular features, in order to identify patient subgroups at higher risk for disease recurrence and death.

TABLE 4. Relationships of p53 status and tumor histotype of the 62 gastric cancer patients

	p53		
	No mutation (%)	Mutation (%)	*P*
Tumor histotype			
Intestinal	33 (73)	17 (100)	
Diffuse	12 (27)	0 (0)	<0.05
Total	45 (72)	17 (28)	

With regard to molecular basis of GC, at least two distinct pathways have been identified: one characterized by the successive accumulation of mutations in oncogenes and tumor suppressor genes, in which p53 plays a relevant role, the other, the MSI pathway, characterized by the accumulation of somatic alteration in the length of simple nucleotide repeats that identify a subset of GC with specific biomolecular features (Fiocca et al., 2001). Reported data (Renault et al., 1996; Halling et al., 1999; Iacopetta et al., 1999; Artunedo et al., 2000; Guo et al., 2000; de Manzoni et al., 2001; Palli et al., 2001; Sud et al., 2001; Czopek et al., 2002; Huiping et al., 2002; Inamori et al., 2002; Laghi et al., 2002; Lee et al., 2002; Takahashi et al., 2002; Yamada et al., 2002) vary considerably with regard to MSI, ranging from 9.5% to 37.8% in GCs.

These variations may be partly explained by the differences between susceptible populations and by different etiological factors, and seem to be clearly linked to the number of cases investigated and to the type and number of markers used in the study. According to precise criteria established by several authors (Boland et al., 1998; Perucho, 1998), the number of GCs with high MSI (MSI-H) might well be lower, agreeing with our own results where we found 14% MSI-H GCs, 9% MSI-L GCs, and 77% MSS GCs.

Mutations in p53 represent one of the critical events in the development of many human neoplasias (Lutz and Nowakowska-Swirta, 2002) and are often associated with the acquisition of greater tumoral invasiveness (Sakurai et al., 1995). GCs show a variable involvement of p53 mutations. Reports published in the last few years, showing a variable p53 mutation rate ranging from 8% to 55% of the cases (Maesawa et al., 1995; Lim et al., 1996; Mazaki et al., 1996; Renault et al., 1996; Ricevuto et al., 1996; Palli et al., 1997; Matturri et al., 1998; Guo et al., 2000; Park et al., 2001; Wang et al., 2001; Kubicka et al., 2002; Berloco et al., 2003; Fricke et al., 2003).

In our own study, 17 of 62 cases studied (27%) presented p53 mutations, mostly within the conserved areas (59%) or in domains, which were important for the functionality of the protein (41% in L3-LSH).

Although several authors report that p53 mutations tended to be located in the upper portion of stomach, associated with advanced age (Palli et al., 1997) or with

TABLE 5. Kaplan–Meier and Cox's proportional hazards analysis of clinico-pathologic and biological variables to predict the hazard ratio of relapse in gastric cancer patients

		Univariate				Multivariate			
Variables	Pts (N)	DFS (%) 2 years	DFS (%) 5 years	O/E	*P*	RR	CI (95%)	β	*P*
Histotype									
Intestinal	50	76	58	0.83					
Diffuse	12	33	11	2.452	<0.01				
TNM stage									
I	12	100	92	0.19		1			
II	25	80	55	0.90		4.93	1.12–21.6	1.59	<0.05
III–IV	25	38	19	2.25	<0.01	13.9	3.14–61.7	2.63	<0.01
Depth of invasion									
PS (−)	37	78	59	0.65					
PS (+)	25	50	34	1.82	<0.01				
Node status									
Node negative	18	89	83	0.55					
Node positive	44	59	34	1.33	<0.05				
P53									
wt	45	69	55	0.94					
mut outside L3/LSH	10	70	70	0.55					
mut in L3-LSH	7	57	0	2.35	<0.05				
Total pts	62	68	51						

Pts, patients; O/E, observed/expected; RR, relative risk; CI, confidence interval; PS(−), invasion into the muscularis propria or into the subserosa without infiltrative growth; PS(+), invasion into the subserosa with infiltrative growth.

advanced stage (Guo et al., 2000); in our own cases, we did not find any significant association between p53 mutations and the traditional clinicopathological variables (age, sex, site and size of the tumor, stage, grading, invasiveness). Moreover, we found no association between these variables and MSI-H, in spite of the fact that several authors have reported higher MSI in intestinal-type tumors (Simpson et al., 2001), in distal (antral) tumors (Ottini et al., 1997), in tumors without lymph node metastases (Huiping et al., 2002; Lee et al., 2002), or in elderly or female patients (Iacopetta et al., 1999; Yamada et al., 2002).

It may often happen that at advanced stages, both histological types (IT and DT) present the same type of genetic alterations (Tamura, 2002), for instance, mutations in APC, KRAS2, p53, or MSI, which are brought about by early events in differentiated GCs. On the other hand, where GCs have developed first as an undifferentiated tumors, they rarely seem to be prone to these types of alterations.

In our own cases, we also found a more frequent high MSI in IT tumors (six of eight MSI-H cases), together with a significant association between p53 mutations and intestinal histotype. In fact, none of the 12 diffuse-type GCs showed p53 alterations (Table 4). These data support the idea that p53 mutations and other specific genetic alterations might be behind the development of intestinal- but not of diffuse-type GCs.

Moreover, as already reported by several other authors (Endoh et al., 2000; Guo et al., 2000), none of the MSI-H cases presented p53 mutations, which suggests that alterations in the DNA repair mechanisms, which give rise to MSI, and p53 mutations may be mutually exclusive indicating the existence of distinct

TABLE 6. Kaplan–Meier and Cox's proportional hazards analysis of clinicopathologic and biological variables to predict death in gastric cancer patients

		Univariate				Multivariate			
Variables	Pts (N)	OS (%), 3 years	OS (%), 5 years	O/E	*P*	RR	CI (95%)	β	*P*
TNM stage									
I	12	100	91	0.20		1			
II	25	75	57	0.85		4.50	1.02–19.9	1.50	<0.05
III–IV	24	36	15	2.42	<0.01	14.3	3.25–63.5	2.66	<0.01
Depth of invasion									
PS (−)	37	78	60	0.61					
PS (+)	24	46	30	1.99	<0.01				
Node status									
Node negative	18	89	83	0.55					
Node positive	43	56	33	1.33	<0.05				
P53									
wt	44	66	56	0.93					
mut outside L3/LSH	10	76	61	0.59					
mut in L3-LSH	7	57	0	2.33	<0.05				
Total pts	61	66	49						

See Table 4 for abbreviations.

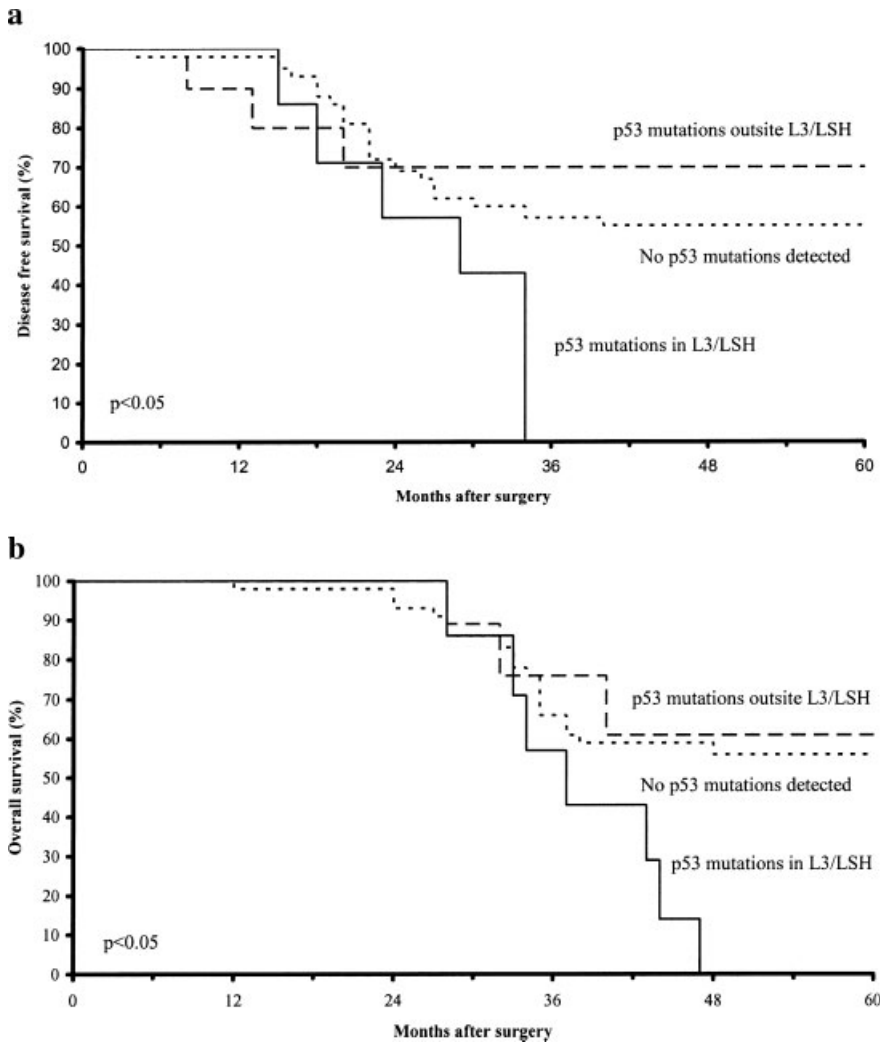

Fig. 2. DFS (**a**) and OS (**b**) of 62 patients with GC according to p53 functional and structural domains.

cancerogenetic pathways for GC (Fiocca et al., 2001). In this respect, it is noteworthy that the majority of MSI-H GCs showed frameshift mutations in genes controlling cellular growth, apoptosis, and DNA repair thus conferring a selective advantage independently of p53 mutations.

In several previous studies (Lim et al., 1996; Yamamoto et al., 1999; Kubicka et al., 2002), the presence of MSI-H has been associated with a more favorable outcome in GC patients; we ourselves, however, in concordance with several other authors (Renault et al., 1996; Halling et al., 1999; Iacopetta et al., 1999; Artunedo et al., 2000; Guo et al., 2000; Palli et al., 2001; Sud et al., 2001; Huiping et al., 2002; Inamori et al., 2002; Lee et al., 2002; Takahashi et al., 2002; Yamada et al., 2002) did not find such an association.

Contradictory findings have been reported (El-Rifai and Powell, 2002) with regard to the presence of p53 alterations and the prognosis of GC patients. A great many studies, however, have used immunohistochemical analysis to detect overexpression of p53 as an indirect means of evaluating mutations of this gene, and this assay does not appear to have consistent prognostic significance in GC patients (Fenoglio-Preiser et al., 2003). The few studies so far conducted to evaluate the prognostic impact of p53 mutations in GCs do not appear to provide any further information regarding this aspect (Lim et al., 1996; Matturri et al., 1998; Kubicka et al., 2002).

In the present study, no significant difference was observed in survival rate between GC patients with such mutations and those without. Nevertheless, since the L3 loop and the LSH motif of p53 contain residues involved in direct DNA contact and in protein stabilization, any alteration within these domains will affect this critical DNA contact and may have a negative influence on cancer therapy involving p53-dependent programmed cell-death (leading to treatment-resistant tumors). This fact suggests that specific mutations may be biologically more significant in terms of disease recurrence and the overall survival rate of patients. Previous studies involving other types of human neoplasias have shown

that specific mutations in the conserved areas of the gene or in the functional domains, as defined by crystallography (Cho et al., 1994), may have more prognostic significance than overall p53 mutations (Borresen et al., 1995; Borresen-Dale et al., 1998; Skaug et al., 2000; Russo et al., 2002).

In this study, univariate analysis shows that patients affected by tumors with p53 mutations in the functional domains L3 and LSH have a considerably shorter DFS and OS period (Tables 5 and 6). Nevertheless, these mutations would seem to be less significant predictive variables than tumor stage ($P < 0.05$ vs. $P < 0.01$), since Cox's proportional hazards analysis demonstrates that only the latter can be considered as significant prognostic factors (OS and DFS) (Tables 5 and 6).

CONCLUSION

Although the role of molecular alterations in GC still remains to be fully defined, it now seems more or less clear that tumors which are apparently identical from the morphological point of view should not be treated as a single type of neoplasia and that biomolecular investigation can help in the choice of a more specific and accurate therapeutic approach. Our study apparently confirms the existence of two different tumorigenetic pathways between intestinal and the diffuse type GCs. Moreover, there would seem to be two different, mutually exclusive, tumorigenesis profiles within different, intestinal-type GCs, one involving p53 mutations and the other alterations of the DNA repair system which accompany MSI.

The role of p53 mutations and MSI-H in the outcome of GC patients, however, is still not fully understood, although our own study seems to suggest that specific mutations in functional domains of p53, rather than any mutations in this gene, may be biologically more significant in terms of disease recurrence and the overall survival rate of patients. If future studies involving a larger number of cases confirm this finding, together with that regarding traditional variables with confirmed prognostic impact (TNM stage), specific p53 mutations may help to identify subgroups of patients at higher risk of relapse or death who might benefit from a more aggressive therapeutic approach.

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