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Technical Advance

Molecular Diagnosis of p53 Mutations in Gastric Carcinoma by Touch Preparation

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Thirty-one tumor samples from selected cases of gastric carcinoma were analyzed for mutations of the p53 tumor suppressor gene. Template DNA was prepared according to the touch preparation procedure, which allowed us to isolate clusters of neoplastic cells out of a stromal cellular background to be used as a template in the amplification of target exons of the p53 locus. In our present study, by polymerase chain reaction/single strand conformation polymorphism analysis we give evidence of p53 mutations occurring in the DNA-binding core domain of the protein (exons 5 through 9), which are clustered in stages III and IV of the disease (six mutations out of seventeen samples; 35%). No p53 mutations were detected in fourteen gastric cancer samples at I and II stages. Beside the use of conventional molecular scanning procedures, our study proposes the application of the touch preparation method to increase the detection of genetic alterations in human solid tumors. (Am J Pathol 1996, 148:405–413)

The identification of a growing number of genes, mainly tumor suppressor genes, that contribute to the development and progression of human solid tumors[1] links the application of molecular diagnosis to clinical practice. Genomic instability induced by alterations of genes that determine replication errors could contribute to the inactivation of tumor suppressor genes and increase the mutation rate.[2–4]

Structural alterations of the p53 product have been frequently detected in a wide variety of human tumors.[5,6] The loss of p53 function depends on recessive or dominant negative mutations, which can affect the DNA-binding domain of the protein (exons 5 to 9), as well as on the interaction with other cellular or viral oncoproteins.[7,8] The wild-type p53 protein exerts pleiotropic effects through the transcriptional activation of different target genes that control important checkpoints in the modulation of cell cycle progression.[9–14] It can also induce a transient suppression of the cellular growth at the G1/S checkpoint[15] and an irreversible induction of the pathways leading to p53-dependent programmed cell death[16,17] and DNA repair. Furthermore, mutated p53-bearing cells have altered controls during the progression through the cell cycle, prevent apoptosis, and may play a role in the mechanisms of resistance to chemotherapeutic genotoxic agents.[18–21]

Gastric carcinoma is the third leading cause of cancer mortality in Italy with 13,700 casualties in 1991.[22] Most of the patients are at high risk of recurrence and show limited survival (10 to 30% at 5 years) at the time

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of diagnosis. Diagnostic procedures do not always give an accurate preoperative evaluation of the extent of the disease.[23] Moreover, the pathological stage does not always identify patients at different risks of recurrence. Mutations of particular genes (p53, APC, c-K-*ras*)[24,25] and microsatellite instability[26–28] have been detected, but their role in the development of gastric cancer remains unclear.[29,30] Furthermore, a more accurate diagnostic approach for evaluating such genetic alterations needs to be developed. As solid tumor tissues display a mixture of malignant and nonmalignant stromal and inflammatory cells that limit the accuracy of the molecular analysis by diluting the neoplastic cell population, we performed the polymerase chain reaction (PCR)/single strand conformation polymorphism (SSCP) analysis of the p53 gene locus using genomic DNA by touch preparation of primary gastric carcinoma samples.[31]

By PCR/SSCP and direct sequencing, of 31 samples tested we observed 6 cases of p53 variants (19% frequency) clustered in patients with stages III and IV of the disease. Molecular diagnosis of p53 mutations was performed by SSCP assay on a tumor specimen derived from a touch-prepared DNA template. Furthermore, comparing the results from templates prepared using the touch preparation or applying standard DNA procedures on macroscopic neoplastic sections, we conclude that the touch preparation procedure has a better grade of resolution in identifying hetero- and/or homozygous alterations of the p53 tumor suppressor gene and we propose the extensive application of this technique in routine molecular diagnosis of genetic alterations in solid tumors.

Materials and Methods

Tissue Sample Recruitment

Fragments of human primary gastric carcinoma and nearby normal mucosa were obtained from surgical resections of 31 untreated patients performed at San Salvatore Hospital, L'Aquila, Italy, and stored at −70°C. Neoplastic samples were submitted to pathological staging according to the American Joint Committee on Cancer[32] and Lauren's histological classification.[33] Detailed clinical and histopathological data on each patient and tumor evaluated are available from the authors upon request.

Touch Preparation of the Neoplastic Tissue and DNA Extraction

Touch preparation of the grossly malignant tissue has been performed according to the procedure of Kovach et al[31] with slight modifications. Briefly, touch preparation consists of a light pressure of the cut surface of a previously dissected neoplastic tissue onto sterile microscope slides, followed by dipping in toluidine blue solution (1% aqueous solution) for 30 minutes and a final washing in double-distilled water (ddH_2O). Slides were viewed under a light microscope with ×25 magnification and freshly bluish stained malignant cells were identified as small clusters, each containing approximately 30 to 50 cells, which were easily removed using the tip of a 2-μl hand-held sterile pipette (Figure 1). Usually, three clusters per sample were picked and transferred into 5 μl of 5% dextrose and centrifuged for 5 minutes at 2000 rpm in a microfuge. DNA was extracted by incubating neoplastic cells with K-buffer (1× PCR buffer, 500 μg/ml proteinase K) for 1 hour at 55°C. A two-step procedure was applied to amplify genomic DNA using pairs of nested primers (Table 1): a first round of amplification with primers complementary to sequences flanking exons IV and IX amplifying a DNA fragment of 1790 bp and the second one with primers specific for each exon (V, VI, VII, VIII, and IX) amplifying regions of less than 250 bp. The first amplification was performed by adding 200 μmol/L dNTPs, 10 pmol of each primer, 1.5 mmol/L $MgCl_2$, and 0.5 U of *Taq* polymerase (Promega, Madison, WI) in a final volume of 25 μl. Thirty-five cycles of denaturation (94°C), annealing (60°C), and extension (72°C) were performed on an automated heat-block thermal cycler (Perkin-Elmer, Emeryville, CA). The DNA extraction from grossly normal and neoplastic mucosa was conducted by standard methods.[34]

Single Strand Conformation Polymorphism

SSCP analysis was accomplished according to an adapted version of the method reported by Gaidano et al.[35] The second amplifications were specific for exons V to IX from the p53 gene using upstream and downstream primers (Table 1) as follows: 1 μl of DNA template from the first amplification was mixed in a solution containing 10 pmol of primers, 2.5 μmol/L dNTPs, a range of 0.6 to 1.5 mmol/L $MgCl_2$ depending on which exon, 1 μCi of [^{32}P]dATP (specific activity 3000 Ci/mmol), 1X PCR buffer (Promega), and 0.5 U of *Taq* polymerase in a final volume of 10 μl. PCR reactions consist of 30 cycles. Then, 2 μl of reaction mix were diluted 1:25 into 0.1% sodium dodecyl sulfate/10 mmol/L EDTA and further mixed 1:1 with 2 μl of sequencing stop solution. Samples were heated at 95°C for 8 minutes, chilled in ice, and immediately loaded (4 μl) onto an 8% polyacryl-

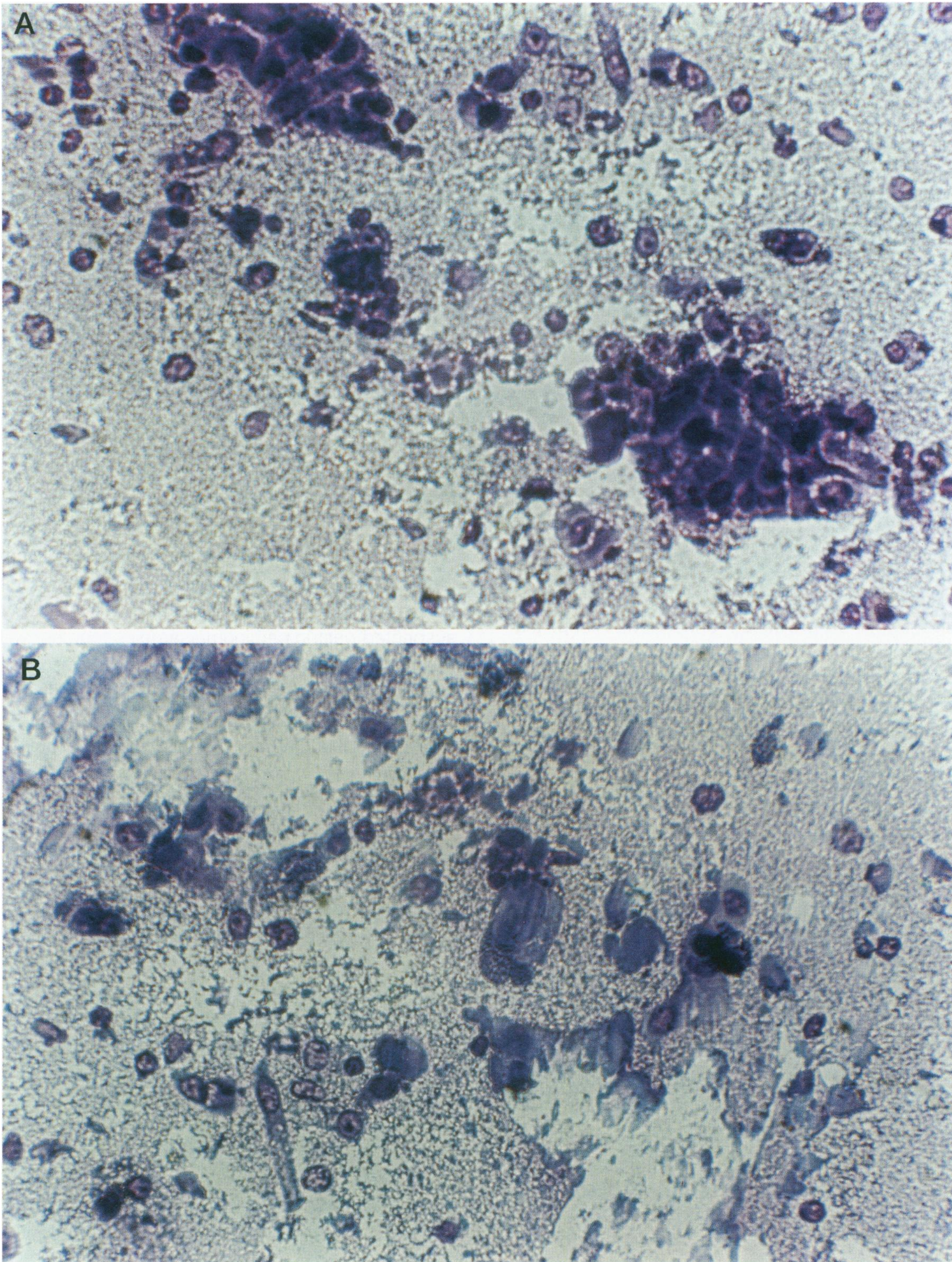

Figure 1. *Touch preparation of gastric cancer cells. The photographs show a light microscope view (magnification, ×25) of gastric cancer tissue by touch preparation.* **A**: *Two discrete clusters of gastric cancer cells.* **B**: *View of the same field after removing the two neoplastic clusters.*

Table 1. *Primer Sequences for p53 Analyses*

Exon	Upstream	Downstream
5–9*	5′-GGAGGTGCTTACACAT-3′	5′-GTTAGCTACAACCAGG-3′
5†	5′-TTCCTCTTCCTACAGTACTC-3′	5′-ACCCTGGGCAACCAGCCCTGT-3′
6†	5′-ACAGGGCTGGTTGCCCAGGGT-3′	5′-AGTTGCAAACCAGACCTCAG-3′
7†	5′-GTGTTATCTCCTAGGTTGGC-3′	5′-GTCAGCGGCAAGCAGAGGCT-3′
8†	5′-TATCCTGAGTAGTGGGTAATC-3′	5′-AAGTGAATCTGAGGCATAAC-3′
9†	5′-GCAGTTATGCCTCAGATTCAC-3′	5′-AAGACTTAGTACCTGAAGGGT-3′

*Primers previously described by Kovach et al.[31] for the first round of amplification from touch preparations.
†Primers previously described by Gaidano et al.[35]

amide gel (19:1, acrylamide:bis) containing 10% glycerol for 12 to 15 hours at room temperature applying 8-watt constant power. SSCP analysis was performed with and without glycerol addition. Autoradiography was performed with an intensifying screen for 24 to 72 hours.

Direct Sequencing

Direct sequencing was performed using amplified DNA from a genomic template extracted by the touch preparation procedure. The second PCR, specific for exons V to IX, was conducted without radioactive tracer as previously described, except that 200 μmol/L dNTPs were used in a final volume of 25 μl for 30 cycles. Amplified fragments were gel purified by the low-melting-point agarose technique. Direct sequencing was performed according to a modification of the method by Neri et al.[36] Briefly, 2 pmol of ^{32}P-end-labeled primers were annealed with 2.5 μl of purified DNA (approximately 100 ng) for 2 minutes at 63°C and dideoxy-NTP reactions were performed according to standard procedures (Sequenase, USB, Cleveland, OH). Electrophoresis was performed under denaturing conditions onto a 6% polyacrylamide gel (29:1) at room temperature applying 60-watt constant power. Autoradiography was performed with an intensifying screen for 24 to 72 hours.

Results

p53 mutations affecting exons V to IX have been scanned by PCR/SSCP assay and direct sequencing from touch preparation of 31 gastric carcinoma samples (Table 2). The molecular analysis by touch preparation has been conducted according to a three-step approach: 1) SSCP analysis of gastric carcinoma cells, 2) confirmatory SSCP analysis of suspected gastric carcinoma samples together with the corresponding nearby normal mucosa, 3) direct sequencing of the mutated cases. Direct sequencing of the suspected sample was performed by using either the upstream or the downstream primer of the involved exon. The results of the SSCP analysis and direct sequencing can be considered as a mean molecular evaluation of three clusters of neoplastic cells. The polymorphic pattern of SSCP and the mutations observed by the direct sequencing were always confirmed twice with additional experiments.

SSCP analysis detected six altered mobility shifts (19% frequency) with the following distribution: exon V, two mutations; exon VI, two mutations; exon VII, one mutation; exon VIII, one mutation. Polymorphisms displayed four heterozygous and two homozygous patterns. Subsequently, SSCP analysis of suspected gastric carcinoma samples together with nearby normal mucosa confirmed the preliminary results and detected a polymorphism also affecting one normal gastric sample (S34).

Direct sequencing from touch-prepared templates displayed a definite detection of the heterozygous polymorphism in four mutated cases. We found the following p53 variants: in exon V, one base deletion at codon 174 from S3 and nine base insertions from S15; in exon VI, two mutations shown by direct sequencing of S35, one base deletion at codon 219 and a nonsense substitution at codon 196 (CGA/TGA); in exon VIII, a mutation at codon 273 (CGT/TGT) revealed by S14, the most frequently described in human gastric carcinoma (Figure 2).

Next, we wanted to assess the specificity of the p53 mutation profiles comparing the SSCP analysis using templates from either touch-prepared or macro- extracted DNA. As shown in Table 3, the touch-prepared samples gave an unequivocal homozygous pattern of mutated p53 in two cases (Figure 3). Exon VII amplification from S19 displayed additional bands of single strand DNA conformation together with the absence of the wild-type pattern due to allelic deletion. Direct sequencing of S19-exon 7 showed a hot spot mutation at codon 248 (substitution CGG/CAG), which is one of the most frequently involved in human cancers and particularly in gastric cancers, even if the substitution reported in other

Table 2. *p53 Mutations in Gastric Cancer from Touch Preparations*

Sample	Histology*	Stage‡	T‡	N	M	p53 mutations				
						Exon	Codon	Nucleotide change	Nature of mutation	Allelic pattern†
S1	D	IIIA	3	1	0					
S2	I	II	2	1	0					
S3	I	IV	2	2	1	5	174	C-deletion	frameshift	Het
S6	D	IV	3	1	1					
S7	D	IIIB	3	2	0					
S8	I	IIIA	3	1	0					
S9	D	II	2	1	0					
S10	D	IV	4	2	0					
S11	I	IB	2	0	0					
S12	D	IIIA	2	2	0					
S14	D	IV	3	0	1	8	273	CGT/TGT	Arg-Cys	Het
S15	D	IIIA	2	2	0	5		9-bp insertion	frameshift	Het
S16	D	IV	4	2	0					
S17	D	IIIB	3	2	0					
S18	D	IV	4	2	0					
S19	I	IIIA	3	1	0	7	248	CGG/CAG	Arg-Gln	Hom
S20	I	II	2	1	0					
S22	D	IIIB	3	2	0					
S23	I	II	2	1	0					
S24	I	IB	2	0	0					
S25	I	IB	2	0	0					
S26	D	IB	2	0	0					
S27	D	IB	2	0	0					
S28	D	IB	2	0	0					
S29	I	II	2	1	0					
S31	D	IB	2	0	0					
S32	I	IA	1	0	0					
S33	D	II	2	1	0					
S34	D	IIIB	3	2	0	6	220	TAT/TCT	Tyr-Ser	Hom
S35	D	IIIB	4	1	0	6	196	CGA/TGA	Arg-Stop	Het
						6	219	C-deletion	frameshift	
S36	D	IIIB	4	1	0					

*I, intestinal; D, diffuse.
†Het, heterozygous polymorphism; Hom, homozygous polymorphism.
‡ Staging of cancers according to UICC (stages I to IV) and AJC (TNM system) classification.

series involves the first base of the codon (CGG/TGG).

Furthermore, SSCP analysis of S34-exon 6 (Figure 4) showed the presence of a heterozygous polymorphism affecting DNA extracted from normal and neoplastic cells. Consequently, direct sequencing of this case confirmed the presence of a heterozygous polymorphism in exon 6 at codon 213 (CGA/CGG), previously described in the Italian population.[37] The analysis of touch-prepared tumor DNA from the same patient showed the loss of such dimorphism at codon 213 and the appearance of a base substitution at codon 220 (TAT/TCT) on the residual allele.

Globally, we detected four base substitutions, three transitions (one at CpG site giving one C/T replacement), and one transversion. Three other mutations consisted of deletions and insertions.

The evaluation of the mutations according to the pathological stage (Table 4) revealed that all of the mutations were found at stages III and IV and none

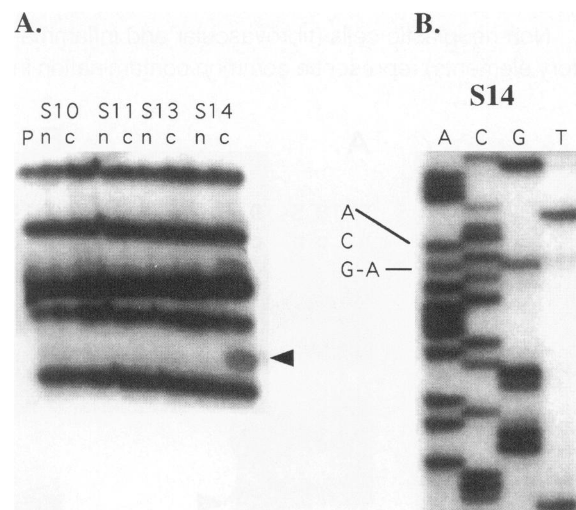

Figure 2. *Heterozygous pattern of p53 mutations from touch-prepared samples.* A: *S14 gastric cancer sample shows a heterozygous SSCP pattern of the exon VIII due to the appearance of extra bands (see* arrowhead*).* B: *Direct sequencing of the antisense strand points out the specific mutation (codon 273) and confirms the heterozygous profile. P, placental genomic DNA used as a control; S10 to S14, samples from gastric tissue; n and c, DNAs from normal and neoplastic gastric cells, respectively.*

Table 3. *Comparison of SSCP Pattern of p53 Mutations from Macroscopic Tissue and Touch-Prepared Template*

	Macroscopic tissue		Touch preparation	
	Heterozygous	Homozygous	Heterozygous	Homozygous
S3	+		+	
S14	+		+	
S15	+		+	
S19	+			+
S34	+			+
S35	+		+	

+, altered p53-SSCP pattern.

were found in the 14 patients at stages I and II of gastric carcinoma.

Discussion

The molecular detection of p53 mutations by PCR/SSCP analysis and direct sequencing from a few clusters of gastric cancer cells showed 6 mutations out of 31 samples analyzed so far (19% frequency). p53 mutations reported by molecular screening procedures (SSCP or DGGE and/or direct sequencing) in gastric carcinoma ranges between 0 and 52% (mean 31%) in published data.[26,27,38–43] In addition, high levels of p53 protein have been detected in 57% of tumor samples by immunostaining.[44] The wide range of p53 variants reported so far in gastric cancer is partially due to the different scanning procedures, as the invariable mixture of malignant and nonmalignant cells in solid tumor tissue is a common condition for misinterpretation of the molecular analysis.

Non-neoplastic cells (fibrovascular and inflammatory elements) represent a common contamination in tumor biopsies. Consequently, the separation of malignant cells from their stromal environment represents the preliminary step in the clinical application of molecular diagnosis of genetic alterations in solid tumors. The cellular morphology accounts for the first criterion in the diagnosis of the malignant phenotype. Furthermore, direct selection of cancer cells from stained preparations of primary tissues is the more reliable approach to obtain a homogeneous tumor cell population. Touch preparation has been used for many years to assist in the hisological diagnosis of carcinomas because it allows the separation of neoplastic cells due to their capacity to adhere to microscopic slides as small, discrete clusters of clonal origin. Cell clusters in touch preparation contain only tumor cells as determined by direct microscopic observation.[31,45] The technique is simple, rapid, and highly selective. The procedure of the detection of p53 mutations from touch preparations of gastric carcinoma was easily feasible, cost limited, and not time consuming.

Our results concerning the detection of p53 mutations in gastric cancer are in the range of those

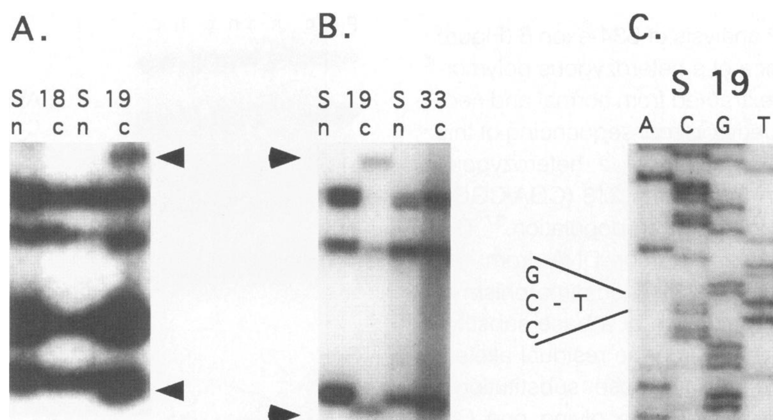

Figure 3. *Resolution of a homozygous pattern of exon VII-p53 variant by touch preparation.* A: *The SSCP assay using DNA from a macrodissection of the tumor tissue displays a heterozygous polymorphism of the S19c sample* (arrowheads) *due to contamination of the stromal or inflammatory cells.* B: *The same analysis performed on touch-prepared template from the same patient (S19) shows the disappearance of the wild-type single strand conformation and a homozygous SSCP profile* (arrowheads). C: *Direct sequencing of the antisense strand highlights the homozygous mutated spot on exon VII (codon 248). Note that a G residue from a very poorly non-neoplastic cell contamination is faintly detected in the direct sequencing probably due to a higher sensitivity of direct sequencing with respect to the SSCP analysis. S18, S19, S33, samples of gastrc tissue; n, normal gastric DNA; c, gastric cancer DNA.*

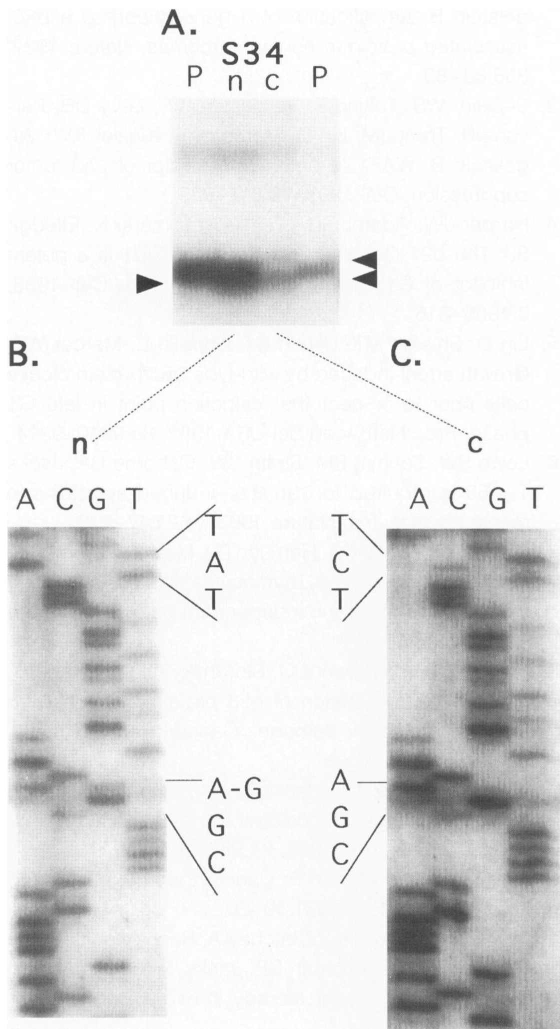

Figure 4. *Exon VI-p53 molecular analysis by touch preparation showing a heterozygous polymorphism on normal cells (n) and a hemizygous somatic mutation on neoplastic cells (c) gaining a deletion of the polymorphic allele.* **A:** *SSCP analysis shows the appearance of a heterozygous polymorphism in normal gastric cells (S34n shows a doublet on the intensity of the band; one* **arrowhead** *on the left) and a different hemizygous SSCP pattern in gastric cancer cells (sample S34c; two* **arrowheads** *on the right). P, placental genomic DNAs used as controls.* **B:** *Direct sequencing of the upstream strand of normal (n) gastric sample S34 confirming the heterozygous polymorphism in exon VI, codon 213 (CGA/CGG).* **C:** *Direct sequencing of the upstream strand of gastric cancer (c) sample S34 showing the disappearance of the polymorphism at codon 213 and the gain of a somatic mutation (codon 220) on the residual allele.*

reported in the literature by PCR/SSCP scanning. This diagnostic approach improves the diagnostic ability of the SSCP to predict the allelic pattern of the p53 gene (mutations and allelic deletions) for clinical purposes (Figures 3 and 4). Touch preparation would improve the accuracy of direct sequencing for the detection of specific gene alterations.

The distribution of mutations in our samples revealed two altered genotypes at codons 273-exon VIII and 248-exon VII, the most frequently mutated sites in human tumors (respectively, 9.6 and 8.8%) and also in gastric cancer.[5,6,46] These hot spot mutations inactivate p53 function by directly interfering with DNA binding.[47] The finding that the majority of the p53 mutations occur in the portion of the DNA-binding domain of the protein confirms the hypothesis that DNA interaction and transactivation are critical points for p53 in mediating tumor suppression. Many of the remaining mutations inactivate p53 most likely by unfolding the conformation structure of the protein and may involve the complete denaturation of the core domain. The four other mutations detected in our study (codons 174, 196, 219, and 220 and a 9-bp insertion at exon V) were not hot spot residues but involve the superficial part of the β-sandwich. The Tyr[220] residue is part of a trio of residues that have mutation rates significantly higher than the rest of the β-sandwich (4.5 *versus* 1%). It appears that they are important for the structure of the short loops at the end of the β-sandwich opposite to the DNA-binding surface.

Table 4. *p53 Mutations According to Pathological Stage*

Stage	Samples	p53 mutations
I	8	0
II	6	0
IIIA	5	2
IIIB	6	2
IV	6	2

The evaluation of the mutations according to the pathological stage (Table 4) revealed that all of the mutations have been observed at stages III and IV of the disease as reported in the literature (6/17, 35%); none has been reported out of the 14 patients at stages I and II of the disease. This result enhances the diagnostic role of the molecular detection of p53 mutations in the advanced phases of gastric cancer, particularly in stages III and IV of the disease.

The detection of chromosome 17p allelic deletion or accumulation of p53 protein showed prognostic implications in different solid tumors (colon, breast, and lung carcinomas) and also in gastric cancer.[44,48] A more accurate detection of the allelic distribution of p53 mutations obtained through the touch preparation technique could have prognostic significance of therapeutic relevance, particularly in the pathological stage with a higher risk of recurrence (stage III). Furthermore, the mutational status of the p53 protein could be a predictor of chemoresistance, as in gastric cancer cell lines the presence of wild-type p53 predicts sensitivity to chemotherapeutic agents.[49]

The molecular diagnosis at the earlier detectable stages is limited by methods concerning scanning of the genetic alterations.[50] New techniques of analyzing the pure neoplastic cell population could enhance the evaluation of the genetic pattern of the different malignant clones constituting the solid tumor tissue, thus improving the early diagnosis and permitting the recognition of minimal residual disease.

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