Comparison of p53 and DNA content abnormalities in adenocarcinoma of the oesophagus and gastric cardia

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Summary This study examined the association between 17p allelic loss, p53 gene mutation, p53 protein expression and DNA aneuploidy in a series of adenocarcinomas arising in the oesophagus and gastric cardia. 17p allelic loss was detected in 79% (15 of 19) of oesophageal and in 83% (29 of 35) of gastric adenocarcinomas. p53 mutations were detected in 70% (14 of 20) and 63% (26 of 41) of oesophageal and of gastric adenocarcinomas respectively. Both tumour types were associated with a predominance of base transitions at CpG dinucleotides. In five cases of oesophageal adenocarcinoma, the same mutation was detected both in tumour and in adjacent dysplastic Barrett's epithelium. Diffuse p53 protein expression was detected in 65% (13 of 20) and 59% (24 of 41) of oesophageal and of gastric tumours, respectively, and was associated with the presence of p53 missense mutation (Chi-squared, P < 0.0001). DNA aneuploidy was detected in 80% (16 of 20) of oesophageal and in 70% (28 of 40) of gastric tumours. No association was found between p53 or DNA content abnormalities and tumour stage or histological subtype. In conclusion, this study detected a similar pattern of p53 alterations in adenocarcinoma of the oesophagus and gastric cardia – molecular data consistent with the observation that these tumours demonstrate similar clinical and epidemiological features.

Keywords: adenocarcinoma; oesophagus; gastric cardia; Barrett's oesophagus; p53

In recent years, the incidence rates of adenocarcinoma of the oesophagus and gastric cardia have increased steadily, while there has been a decrease in the proportion of tumours arising in the distal stomach (Powell and McConkey, 1990; Blot et al, 1991). Gastric adenocarcinomas represent a heterogeneous group of tumours. Adenocarcinomas arising in the proximal stomach (gastric cardia) were reported to demonstrate biological and epidemiological features distinct from those arising in the distal stomach (gastric antrum) (Sidoni et al, 1989; Blot et al, 1991). Conversely, adenocarcinoma of the gastric cardia was reported to demonstrate clinicopathological features similar to those of oesophageal adenocarcinoma (Kalish et al, 1984; Wang et al, 1986). These data suggest that tumours arising in different anatomical sites in the stomach are associated with distinct aetiologies, while adenocarcinomas arising in the oesophagus and gastric cardia may share similar aetiologies.

The specific aetiological factors underlying the increasing incidence of adenocarcinoma of the oesophagus and gastric cardia remain unresolved. Barrett's oesophagus, a condition in which the squamous epithelium normally lining the lower oesophagus is replaced by a metaplastic columnar epithelium, represents one known risk factor for oesophageal adenocarcinoma. This condition arises in 10–12% of patients with chronic gastro-oesophageal reflux (Winters et al, 1987). The estimated risk of a patient with Barrett's oesophagus developing adenocarcinoma is 30 to 40 times higher than in the general population (Spechler et al, 1984). Tumour development in these patients is proposed to occur via a series of dysplastic cell changes, recognized histologically as a metaplasia–dysplasia–carcinoma sequence (Thompson et al, 1983).

The p53 gene, localized to chromosome 17p13 (McBride et al, 1986), encodes a 53-kDa nuclear phosphoprotein that functions in a signal transduction pathway, causing an arrest of cells in the G1 phase of the cell cycle in response to DNA damage (Kastan et al, 1991). Allelic loss on chromosome 17p and p53 gene mutations are among the most common genetic abnormalities documented in human cancers (Greenblatt et al, 1994). The majority of p53 mutations have been reported to occur in exons 5–8, corresponding to the evolutionarily conserved domains of the protein (Soussi et al, 1990; Greenblatt et al, 1994). Mutations in these domains were found to be associated with loss of wild-type p53 function, including growth suppression (Martinez et al, 1991). Inactivation of p53 was reported to be associated with the development of genomic instability and DNA aneuploidy (Livingstone et al, 1992; Yin et al, 1992; Carder et al, 1993).

p53 and DNA content abnormalities have been implicated in the development of both oesophageal and gastric adenocarcinoma (Reid et al, 1987; Yonemura et al, 1992; Blount et al, 1993; Renaud et al, 1993; Hamelin et al, 1994). However, molecular studies in which gastric tumours were analysed with respect to anatomical site of origin (cardia vs antrum) have reported a number of site-specific differences. Significantly higher levels of p53 protein expression (Flejou et al, 1994) and DNA aneuploidy (Johnson et al, 1993; Flejou et al, 1994) have been reported in gastric cardia tumours compared with tumours arising in the gastric antrum. These molecular data are consistent with the observation that proximal and distal gastric tumours display distinct epidemiological features (Blot et al, 1991). Conversely, adenocarcinoma of the oesophagus and gastric cardia demonstrate similar clinical and epidemiological features (Wang et al, 1986; Blot et al, 1991). The aim of the present study was, firstly, to document the pattern of p53 and DNA content abnormalities in a homogenous series of adenocarcinomas arising in the gastric cardia and to compare the pattern with that detected in a series of oesophageal adenocarcinomas.
Table 1  PCR amplification and sequencing primers for p53, exons 5–8

| p53  | Primer sequences                  | Application: PCR and/or sequencing* |
|------|----------------------------------|-----------------------------------|
| Exon 5 | 5'-TGTTCACCTTGCCACTGACT-3' | Left PCR and sequencing primer   |
|       | 5'-AGCAATCAGTGGAAATCA-3'     | Right PCR and sequencing primer  |
| Exon 6 | 5'-TGTTGGCCAGCGTCCCG-3'      | Left PCR and sequencing primer   |
|       | 5'-GAAGGGCAGACTGACAAC-3'     | Right PCR and sequencing primer  |
| Exon 7 | 5'-CTTGGCAAGTCTGCCCAAA-3'     | Left PCR and sequencing primer   |
|       | 5'-AGGGGTCAGCGCAAGCAGA-3'     | Right external PCR primer        |
|       | 5'-TGTCAGGAGTGCAAGTGGC-3'     | Right internal sequencing primer |
| Exon 8 | 5'-TTCTTATTGCTTCTTGCTC-3'     | Left PCR and sequencing primer   |
|       | 5'-AGGATATGCGACCTTTGG-3'      | Right PCR and sequencing primer  |

*Sequence analysis of exons 5, 6 and 8 used the same primers as those used for PCR amplification. For exon 7, an internal primer was used in the reverse direction to give optimal sequencing profiles.

Table 2  17p allelic loss, p53 gene mutation, p53 protein expression and DNA content in Barrett’s epithelium and oesophageal adenocarcinoma

| Patient no. | Stage* | Histology* | LOH at 17p13† | p53 Mutation analysis | Codon | Nucleotide change | Nature of nucleotide substitution | Amino acid change | ICC* | DNA content | DI† |
|-------------|--------|------------|---------------|------------------------|-------|------------------|-----------------------------------|------------------|------|-------------|-----|
| 104         | I      | W/MD       |               |                        |       |                  |                                    |                  |      |             |     |
| 108         | I      | MD         |               |                        |       |                  |                                    |                  |      |             |     |
| 90          | II     | WD         | H             | 273                    | CGT   | CAT              | G:C to A:T†                       | Arg → His        | +++  | Aneuploid   | 3.2 |
| 36          | II     | WD         | H             | -ve                    |        |                  |                                    |                  |      |             |     |
| 88          | II     | W/MD       | b             | 282                    | CGG   | IGG              | G:C to A:T†                       | Arg → Trp        | +++  | Aneuploid   | 1.8 |
| 110         | II     | MD         | ab            | H                      | -ve   |                  |                                    |                  |      |             |     |
| 112         | II     | MD         | b             | 158                    | CQC   | CTC              | G:C to T:A                        | Arg → Leu        | +++  | Aneuploid   | 1.4 |
| 125         | II     | MD         | b             | -ve                    |        |                  |                                    |                  |      |             |     |
| 76          | II     | MD         | a             | 282                    | CGG   | TGG              | G:C to A:T†                       | Arg → Trp        | +++  | Aneuploid   | 1.5 |
| 22          | II     | PD         | b             | 7                      | 248   | CGG              | G:C to A:T†                       | Arg → Trp        | +++  | Aneuploid   | 1.3 |
| 120         | II     | PD         | a             | -                      | N.D.  |                  |                                    |                  |      |             |     |
| 123         | II     | PD         | ab            | ab                     | 266   | GQA              | G:C to A:T                        | Gly → Glu        | +++  | Diploid     | 2.3 |
| 109         | II     | PD         | b             | -                      | 175   | CQC              | C:A                              | Arg → His        | +++  | Diploid     | 1.0 |
| 54          | III    | MD         | ab            | H                      | -ve   |                  |                                    |                  |      |             |     |
| 99          | III    | MD         | H             | 248                    | CGG   | TGG              | G:C to A:T†                       | Arg → Trp        | +++  | Aneuploid   | 1.4 |
| 124         | III    | MD         | b             | 8                      | 285   | GAG              | A:A                              | Glu → Lys         | +++  | Aneuploid   | 1.4 |
| 29          | III    | MPD        | ab            | ab                     | 285   | GAG              | A:A                              | Glu → Lys         | +++  | Aneuploid   | 1.4 |
| 50          | III    | PD         | H             | 7                      | 242   | TGG              | C:T                             | Cys → Phe         | +++  | Aneuploid   | 1.7 |
| 74          | III    | PD         | b             | 7                      | 248   | CGG              | C:A                              | Arg → Gin         | +++  | Aneuploid   | 1.6 |
| 43†         | III    | PD         | b             | -                      | -ve   |                  |                                    |                  |      |             |     |

*TNM − stage I: 1, 0, 0; stage II: 2, 0, 0/3, 0; stage III: 3, 1, 0/4, 0. The tumours were classified according to the procedure of Lauren (1965). †Tumour no. 43 was a diffuse-type adenocarcinoma. All other tumours were intestinal-type adenocarcinoma arising on a background of Barrett’s metaplasia. WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; HGD, high-grade dysplasia; LGD, low-grade dysplasia; IM, intestinal metaplasia. *a, Upper allele retained; b, lower allele retained; ab, heterozygous with no loss; H, homozygous; --, not determined. ‡Exon exhibiting band shift. --, band shift not detected. †G:C to A:T base transitions occurring at CpG dinucleotides. ‡Predicted change in amino acid sequence as a result of mutation. *ICC, immunohistochemistry: +, < 10% positive cells, focal staining pattern; ++, 10–50% positive cells; ++++, > 50% positive cells, diffuse staining pattern; --ve, negative. †DI, DNA index.
Secondly, a number of studies have implicated p53 inactivation in the malignant transformation of Barrett’s oesophagus (Hamelin et al., 1994; Neshat et al., 1994; Gleeson et al., 1995). In order to confirm and extend these observations, this study assessed the involvement of p53 in premalignant Barrett’s epithelium.

**MATERIALS AND METHODS**

**Tissue collection and DNA extraction**

Matched normal and tumour tissue samples were obtained intraoperatively from 20 patients with Barrett’s-associates oesophageal adenocarcinoma and from 41 patients with adenocarcinoma of the gastric cardia. Barrett’s-associates adenocarcinomas were categorized as those tumours with histological evidence of having arisen against a background of Barrett’s epithelium. All oesophageal cases were associated with Barrett’s metaplasia extending greater than 3 cm into the oesophagus, and all tumours exhibited predominant localization in the oesophagus. The gastric cardia tumours were located near the gastro-oesophageal junction. None had associated Barrett’s epithelium. In six cases of Barrett’s-associates adenocarcinoma, residual Barrett’s epithelium adjacent to adenocarcinoma was obtained for analysis. The tissue was snap frozen in liquid nitrogen and stored at −70°C. The tissue samples were histologically assessed...
by a consultant pathologist (JMS) and classified according to the procedure of Lauren (1965). Tumour stage was assessed using the American Joint Committee on Cancer (A.J.C.C.) and the International Union against Cancer (U.I.C.C.) criteria for pathological staging (Hermanek and Sobin, 1987; Beahrs et al, 1988). Cryostat sectioning was carried out to select tumour-rich tissue for analysis, and microdissection was used to isolate all preneoplastic lesions. DNA was extracted from matched normal and tumour tissue samples as described previously (Gleeson et al, 1995).

Allelic loss analysis

To detect 17p allelic loss in the tumour tissue, two dinucleotide repeat polymorphisms, D17S513 and D17S796, mapping to 17p13 were analysed (Oliphant et al, 1990; Gyapay et al, 1994). Polymerase chain reaction (PCR) amplification and microsatellite analysis were carried out as described previously (Gleeson et al, 1995). LOH was scored by direct visual comparison of the relative allelic ratios present in matched normal and tumour DNAs. Allele loss was scored if one allele was absent or exhibited altered signal intensity in tumour DNA relative to the allelic ratio of normal DNA.

Mutational analysis of p53, exons 5–8

For PCR amplification of p53, exons 5–8, primers homologous to sequences in the adjacent introns were used (Hsu et al, 1991, Table 1). Genomic DNA (300 ng) was incubated in a total volume of 100 µl of PCR buffer [50 mM potassium chloride, 10 mM Tris-HCl, pH 9.0, 1 mM magnesium chloride (1.5 mM for exons 5 and 8), 0.1% Triton X-100] containing 0.25 µM of each primer, 0.2 mM dNTPs and 5 units of Taq polymerase (Promega). There was an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C (62°C for exon 6) and 30 s at 72°C, with a final extension step of 72°C for 5 min.

A number of p53 mutations (n = 19) were initially identified by direct DNA sequencing on a subgroup of 35 tumours. Sequence analysis was carried out as described previously (Gleeson et al, 1995). These 19 control mutations were analysed to select optimal gel conditions for mutation detection by non-isotopic single-strand conformational polymorphism (SSCP). For each exon, one set of gel conditions was identified that detected all known sequence variants in that exon. All exons were analysed on 1-mm-thick 8% polyacrylamide gels with 2.6% cross-linking. The optimal conditions selected were 4°C without glycerol (exon 6), 24°C without glycerol (exon 5) and 24°C with 5% glycerol (exons 7 and 8).
Table 4 Association between p53 gene mutation and 17p allelic loss in oesophageal and gastric adenocarcinoma

| p53 Gene mutation | 17p Allelic loss |
|-------------------|-----------------|
|                   | LOH | No LOH | Fisher exact test |
| Mutation          | 33  | 3     |                  |
| No mutation       | 11  | 7     | \( P = 0.02 \)   |

Table 5 Association between p53 gene mutation and p53 protein expression in oesophageal and gastric adenocarcinoma

| p53 Gene mutation | p53 Protein expression | Diffuse | Focal/ Negative | Chi-squared |
|-------------------|------------------------|---------|----------------|-------------|
| Mutation          |                        | 31      | 9              | \( P = 0.003 \) |
| No mutation       |                        | 6       | 12             |             |

Table 6 Association between p53 missense mutation and p53 protein expression in oesophageal and gastric adenocarcinoma

| p53 Missense mutation | p53 Protein expression | Diffuse | Focal/ Negative | Chi-squared |
|-----------------------|------------------------|---------|----------------|-------------|
| Missense mutation     |                        | 31      | 0              | \( P < 0.0001 \) |
| No missense mutation  |                        | 6       | 21             |             |

Aliquots of PCR product (5 μl) were denatured in alkali buffer (10× stock: 500 mM sodium hydroxide, 10 mM EDTA) at 42°C for 30 min and immediately placed on ice. Loading buffer [10× stock: 0.5% (w/v) bromophenol blue, 0.5% (w/v) xylene cyanol in deionized formamide] was added and the samples were loaded to the appropriate gel. Electrophoresis was performed at 400 V for 3–6 h using 1 × TBE buffer. Electrophoresis was carried out using a Protean II vertical electrophoresis tank (Biorad), which was modified to allow buffer recirculation through a closed-circuit loop placed in a temperature-regulated water bath, allowing accurate and reproducible temperature control. After electrophoresis, the gel was fixed in an aqueous solution of 10% (v/v) ethanol and 0.5% (v/v) acetic acid for 3 min (×2). The gel was stained in an aqueous solution of 0.1% (w/v) silver nitrate for 15 min and visualized by immersion in an aqueous solution of 0.1% (w/v) sodium hydroxide and 0.1% (w/v) formaldehyde for 20 min. The gel was fixed for 10 min in an aqueous solution of 0.75% (w/v) sodium carbonate. Band shifts were identified by direct comparison of the pattern of single-stranded bands in control and tumour DNA from the same individual. SSCP analysis, using the optimized gel conditions, was used to screen for p53 mutations in the remaining 26 cases. Sequence analysis was used to confirm and identify the mutation present in each sample exhibiting an altered mobility shift.

Immunohistochemistry

Paraffin sections (4 μm) were dewaxed in xylene, rehydrated through descending grades of alcohol and immersed in 3% (v/v) alcoholic hydrogen peroxide for 10 min. The sections were immersed in 0.1 M sodium citrate buffer, pH 6.0, and microwaved for 30 min. The sections were stained with the DO7 antibody (Novocastra) at an optimal dilution of 1:50 for 30 min at room temperature and then rinsed in TBS to remove unbound primary antiserum. A biotinylated anti-mouse secondary antiserum (Dako) was incubated at an optimal dilution (1:200 in 1:25 normal human serum) for 30 min at room temperature. The sections were rinsed in TBS and the bound antiserum was visualized using the streptavidin–biotin complex immunoperoxidase protocol, using diaminobenzidine as a chromogen (Dako, UK). Sections were counterstained in Harris’s haematoxylin and mounted with DPX mounting medium (Diachem). Sections demonstrating nuclear staining in greater than 50% of tumour cells were categorized as diffuse staining, while sections demonstrating nuclear staining in less than 10% of tumour cells were categorized as focal staining. A known strongly staining case was used as a positive control. Omission of the primary antiserum served as a negative control.

Flow cytometry

Paraffin sections (50 μm) were processed for flow cytometry using a modification of the method described by Hedley et al. (1983). Nuclear suspensions were stained with propidium iodide solution (propidium iodide (3 mg per 100 ml of phosphate-buffered saline) containing 10 mg of Ribonuclease A and 0.5 ml of 0.5% Triton X-100) at 4°C for 30 min. Samples were analysed on a Coulter Epics Elite Flow Cytometer equipped with a 15-mW Argon laser, excitation beam 488 nm. The DNA histogram upon which analysis was performed in each case was gated on Forward Angle Light Scatter (FALS) to minimize doublets and clumps, but included a proportion of debris. The histograms were analysed using Multicycle Software (Phoenix Flow Systems). This analysis allowed debris and sliced nuclei to be accounted for and to be subtracted from the histograms. Histograms that showed a single G1/G2 peak with a corresponding G2 + M peak were classified as DNA diploid. Diploid histograms were only accepted if the coefficient of variation (CV) at half the peak height was less than 10%. DNA aneuploidy was defined as the presence of an additional G1/G2 peak that contained >10% of the total cell population. The DNA index, defined as the modal DNA content of the aneuploid cell population divided by the modal DNA content of the diploid cell population, was calculated for each aneuploid histogram.

RESULTS

The mean age at diagnosis with oesophageal adenocarcinoma was 61 years (range 31–75 years), and the patient group demonstrated a male–female ratio of 17:3. The tumour series included two stage I tumours, 11 stage II tumours and seven stage III tumours. With respect to histological subtype, the tumours included 19 cases of the intestinal type (well–moderately differentiated, \( n = 12 \); poorly differentiated, \( n = 7 \)) and one poorly differentiated diffuse-type adenocarcinoma (Table 2). The mean age at diagnosis with adenocarcinoma of the gastric cardia was 63 years (range 44–80 years), and the patient group exhibited a male–female ratio of 31:10. The tumour group included 11 stage II tumours and 30 stage III tumours. Histological assessment identified 32 gastric adenocarcinomas of the intestinal type (well–moderately differentiated, \( n = 17 \); poorly differentiated, \( n = 15 \)) and nine gastric adenocarcinomas of the diffuse type (Table 3).

Allelic loss on chromosome 17p was investigated using two highly informative dinucleotide repeat polymorphisms mapping to
Representative examples of allelic loss at the D17S796 microsatellite are shown in Figure 1.

Mutational analysis of p53, exons 5–8, resulted in the detection of mutations in 70% (14 of 20) of oesophageal adenocarcinomas (Table 2), eleven of which have been documented previously (Gleeson et al, 1995). These included 12 missense mutations, one non-sense mutation and one frameshift mutation, the last being a 2-bp deletion in exon 6. Eighty-five per cent (11 of 13) of the single-base substitutions were G:C to A:T base transitions, with 69% (9 of 13) occurring at CpG dinucleotides. The remaining two single-base changes were G:C to T:A base transversions. Mutations were detected in 63% (26 of 41) of gastric adenocarcinomas (Table 3). These included 19 missense mutations, three non-sense mutations, three splice site mutations and one deletion. Of the 22 single-base substitutions in coding sequence, 82% (18 of 22) were base transitions (17 G:C to A:T, one A:T to G:C), with 55% (12 of 22) occurring at CpG dinucleotides. The remaining four single-base substitutions were base transversions (two G:C to T:A, one A:T to T:A, one A:T to C:G). A graphical representation of the mutational profiles detected in oesophageal and gastric adenocarcinoma is shown in Figure 2.

Premalignant Barrett’s epithelium adjacent to oesophageal adenocarcinoma was available for analysis in six cases (nos. 22, 29, 76, 108, 124 and 125). Sequence analysis did not detect a mutation in case no. 125. In the remaining five cases, the same p53 mutation was detected both in high-grade dysplasia and in adjacent tumour tissue. In case no. 29, the same mutation was also detected in low-grade dysplasia but not in Barrett’s intestinal metaplasia (Figure 3). In case no. 108, low-grade dysplasia and Barrett’s intestinal metaplasia, negative for dysplasia, were not found to contain the p53 mutation detected in the adjacent high-grade dysplasia and tumour (Table 2).

Overall, 33 tumours with 17p allelic loss were found to contain a p53 mutation. A further 11 cases exhibited 17p allelic loss, however mutations were not detected in exons 5–8 of the p53 gene. p53 mutations were detected in three cases with retention of heterozygosity on 17p and in four non-informative cases. Mutations were not detected in seven cases that retained heterozygosity on 17p nor in three non-informative cases. Statistical analysis revealed an association between 17p allelic loss and p53 gene mutation (Table 4, Fisher exact test, P = 0.02).

p53 protein expression was assessed using the DO7 antiserum, and representative examples of the p53 immunostaining patterns observed are shown in Figure 4. Weak cytoplasmic staining was detected in focal cells in the basal layers of adjacent gastric mucosa, squamous epithelium of the oesophagus and non-dysplastic Barrett’s epithelium. This staining was readily distinguished from the specific p53 nuclear immunostaining detected in dysplastic epithelium and carcinoma. Omission of the primary antiserum resulted in complete abolition of nuclear immunostaining. Diffuse p53 protein expression was detected in 65% (13 of 20) of oesophageal adenocarcinomas and in 59% (24 of 41) of gastric adenocarcinomas. A further 21 cases exhibited focal staining or negative p53 immunoreactivity. The remaining three cases demonstrated immunoreactivity in 10–50% of tumour cells. The staining pattern in these cases was not easily classified as either diffuse (Figure 4B) or focal (Figure 4A).

Figure 4 Representative examples of immunostaining patterns obtained with the DO7 antiserum. (A) +, Focal staining of individual cell nuclei in dysplastic Barrett’s epithelium (original magnification x 400); (B) ++, diffuse immunostaining of a moderately differentiated gastric adenocarcinoma, case no. 92 (original magnification x 250); (C) ++++, diffuse immunostaining in dysplastic Barrett’s epithelium, case no. 29 (original magnification x 100). Intervening non-malignant stroma is immunonegative.


cytosomic 17p13. For oesophageal adenocarcinoma, 95% (19 of 20) of samples were informative at either one or both of the polymorphic loci analysed, and allelic loss was detected in 79% (15 of 19) of informative cases (Table 2). Allelic loss was detected in 83% (29 of 35) of informative cases of gastric adenocarcinoma (Table 3). With the exception of case no. 94, allele loss scores were concordant in all cases for which both loci were informative.
31 missense mutations exhibited diffuse p53 protein expression, while nine non-sense or truncating mutations were immunonegative. Statistical analysis revealed a highly significant association between diffuse p53 protein expression and p53 missense mutation in these tumours (Table 6; Chi-squared, $P < 0.0001$).

There were six cases with premalignant Barrett's epithelium adjacent to carcinoma, and in all cases the immunostaining pattern in the dysplastic Barrett's epithelium was concordant with that detected in the tumour tissue. Two cases (no. 108 and no. 125) showed no immunostaining in either Barrett's epithelium or in adjacent carcinoma. In the remaining four cases (nos. 22, 29, 76 and 124) diffuse p53 expression was detected in both premalignant and malignant lesions; in each of these cases, sequence analysis confirmed the presence of a missense mutation in both dysplastic and invasive lesions (Table 2). Figure 4C shows an example of diffuse p53 protein expression detected in dysplastic Barrett's epithelium and in adjacent carcinoma.

DNA aneuploidy was detected in 80% (16 of 20) of oesophageal tumours (Table 2) and in 70% (28 of 40) of gastric tumours (Table 3). Figure 5 shows representative examples of diploid and aneuploid flow cytometric profiles. Allelic loss on 17p was associated with the occurrence of DNA aneuploidy (Fisher exact test, $P = 0.02$). There was no association between DNA aneuploidy and p53 gene mutation or p53 protein expression.

There was no significant association between p53 gene abnormalities or DNA content abnormalities and tumour stage or histological subtype in either oesophageal or gastric adenocarcinoma.

**DISCUSSION**

The present study investigated the association between 17p allelic loss, p53 gene mutation and p53 protein expression in a series of adenocarcinomas arising in the oesophagus and gastric cardia. The association between p53 gene abnormalities and DNA content in these tumours was also assessed.

Uniformly high levels of 17p allelic loss, p53 gene mutation, p53 protein expression and DNA aneuploidy were detected in both adenocarcinomas of the oesophagus and gastric cardia. Similarly, previous studies have documented 17p allelic loss in 69–100% of oesophageal adenocarcinomas (Blount et al, 1991; Huang et al, 1992). p53 gene mutations were reported in 55% (6 of 11) and 88% (15 of 17) of oesophageal adenocarcinomas (Hamelin et al, 1994; Neshat et al, 1994), while p53 protein expression was detected in 53–87% of cases (Flejou et al, 1993; Hamelin et al, 1994; Hardwick et al, 1994). The presence of aneuploidy in Barrett's oesophagus was reported to occur with increasing frequency during the histological progression from metaplasia to dysplasia and carcinoma (Reid et al, 1987; Haggitt et al, 1988). Studies have consistently documented high levels of DNA aneuploidy in oesophageal adenocarcinoma, typically ranging from 80% to 100% of cases (Reid et al, 1987; Nakamura et al, 1994).

A number of studies have reported a close association between p53 overexpression in adenocarcinoma and in adjacent highly dysplastic Barrett's epithelium (Flejou et al, 1993; Hardwick et al, 1994). Consistent with these observations, the present study identified five cases in which the same p53 gene mutation was present in high-grade dysplasia and in adjacent carcinoma, suggesting that p53 mutation preceded the development of invasive carcinoma in these patients. Furthermore, in one of these cases (no. 29), the same p53 gene mutation was also detected in coexisting low-grade dysplasia, but not in adjacent non-dysplastic Barrett's epithelium.

The detection of identical p53 mutations in high-grade dysplasia and adjacent adenocarcinoma has been reported in two independent studies (Hamelin et al, 1994; Neshat et al, 1994). One study did not detect mutations in areas of low-grade dysplasia (no. 3) and non-dysplastic Barrett's epithelium (no. 6) adjacent to carcinoma (Hamelin et al, 1994). Similarly, in the present study, there was one case (no. 108) in which the p53 mutation detected in high-grade dysplasia and tumour was not detected in adjacent low-grade dysplasia and non-dysplastic Barrett's epithelium. Therefore, to date, p53 protein expression and p53 gene mutation have been detected more frequently in high-grade dysplasia than in low-grade dysplasia, suggesting that p53 gene mutation may be associated with increasing severity of dysplasia.

Between 37.5% and 68% of gastric adenocarcinomas have been reported to demonstrate 17p allelic loss (Sano et al, 1991; Seruca et al, 1992; Ranzani et al, 1993). These values are lower than the value of 83% noted in the present series of adenocarcinomas arising in the gastric cardia. It is possible that site-specific differences (cardia vs antrum) may account for this higher frequency of allelic loss. Studies have reported p53 mutations in 35–58% of gastric carcinomas (Renault et al, 1993; Uchino et al, 1993; Shiao...
et al, 1994; Hongyo et al, 1995), and p53 protein expression has been detected in 46–61% of tumours (Martin et al, 1992; Flejou et al, 1994; Hongyo et al, 1995). DNA aneuploidy has been reported in 40–50% of tumours (Yonemura et al, 1992; Brito et al, 1993). However, studies in which tumours were analysed with respect to anatomical site of origin have reported a number of site-specific differences. A significantly higher level of p53 expression was observed in gastric cardia tumours (56%, 20 of 36) compared with tumours arising in the antrum (27%, 8 of 30) (Flejou et al, 1994). Furthermore, a higher frequency of DNA aneuploidy was detected in adenocarcinomas arising in the gastric cardia (Johnson et al, 1993; Flejou et al, 1994). For example, one study reported aneuploidy in 76% (25 of 33) of adenocarcinomas arising in the gastric cardia, compared with 30% (8 of 27) of adenocarcinomas arising in the gastric antrum (Flejou et al, 1994). These molecular data are consistent with reports that proximal and distal gastric tumours display distinct clinical and epidemiological features. In general, carcinomas of the gastric cardia have a poorer prognosis than those of the gastric body, and their relative incidence appears to be increasing (Sidoni et al, 1989; Blot et al, 1991). Conversely, adenocarcinoma of the oesophagus and gastric cardia have been reported to demonstrate similar clinicopathological parameters. Both tumour types have demonstrated a parallel increase in incidence rates (Blot et al, 1991). These tumours have been consistently associated with a lower mean age, higher male–female ratio and greater frequency of hiatal hernia than distal gastric tumours (Kalish et al, 1984; Wang et al, 1986). The observation in this study of similar frequencies of p53 gene abnormalities and DNA aneuploidy in these tumour types is consistent with the hypothesis that they share a common aetiology.

The occurrence of p53 mutations early in the development of oesophageal and gastric adenocarcinoma (Shiao et al, 1994) suggests that the gene plays a central role in the control of normal cell division at these sites. The oesophagus and stomach are easily accessible and are likely to be exposed to many environmental insults. p53-dependent pathways may play a vital role in preserving molecular integrity by allowing damaged cells to undergo repair or apoptosis. Cells that have acquired a p53 mutation may have a selective advantage, being able to proliferate under conditions of DNA damage that would be inhibitory in cells with wild-type p53. These actively proliferating cells may be the precursors of neoplastic clones.

The detection of frequent mutations in a defined sequence has been identified as a feature of mutational specificity (Thilly, 1990). It has been proposed that the comparison of p53 mutation spectra in tumours of different origin may reveal similarities or differences concerning the endogenous and exogenous molecular processes contributing to tumour development (Greenblatt et al, 1994). The observation of similar p53 mutation spectra in adenocarcinoma of the oesophagus and gastric cardia further suggests similarities in the aetiologies of these cancers. Both tumour groups demonstrated a predominance of base transitions at CpG dinucleotides and a low frequency of base transversions. G:C to A:T base transitions at CpG dinucleotides are thought to result from spontaneous deamination of 5′-methylcytosine, resulting in C to T replacements (Couloumbe et al, 1978). Other studies have reported a predominance of base transitions at CpG dinucleotides in Barrett’s-associated adenocarcinoma (Hamelin et al, 1994; Nesbat et al, 1994). Furthermore, this spectrum of p53 mutations is similar to that previously described for both colorectal cancer (Baker et al, 1990) and gastric cancer (Renault et al, 1993; Uchino et al, 1993). It has been reported that base transitions at CpG dinucleotides account for 62.5% (20 of 32) and 67% (8 of 12) of single-base substitutions in colorectal carcinoma respectively (Baker et al, 1990; Renault et al, 1993). Conversely, although a study by Hongyo et al (1995), in a high incidence area of gastric cancer in Italy, detected G:C to A:T transitions in 93% (26 of 28) of cases with single-base substitutions, only 18% (5 of 28) occurred at CpG dinucleotides. The different mutation spectrum in the study of Hongyo et al (1995) may reflect site-specific differences (cardia vs antrum) or regional exposure to particular environmental agents.

It is generally accepted that the majority of missense mutations generate a mutant protein with increased protein stability, thereby facilitating detection by immunohistochemical techniques (Cripps et al, 1994). Consistent with this, a highly significant association was observed between missense mutation of the p53 gene and diffuse p53 protein expression in both oesophageal and gastric adenocarcinoma, with all 31 cases with missense mutations exhibiting diffuse protein expression. A previous study of oesophageal adenocarcinoma also reported a close correlation between missense mutation and p53 protein expression (100% agreement, 10 of 10) (Hamelin et al, 1994).

In the present study, there were six cases with diffuse p53 protein expression that were not found to contain a mutation in exons 5–8 of the p53 gene. There are a number of possibilities that may account for the apparent discordance in these samples. Firstly, mutations may have been missed by the screening techniques used in the present study. Secondly, missense mutations may lie in regions of the gene not screened in the present study. A review of 50 studies that carried out sequencing of the entire coding region of the p53 gene reported that 13% of mutations, up to 30% of which were missense mutations, were located outside exons 5–8 (Greenblatt et al, 1994). A third possibility is that mechanisms other than mutation resulted in the inactivation and stabilization of p53 protein. Binding to viral oncoproteins, e.g. the adenovirus E1B 55-kb protein, and cellular oncoproteins, e.g. mdm2, has been shown to result in p53 stabilization and in the inactivation of wild-type p53 function (Yew and Berk, 1992; Wu et al, 1993). In these instances, p53 overexpression represents the functional, but not the structural, inactivation of the p53 gene. In this respect, it was suggested that ‘false positives’ may not be misleading in the biological sense if the stabilization of p53 occurs via a mechanism that also abolishes its function (Wyntford-Thomas, 1992).

None of 21 tumours with focal or negative immunostaining were found to have a missense mutation, suggesting that focal immunopositivity is not an indicator of mutational stabilization of the p53 protein. However, truncating p53 mutations (non-sense mutations, insertions/deletions and splice site mutations) were detected in 14% (2 of 14) of oesophageal adenocarcinomas and in 27% (7 of 26) of gastric adenocarcinomas studied. All nine truncating mutations were immunonegative. These data indicate that a proportion of immunonegative tumours may contain a mutation that does not result in protein stabilization. It has been reported that truncating mutations may account for as much as 37.5% of mutations in oesophageal adenocarcinoma (Hamelin et al, 1994) and 27% of mutations in gastric adenocarcinoma (Renault et al, 1993; Uchino et al, 1993). In these cases, immunohistochemical assessment of p53 protein expression as a sole indicator of the presence of p53 gene mutation would result in an underestimation of mutation frequency (false negatives). The observation of
apparent discordance between molecular and immunohistochemical analysis of p53 gene mutation indicates that such techniques should be regarded as complementary.

The acquisition of chromosomal rearrangements following the loss of p53 function has been observed in a number of in vitro systems (Livingstone et al, 1992; Yin et al, 1992). This suggests a potential association between p53 gene abnormalities and the development of genetic instability (Livingstone et al, 1992). Evidence to support this hypothesis was provided by studies on colorectal carcinoma in which DNA aneuploidy was shown to be associated with 17p allelic loss (Offerhaus et al, 1992) and increased p53 protein expression (Carter et al, 1993). Furthermore, Carter et al (1993) reported that the mean DNA index in aneuploid tumours with stabilized p53 protein was significantly higher than that in those aneuploid cases without stabilized protein. In this study, statistical analysis demonstrated evidence of an association between the presence of 17p allelic loss and DNA aneuploidy (Fisher exact test, \( P = 0.02 \)). However, the presence of p53 gene mutation or p53 protein expression was not found to be significantly associated with the occurrence of DNA aneuploidy in oesophageal and gastric adenocarcinoma. Furthermore, the mean DNA index in aneuploid tumours with a p53 mutation was not significantly higher than that in aneuploid cases without a p53 gene mutation. It is uncertain to what extent the association between 17p allelic loss and abnormal DNA content reflects the involvement of p53 inactivation in the development or establishment of aneuploid cell populations.

For both the oesophageal and gastric tumour series studied, neither 17p allelic loss, p53 gene mutation, p53 protein expression nor DNA aneuploidy exhibited an association with either tumour stage or histological subtype. This may reflect, in part, the predominantly advanced stage of tumours in the present series. Similarly, other studies on gastric adenocarcinoma have reported no association between DNA ploidy and depth of tumour invasion or lymph node metastasis (Yonemura et al, 1992; Brito et al, 1993). Studies have reported an equal prevalence of p53 mutations in early- and late-stage gastric carcinoma (Uchino et al, 1993; Hongyo et al, 1995), and one study has reported the detection of the same p53 mutation in both tumour and adjacent dysplastic epithelium in five cases of gastric adenocarcinoma (Shiao et al, 1994). In oesophageal adenocarcinoma, no association has been reported between p53 protein expression and tumour differentiation grade, tumour stage (Flejou et al, 1993; Krishnadath et al, 1995) or lymph node status (Flejou et al, 1993). Allelic loss on 17p, p53 gene mutation and p53 protein expression have been documented in dysplastic Barrett’s epithelium (Flejou et al, 1993; Blount et al, 1994; Hardwick et al, 1994) and in both early and advanced adenocarcinomas (Blount et al, 1991; Flejou et al, 1993; Hamelin et al, 1994). These data suggest that p53 gene abnormalities may occur early in the development of oesophageal adenocarcinoma. The value of p53 as an intermediate biomarker in the identification of patients at increased risk of malignant transformation in Barrett’s oesophagus awaits the outcome of prospective follow-up studies.

In conclusion, the present study demonstrated uniformly high levels of p53 gene abnormalities and DNA aneuploidy in adenocarcinoma of the oesophagus and gastric cardia. Both oesophageal and gastric tumours exhibited similar p53 mutation profiles, with both tumour types being associated with a high frequency of base transitions at CpG dinucleotides. The detection of similar molecular alterations in adenocarcinoma of the oesophagus and gastric cardia is consistent with the observation that these tumours also exhibit similar clinical and epidemiological features. There was a highly significant association between p53 missense mutation and the presence of diffuse p53 protein expression in these tumours, supporting the rationale for using immunohistochemical methods as an indicator of missense mutation. However, truncating mutations, which may account for a significant proportion of p53 mutations in oesophageal and gastric tumours, cannot be detected by immunohistochemical techniques. Allelic loss on 17p, p53 gene mutation and p53 protein expression were each detected in premalignant Barrett’s epithelium. Future prospective studies should determine the clinical relevance of p53 gene alterations as an independent marker of Barrett’s patients at an increased risk of neoplastic transformation.

**ABBREVIATIONS**

LOH, loss of heterozygosity; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism

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