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Frequency and spectrum of p53 mutations in gastric cancer – a molecular genetic and immunohistochemical study

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Abstract The *p53* tumour-suppressor gene plays an important role in gastric carcinogenesis. In an analysis of the spectrum of mutations of the *p53* gene seen in 56 primary gastric carcinomas of various types and grades of differentiation, the entire coding sequence (exons 2–11) of the *p53* gene was screened by single-strand conformation polymorphism analysis and direct genomic sequencing of polymerase chain reaction products. Intragenic restriction site polymorphisms and the probe YNZ22 were used for the detection of loss of heterozygosity (LOH) of the *p53* gene locus on chromosome 17p. p53 overexpression was studied with the anti-p53 antibody CM-1. A total of 21 somatic alterations of the *p53* gene were found. Twenty were base-pair substitutions, and one was an eight base-pair deletion. Six tumours with *p53* mutations revealed LOH. Abnormalities in p53 expression were found in 17 tumour samples, of which 16 had gene mutations. The spectrum of mutations observed was consistent with the predicted spectrum for dietary mutagens associated with the metabolism of nitrogenous compounds, resulting in deamination of nucleic acids. Our findings suggest that *p53* could be a primary target for mutations associated with dietary carcinogens in gastric carcinogenesis.

Key words Gastric cancer · *p53* tumour-suppressor gene · Mutation spectrum · Dietary mutagens · Immunohistochemistry

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Introduction

Gastric cancer is one of the most prevalent malignancies in the world, but little is known about the genetic alterations associated with its development and progression. Few molecular genetic studies have been performed to clarify the mechanisms of the carcinogenesis of these malignant lesions. The *ras* family of oncogenes are the most frequently encountered transforming genes detected by NIH3T3 transfection assay in human cancers [5, 59]. However, various studies have shown point mutations of the *ras* genes in less than 5% of stomach cancers [6, 17]. Further studies have suggested that alterations of multiple genes may be involved in gastric tumorigenesis. Amplification of the c-*myc* gene, c-*erb*B-2 gene and K-*sam/bek* gene have been found in various types of gastric cancer, although with a relatively low frequency [14, 37, 63]. Somatic mutations of the APC gene have been demonstrated in well differentiated adenocarcinomas and signet-ring cell carcinomas, but not in poorly differentiated gastric cancers [38].

The *p53* tumour suppressor gene on chromosome 17p encodes a nuclear 393-amino acid phosphoprotein thought to regulate cell cycle progression [26, 27]. Point mutations, deletions or rearrangements of the *p53* gene may result in a protein with altered biological properties, including increased stability and accumulation of the mutant protein that is detectable by immunohistochemical methods [12, 23, 56]. Mutant p53 protein is characterized by a loss of antiproliferative activity and may have a dominant negative effect on the remaining wild-type p53 by complex formation [33]. Various studies have indicated that *p53* gene mutations occur most often in the evolutionarily conserved domains affecting exons 5 to 9 [27, 51]. More recently, studies have provided evidence that genetic alterations of the *p53* tumour suppressor gene may play an important role in the development of human gastric cancers [44, 47, 48, 49, 64]. However, the role of the *p53* gene in gastric cancer is still controversial; the reported frequency of genomic *p53* mutations varies from 0% to 64% [21, 31, 54, 60], and immunohis-

tochemical studies have revealed p53 protein accumulation in up to 61% of cases [48]. In addition, p53 protein accumulation in tumour cells in gastric and oesophageal cancer has been demonstrated to correlate with a poor prognosis of the disease [18, 29, 50, 52]. Here, we describe a comprehensive analysis of *p53* alterations in 56 malignant gastric tumours evaluating genomic DNA alterations by single strand conformation polymorphism (SSCP) analysis from exon 2 to exon 11 (the entire coding sequence of the *p53* gene) followed by DNA sequencing of variants. Detection of loss of heterozygosity (LOH) of 17p was carried out by polymerase chain reaction (PCR) analysis of intragenic *p53* polymorphisms [10]. Immunohistochemical analysis was performed to investigate p53 protein accumulation.

Materials and methods

The study included 56 unselected cases of patients with primary adenocarcinoma of the stomach. Tissues were collected at surgery, snap frozen and stored at −80° C until use. Histopathological classification was performed according to Lauren [25] and Ming [34], and criteria established by the World Health Organisation [41]. Frozen sections were stained with haematoxylin and eosin to select tissue for molecular genetic studies with the smallest possible admixture of nonmalignant cells, and the normal cell fraction was estimated for each sample. Frozen normal (tumour-free) material from 19 patients and formalin fixed and paraffin embedded normal tissues from 20 further patients were available for study.

High molecular weight DNA was prepared from frozen material by proteinase K digestion and phenol/chloroform extraction as described elsewhere [28]. DNA was precipitated in ethanol and resuspended in sterile water for storage at 4° C. Formalin fixed and paraffin embedded specimens were cut in 5 µm sections. Excess of paraffin was removed before proteinase K digestion was carried out overnight at 55° C. After digestion residual paraffin could be removed from the top of the digestion mixture with a pipette tip. DNA was subsequently extracted by standard phenol/chloroform methods as described above.

Primers used for amplification and sequencing were synthesized by the phosphoramidite method using biotin-phosphoramidite for 5′ biotinylated oligonucleotides by an automated oligonucleotide synthesizer (Genassembler, Pharmacia Biotech). All primers were used without further purification.

For analysis of genomic DNA by SSCP, p53 exons 2–11 were amplified in 14 different PCRs as previously described [57]. Briefly, each PCR in a 50 µl mixture contained: 50 ng of genomic DNA, 20 pmol of each primer, 1.0–2.5 mM magnesium chloride, 20 µM each of dATP, dTTP, dGTP, 2 µM dCTP, 20 mM TRIS (pH 8.4 or 8.6), 50 mM potassium chloride, 50 µg/ml bovine serum albumin, 0.5 units *Taq* polymerase (Perkin-Elmer Cetus) and 0.1 µl (7 nmol/l) [alpha-^{32}P]dCTP (3000 Ci/mmol). DNA was amplified with 30–35 cycles of PCR (75 s at 94° C, 90 s at 52–60° C and 120 s at 71° C) using a programmable thermal controller (MJ Research, Cambridge, Mass.). Amplified fragments from exons 2, 4 and 10 were digested with an appropriate restriction endonuclease to increase the sensitivity of the SSCP analysis. PCR products were diluted 1:6 with a loading solution, denatured at 96° C for 5 min, and loaded twice on 9% nondenaturing polyacrylamide gels, one of which contained 10% glycerol. Electrophoresis was performed at ambient temperature for 8–16 h. Autoradiography was carried out without an intensifying screen for 12–96 h [42].

Direct genomic sequencing of double stranded PCR fragments was performed using protocols previously described [61]. In short, amplified DNA samples were purified through Sepharose CL-6B (Pharmacia-LKB) and combined with 7 pmol of ^{32}P-end-labelled primer. Primer-template mixtures were heat-denatured and sequencing reactions were carried out using the sequenase enzyme (US Biochemical). Electrophoresis was performed on 6% denaturing polyacrylamide gels. Autoradiography was carried out for 12–24 h without an intensifying screen.

To detect LOH, amplification of the variable number of tandem repeats (VNTR) region YNZ.22, the *Msp*1 enzyme restriction site in *p53* intron 6 and the *Acc*II polymorphism in *p53* exon 4, was carried out as previously described by Dockhorn-Dworniczak et al. [10]: 20 µl final volume with the same buffer conditions as described above contained 10 pmol of each of the primers, 0.5 U

Table 1 Histological tumour type and molecular genetics and immunohistochemical results [*A* adenocarcinoma, *pd* poorly differentiated, *md* moderately differentiated, *wd* well differentiated, *in* intestinal type, *di* diffuse type, *mi* mixed type, *Ba* Barrett's esophagus, *an* anaplastic carcinoma, *as* adeno-squamous carcinoma, + focal nuclear positivity (<20% positive cells), ++ moderate nuclear positivity (20–50% positive cells), +++ high nuclear positivity (51–100% positive cells)]

Case number	Tumor-type	Exon	Codon	Conserved domain?	Nucleotide charge	Amino acid change	Immunostaining
0655	A; pd; in	5	133	Yes	ATG to AAG	Met to Lys	+++
0624	A; md; in	5	136	Yes	CAA to TAA	Gln to "stop"	Negative
0638	A; md; in	5	147	No	GTT to GGT	Val to Gly	++
0662	A; pd; di	5	160	No	ATG to AAG	Met to Lys	++
0653	A; md; in	5	165	No	CAG to CGG	Gln to Arg	+
0630	A; pd; di	5	168	No	CAC to AAC	His to Asn	++
0643	A; pd; mi	5	173	Yes	GTG to TTG	Val to Leu	+++
0652	A; pd; mi	5	176	Yes	TGC to TTC	Cys to Phe	+
0623	A; wd; Ba	6	194	No	CTT to TTT	Leu to Phe	+++
0635	A; pd; mi	6	196	No	CGA to TGA	Arg to "stop"	Negative
0657	A; pd; an	6	196	No	CGA to TGA	Arg to "stop"	Negative
1040	A; md; in	7	246	Yes	ATG to ATA	Met to Ile	Negative
0637	A; md; in	7	248	Yes	CGG to TGG	Arg to Trp	+++
0627	A; pd; di	7	248	Yes	CGG to TGG	Arg to Trp	+
1024	A; pd; as	7	248	Yes	CGG to TGG	Arg to Trp	+++
1026	A; pd; di	7	248	Yes	CGG to TGG	Arg to Trp	+
1032	A; pd; di	7	248	Yes	CGG to TGG	Arg to Trp	+++
0660	A; md; in	8	269–271	No	Eight base pair deletion	"Stop" at codon 305	++
0641	A; pd; di	8	280	Yes	AGA to GGA	Arg to Gly	+++
1041	A; md; in	8	282	Yes	CGG to CCG	Arg to Pro	+
1038	A; md; mi	8	285	Yes	GAG to AAG	Glu to Lys	Negative

ampli-*Taq* polymerase (Perkin-Elmer Cetus) and 40 ng of template. Initial denaturation was performed at 94° C for 4 min followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 63° C for 1 min and extension at 72° C for 5 min. Two microlitres of the reaction mixtures were analysed on native 15% polyacrylamide gels without further purification. Polyacrylamide gel electrophoresis was carried out on a vertical electrophoresis system (Multiphor, Pharmacia, Sweden) with ultrathin gels baked on GelBond Pag (FMC, Rockland, Me.). Electrophoresis was performed in a discontinous buffer system with 35 mM sulphate-borate (pH 9.0) as the leading-trailing ion and 141 mM TRIS-borate (pH 9.0) as the trailing ion at 15° C and 10 mA for 1 h. Bands were visualised by silver staining according to standard protocols.

Sections from frozen material and paraffin-embedded tissues were cut at a thickness of 4 µm, mounted on silane coated glass slides and air dried overnight at room temperature. After dewaxing in xylene and rehydration in a series of ethanols, wet autoclave pretreatment was carried out as described by Bankfalvi et al [2]. The primary antibody CM-1 (Medac) was applied overnight at 4° C in a humidified chamber (dilution; 1:20.000 in 0.6% bovine serum albumin), followed by a goat-anti-rabbit bridging antibody (1:30; 30 min at room temperature; Dako) and a polyclonal rabbit-alkaline phosphatase-anti-alkaline phosphatase-complex (1:100; 60 min at room temperature; Dianova). Subsequently the enzyme reaction was developed for 25 min at room temperature in a freshly prepared newfuchsin solution containing naphtol-As-biphosphate. Finally the sections were counterstained with haematoxylin and mounted with Kayser's glycerine gelatine [2].

Results

Screening for point mutations of the p53 gene

We found a total of 21 (37.5%) mutations in 56 samples we have studied (Table 1). Twenty mutations were base-

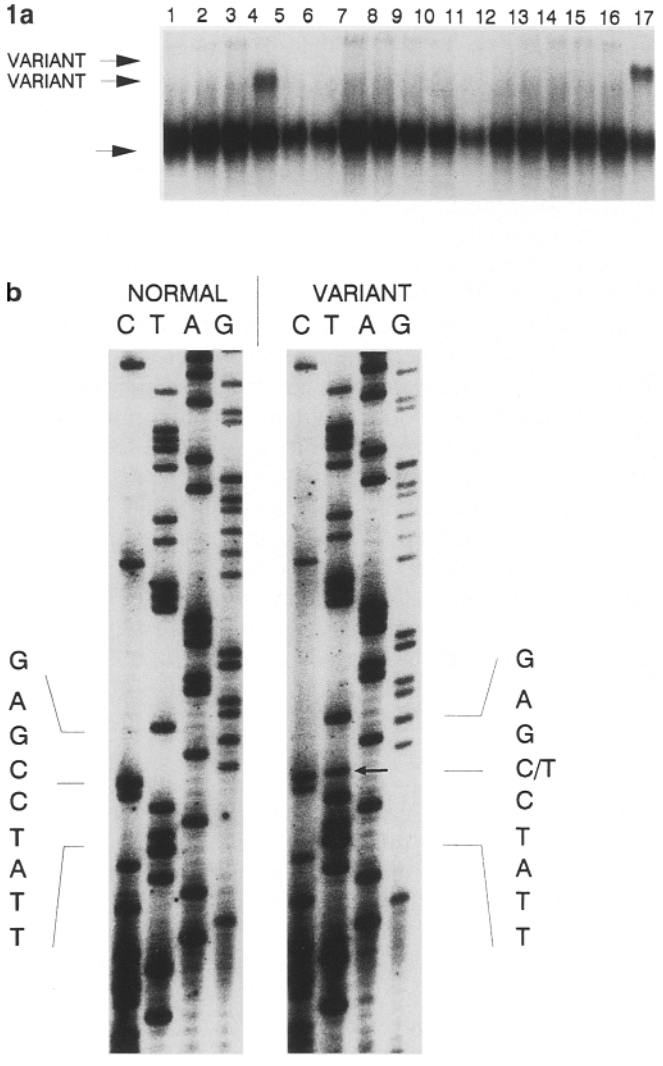

Fig. 1 **a** Single-strand conformation polymorphism (SSCP) analysis of exon 6 of the *p53* gene. In lane 4 (sample 657) and lane 17 (sample 1029), the higher bands are variant alleles of the *p53* gene. **b** Direct genomic sequence analysis of the *p53* gene in sample 657, derived from an anaplastic adenocarcinoma. A heterozygous transition from cytosine (*C*) to thymine (*T*) at codon 196 caused a premature "stop" where arginine normally occurs in the p53 protein

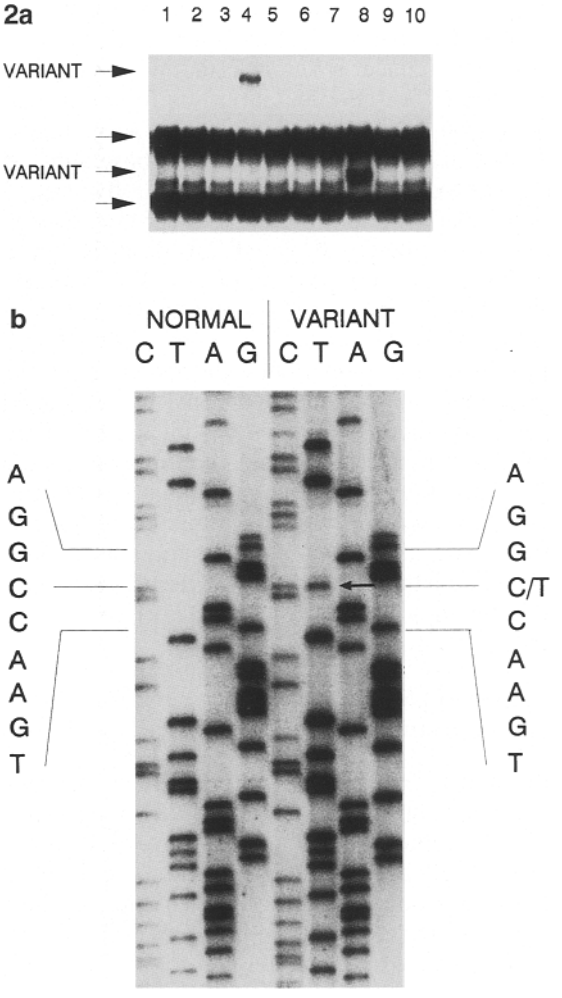

Fig. 2 **a** SSCP analysis of exon 7 of the *p53* gene. Two of these ten samples, in lane 4 (sample 1024) and lane 8 (sample 1036), show variant bands in addition to the normal alleles of the *p53* gene. **b** DNA sequence analysis of sample 1024 from Figure 2a (*variant*) and non-tumorous gastric tissue (*normal*) from the same patient, suffering from a poorly differentiated adeno-squamous carcinoma of the cardia. A heterozygous transition from cytosine (*C*) to thymine (*T*) at codon 248 caused a tryptophan codon to be substituted for the normal arginine codon. Since this variant is not present in the non-tumorous gastric tissue from this patient, the mutation was a somatic event

pair substitutions and 1 was an eight base-pair deletion. All of these genetic alterations occurred within exons 5–8 of the *p53* gene. Figures 1 and 2 demonstrate examples of results obtained by SSCP analysis and subsequent direct genomic sequencing of samples that showed an altered electrophoretic behaviour in the SSCP analysis. Seventeen out of the twenty-one mutations resulted in missense errors leading to a change in the amino acid sequence of the *p53* protein. Three base-substitutions created a new stop-codon. One additional nonsense mutation was due to a deletion of eight bases. Among the base substitutions, there was a predominance of transitions, 13 of 20 (65%), in which a purine is substituted for a purine or a pyrimidine for a pyrimidine. In 7 of 20 cases a transversion has occurred by substitution of a purine for a pyrimidine or vice versa. Eleven of thirteen (85%) transitions were G:C to A:T changes, from which 7 occurred at CpG dinucleotide pairs. The nature of all base substitutions of the *p53* gene in gastric cancer is summerized in Table 2. In codon 196 in exon 6 the same mutation was found in two samples. In codon 248 in exon 7, which is a known mutational hot-spot in the *p53* gene, the same base substitutions occurred in five samples. Furthermore, in five samples an amino acid substitution in codon 72 (proline to arginine) in exon 4 could be found, which was described previously as a frequent neutral polymorphism [30]. Another three samples carried a silent mutation at codon 213 in exon 6 (213^{Arg} to 213^{Arg}).

In order to distinguish between genetic alterations in the germline and somatic mutations, we examined and compared non-neoplastic tissues with the respective tumour tissue samples. The same exon in which the mutation was found in the respective tumour was PCR-amplified and analysed by direct genomic sequencing in the non-neoplastic tissue. Since no genetic alterations could be detected in the non-neoplastic tissues, all mutations found in the tumours were of somatic origin.

Association of p53 gene mutations with loss of heterozygosity

For the detection of LOH, normal tissue material was amplified for the VNTR region YNZ22, an intragenic *p53 Msp*I enzyme restriction site polymorphism in intron 6, and an *Acc*II polymorphism in amino acid residue 72 in exon 4, to evaluate whether the samples are informative (heterozygous). Out of 39 samples from which normal tissues were examined, a total of 23 (59%) were heterozygous and thus informative. When compared with the amplification of the respective tumour material LOH was found in a total of six samples or 26.1% (Table 3). Loss of heterozygosity has only occurred in those tumours with *p53* mutations [10].

Immunohistochemical analysis

Nuclear staining of cells in immunohistochemistry was considered positive for abnormal p53 accumulation. The immunohistochemical reactions for p53 were scored according to the following categories: N, negative; ISC, nuclear staining in isolated single tumour cells; +, focal nuclear positivity, <20% positive tumour cells; ++, moderate nuclear positivity, 20%–50% positive tumour cells; +++, high nuclear positivity, 51–100% positive tumour cells. Staining with the polyclonal antibody CM-1 was observed in 17 of the 56 tumours (30%) examined. The details regarding the staining pattern and type of tumour are summarized in Table 1. Of the 21 tumours carrying *p53* mutations confirmed by direct genomic sequencing, 16 (76%) were positive by immunohistochemistry (Figs. 3, 4). Of the five samples with *p53* mutations but negative immunhistochemical results, three carried mutations leading to premature "stop" codons, resulting in truncated protein products. One sample (number 629) revealed moderate nuclear positivity by immunohistochemistry, but no *p53* gene mutation could be detected.

Table 2 Nature of base substitutions of the *p53* gene mutations in gastric cancer

Substitutions	Number (%)
Transitions	13 (65)
G:C to A:T	11 (55)
A:T to G:C	2 (10)
Transversions	7 (35)
G:C to T:A	3 (15)
G:C to C:G	1 (05)
A:T to C:G	1 (05)
A:T to T:A	2 (10)

Table 3 Loss of heterozygosity at the *p53* and YNZ22 locus

Polymorphic marker	Number tested	Informative (heterozygous)	Loss of heterozygosity
Msp I	39	9 (23%)	3
Acc II	39	12 (31%)	3
YNZ22	39	11 (23%)	3
Total	39	23 (59%)	6 (26%)

Discussion

Previous studies of gastric cancer have suggested that mutations in the *p53* tumour-suppressor gene may play a role in the aetiology and/or progression of this disease. Because only a small number of samples were examined in former studies, the frequency of mutations in *p53* remains unclear and even contradictory. The data presented in this investigation are based on 56 samples of primary gastric cancer and of non-tumour gastric tissues that were available from the patients. In comparison with the reported frequency of *p53* mutations in gastric cancer, ranging from 0% to 64% [21, 31, 54, 60], the data presented here suggests a frequency of approximately 38%. Because the samples examined in this study have not been preselected by such criteria as family history of gastric cancer or anploidy versus diploidy of the tumour cells, the frequency of 38% may represent the number of

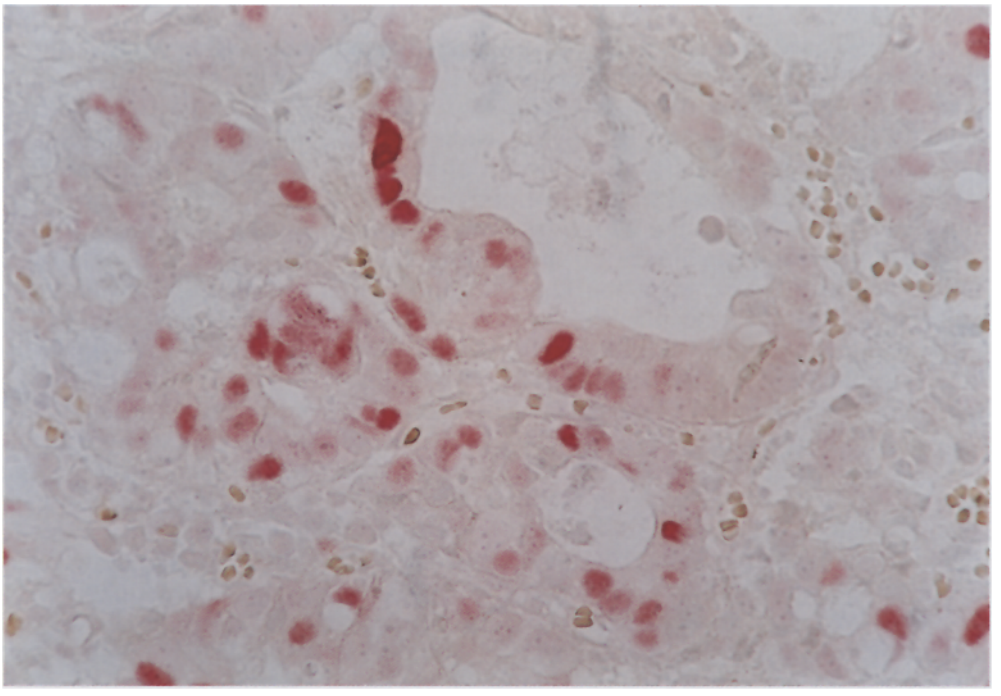

Fig. 3 High nuclear positivity for the anti-p53 antibody CM-1 in a moderately differentiated adenoncarcinoma of the intestinal type (case 0637; ×400). In this sample, a somatic *p53* mutation could be found in codon 248 (Table 1)

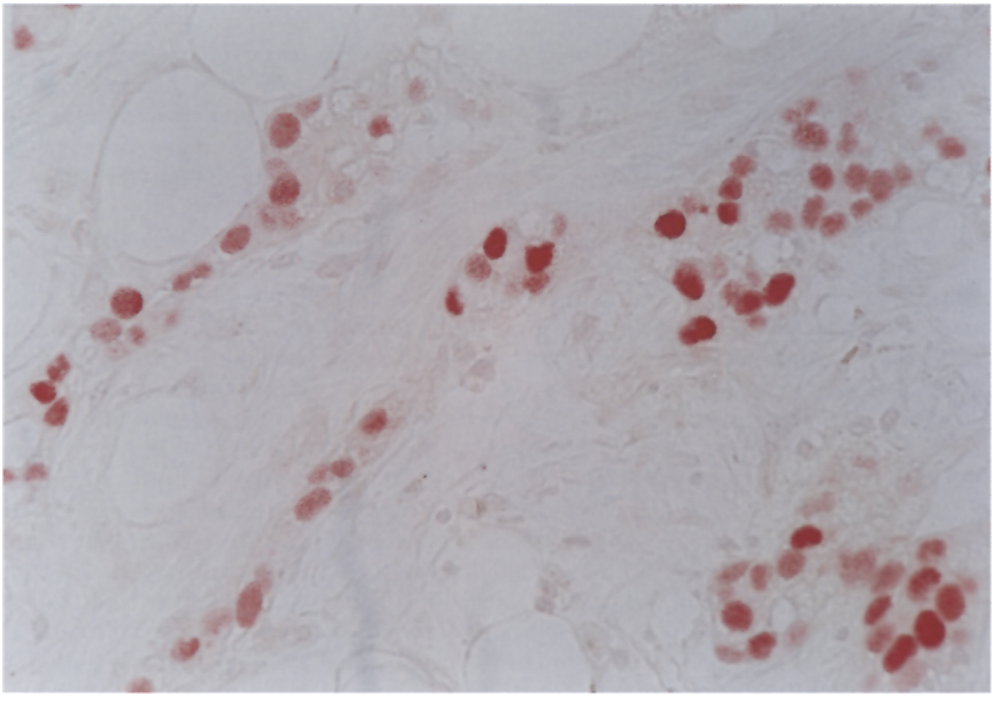

Fig. 4 Poorly differentiated adenocarcinoma of the mixed type (case 0643), revealing high nuclear positivity. The staining is confined to the nuclei of the cells (×400). This sample carried a somatic *p53* mutation in codon 173 (Table 1)

p53 mutations in spontaneous primary gastric cancer in the population of northwestern Germany.

Among the 21 mutations described above, 13 occurred within four of the five domains of high homology among different species; 12 of these were missense mutations, 1 was a nonsense mutation. The remaining eight mutations (five missense mutations, three nonsense mutations) were found outside the conserved domains, but seven of them occurred at codons that are identical in human, monkey, mouse and rat. Tumour mutations appear to happen almost exclusively at amino acids that are completely conserved during evolution at least in mammalians, regardless of the amino acid conservation of surrounding residues [16].

In contrast to these findings, the frequent neutral polymorphism at codon 72 (although substituting the amino acid proline to arginine) [30] occurs at a position in the *p53* gene where the sequence even differs among mammalians.

Two codons (196 and 248) of the *p53* gene were mutated more than once in the gastric cancer samples. Mutations in codon 196 (CGA to TGA causes arginine to

"stop") were found in two cases in this study. Mutations in codon 196 have been described before for other types of cancers, like malignant fibrous histiocytoma [57] and colorectal carcinoma [1]. Codon 248 is known as a "hot-spot" [16] for *p53* mutations in different malignancies: the substitution of thymine for cytosine (CGG to TGG) resulting in an amino acid exchange from arginine to tryptophan has been reported with a relatively high frequency in colorectal carcinomas [1, 40], ovarian cancer [24] as well as gastric carcinomas [21, 32, 60]. In this study, five (24%) of 21 mutations occurred at codon 248, suggesting an important role of the encoded amino acid in the function of the wild-type p53 product. As suggested by a previous study, *p53* gene mutations at codon 173 seem to be rare mutations which have been described once in a tumour [57] other than gastric cancer. In contrast to former reports [32], the results presented in this study indicate that codon 173 is unlikely to be a "hot-spot" for *p53* mutations in gastric cancer. Only one mutation found in this study involves codon 173 (CTG to TTG causes valine to leucine). Furthermore, the spectrum of mutations observed at this codon in gastric cancer differs from one another: mutations resulting in amino acid substitutions from a) valine to alanine (GTG to GCG), b) valine to methionine (GTG to ATG) and c) valine to valine (GTG to GTA) have been reported [32, 49, 54, 60].

The mutation spectrum described in this study supports the supposition that physical and functional aspects of *p53* mutations may be highly variable from one tumour type to another [13], due to either functional selection in some tissues or alternatively due to divergent mutagen exposure in different organ systems. Although some codons seem to be a more frequent target for mutations, it was not possible to determine a mutation pattern for gastric cancer with particular codons to be specifically mutated only in this disease.

Analysis of specific gene lesions in many human tumours has indicated that cancer is the result of an accumulation of discrete genetic changes over a long period of time [4, 11, 58]. A variety of carcinogens can generate the mutations in protooncogenes and tumour suppressor genes that have been found in human malignancies. Nitrogenous compounds and their metabolites have been accused for many years to play an important role in gastric carcinogenesis [35, 53]. They are able to deaminate purines, pyrimidines and different forms of DNA and RNA under acidic conditions. Protonation of nitrite leads to formation of dinitrogen trioxide, an electrophilic nitrosating agent that can deaminate aromatic amines via their aryl diazonium ions; this is known as acid-catalyzed nitrite transformation in nucleic acids. Types of mutations that may arise from deamination of DNA bases are summarized in Table 4 [39].

Thirteen (65%) of the 20 point mutations in the *p53* gene detected in the gastric cancer samples were a result of deamination of nucleic acids. Seven of these mutations occurred at CpG dinucleotides (cases 635, 657, 637, 627, 1024, 1026, and 1032 in Table 1). CpG dinucleotide pairs are a hot-spot for inherited mutations in humans, accounting for approximately 35% of the point mutations causing human genetic disorders [7]. Regarding the frequency (7 of 20 or 35%) of point mutations involving CpG dinucleotide pairs in this study, this result is identical with the published expected frequency. The most likely possibility for the underlying mechanism is hydrolytic deamination of 5-methylcytosine at methylated CpG pairs, resulting in a transition mutation where thymine is substituted for 5-methylcytosine [9]. Evidence has been reported previously suggesting that the apparent mutational hot-spot at *p53* codon 248 (and also codons 175 and 273) may arise due to this mechanism at methylated CpG pairs. In the 2,362 nucleotides of the *p53* double-stranded coding sequence are 82 CpG pairs, which means that approximately 3.5% of the entire sequence contributes to about one third of the point mutations. Therefore 5-methylcytosine should be considered an "endogenous mutagen" in the *p53* gene [45]. Interestingly, methylating nitrosamines [15] may influence the level of methylation at 5-methylcytosine, and furthermore there is good evidence that nitric oxide is responsible for the deamination of 5-methylcytosine to thymine. The other six point mutations in the *p53* gene (cases 624, 653, 643, 652, 623, and 641 in Table 1) are also consistent with the predicted types of mutations that potentially arise from deamination of DNA bases (Table 4). The data from this study therefore strongly supports the supposition that the *p53* gene may be one of the important molecular targets for deamination by nitric oxide in gastric cells.

N-nitroso compounds may function as mutants in different ways: exogenic factors as tobacco and tobacco smoke, containing N-nitroso compounds, may facilitate the transition from chronic atrophic gastritis/intestinal metaplasia to dysplasia in the carcinogenic process [22]. *Helicobacter pylori* – not found in normal stomachs, but very frequently present in chronic gastritis – is probably a source of active inflammation lasting for decades. Excessive salt intake leads to mucosal damage and inflammatory changes associated with its repair, inducing both gastritis and atrophy. Chronic atrophic gastritis leads to higher gastric pH as a result of the loss of acid-secreting parietal cells, facilitating proliferation of anaerobic bacteria which reduce nitrate (in foods) to nitrite [8]. Furthermore, cells of the inflammatory infiltrate like macrophages and polymorphonuclear leukocytes, are known to release nitric oxide themselves [39]. As pointed out before, nitric oxide could induce mutations which alter the

Table 4 Types of mutations that potentially arise from deamination of DNA bases

Conversion	Mutation type
5-Methylcytosine to thymine	G:C to A:T
Cytosine to uracil	G:C to A:T
Adenine to hypoxanthine	A:T to G:C
Guanine to xanthine to apurinic site	G:C to T:A
Adenine to hypoxanthine to apurinic site	A:T to T:A

nucleic acids of the *p53* gene by deamination. Among the samples with possible deamination, the histological types include five carcinomas of the diffuse type (cases 627, 635, 641, 1026, 1032), three carcinomas of the intestinal type (cases 624, 637, 653), two carcinomas of the mixed type (643, 652), 1 adenocarcinoma in Barrett's oesophagus (case 623), 1 anaplastic (case 657) and 1 adenosquamous (case 1024) carcinoma. Although the histology of these tumours is different at the time of diagnosis, they may have had common features in the precancerous or early stages of malignancy. Regarding the theory of possible mechanisms causing deamination of nucleotides, it is known that the intestinal type of gastric carcinoma can derive from chronic atrophic gastritis [43], and also the Barrett syndrome is supposed to derive from an inflammatory, metaplastic process [3]. All of the diffuse and anaplastic carcinomas examined in this study were poorly differentiated; but as seen in the mixed type carcinomas, consisting of both intestinal and diffuse carcinoma parts, it may be possible that in the process of dedifferentiation the whole carcinoma becomes diffuse or anaplastic. In some of those carcinomas precursor lesions like chronic atrophic gastritis are still present.

In conclusion, deamination of certain nucleotides by carcinogenic agents as nitric oxide, causing point mutations in the *p53* tumour suppressor gene, may play an important role in at least some of the gastric carcinomas, independent from their histology and differentiation at the time of diagnosis.

Employing intragenic *p53* gene polymorphisms, we detected LOH in 6 tumours (26.1%) of 23 informative heterozygous cases. All six samples with LOH also showed mutations in the remaining *p53* allele, whereas no LOH was detected in the remaining informative tumour samples in which *p53* mutations were found. This data suggests that in gastric carcinomas, as described previously in colorectal carcinomas [1], missense mutations may precede allelic deletions. The detection of mutant *p53* in gastric adenomas [55] supports the hypothesis that *p53* gene mutations may be an early event in gastric carcinogenesis. In this scenario, a single specific mutation resulting in a mutant protein creates a growth advantage through a "dominant negative" effect, by which the mutant protein drives the wild-type protein into the mutant conformation during translation [33]. However, cells with one mutant allele may lose the remaining normal allele through a "second hit" during a phase of rapid proliferation. Thus, a quantitative decrease of p53 protein might be enough to endow cells with a growth advantage.

In different previous studies, immunohistochemistry alone was used for the detection of abnormal p53 and its prognostic significance in gastric carcinoma [19, 29, 52], resulting in up to more than 60% positive cases in some studies. The immunohistological detection of p53 in tumour cells was initially reported to be directly associated with *p53* mutations [46]. Our findings, however, have shown that there is only a limited coincidence of p53 immunopositivity and mutations in the *p53* gene: p53 protein is undetectable in most tumours with chain-terminating (nonsense) or frame-shift (deletions or insertions) mutations in the exons or adjacent to splice sites, because the protein product is absent or truncated. These mutations are probably missed if immunohistochemistry alone is used as a screening technique. Previous studies suggest that approximately 20% or more of the tumours with a mutant p53 are false negative by exclusive immunohistochemical studies [20]. In our study, three samples with premature stop codons (cases 624, 635, 657) were negative for immunostaining. In these cases the premature "stop" occurred through a mutation in the N-terminal region or the middle of the coding sequence, resulting in a protein with less than 50% of its normal primary structure. One other samples (case 660) with a "stop" at codon 305 closer to the c-terminus of the protein revealed positive immunohistochemistry. However, one positively staining sample (case 629) did not bear a detectable *p53* gene mutation in the whole coding sequence. One possible explanation is that posttranslational mechanisms stabilize the wild-type p53 by complex formation and lead to functional inactivation as described as an example for the *mdm*-2 oncogene product [36]. Even though immunohistochemical techniques preserve morphological details and are cheaper and less labour intensive than molecular genetic approaches, they cannot detect all types of mutations and vary in sensitivity and specificity for different kinds of *p53* alterations [62].

In conclusion, the spectrum of *p53* mutations in primary gastric carcinomas observed in this study appeared to result from deamination of nucleic acids and was consistent with the predicted spectrum for dietary mutagens associated with the metabolism of nitrogenous compounds. Especially CpG dinucleotides in the *p53* gene seem to be a primary target for mutations associated with dietary carcinogens. All mutations found in this study were of somatic origin. Loss of heterozygosity of the *p53* gene was detected only in tumours bearing gene mutations. Immunohistochemistry using the polyclonal anti-p53 antibody CM-1 reveals only limited coincidence between immunopositivity and gene mutations because most nonsense mutations and frameshift errors do not lead to detectable p53 protein expression.

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