Expression of p53 and bcl-2 and response to preoperative chemotherapy and radiotherapy for locally advanced squamous cell carcinoma of the oesophagus

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Abstract

Aims—To investigate the immunohistochemical expression of p53 and bcl-2 proteins in squamous cell carcinoma (SCC) of the oesophagus and to assess whether expression of these oncoproteins can be used to stratify patients into groups with a favourable or unfavourable response to preoperative chemo/radiotherapy.

Methods—The initial diagnostic biopsy and the corresponding resected samples were obtained from 22 consecutive patients with SCC. All patients underwent preoperative chemo/radiotherapy. Tumour sections were incubated with a monoclonal antibody directed against p53 (DO-7). Twenty four non-neoplastic oesophageal biopsy specimens immunostained for p53 served as controls. Twelve randomly chosen sections from the 22 SCC samples were immunostained to test for bcl-2 protein expression.

Results—After chemo/radiotherapy, 12 (55%) of the 22 patients had no evidence of tumour in the resected oesophagus. Before chemoradiotherapy, however, 17 (77%) patients were p53 positive. After treatment, residual carcinoma was detected in seven (41%) of the 17 p53 positive patients. All non-responsive cases had the same p53 immunopattern as before treatment. Bcl-2 immunoprotein expression was detected in six (50%) of 12 patients. Residual tumour was detected in the residual oesophagus in two (33%) of the six bcl-2 positive patients. After treatment, bcl-2 expression was no longer detected in the residual neoplastic cells of a previously bcl-2 positive tumour. Using Fisher's exact test no significant association was found between oncoprotein expression and response to preoperative treatment.

Conclusion—This study confirms the observation that p53 protein is frequently expressed in SCCs of the oesophagus, probably as a result of a mutation of the TP53 gene. However, no significant association was found between oncoprotein expression and response to chemo/radiotherapy. Anticancer agents do not seem to modify the expression of p53 and bcl-2 proteins.

Keywords: p53, bcl-2, immunohistochemistry, squamous cell carcinoma of the oesophagus, preoperative chemo/radiotherapy.

Response or resistance of neoplastic cells to cancer therapy depends on the presence of a wide variety of mechanisms that, respectively, can induce or prevent cell death.¹⁻³ A recent report by Lowe *et al*⁴ suggested that the effect of antitumour agents, such as ionising radiation and the common anticancer drugs, could be modulated by cellular propensity to undergo apoptosis. The term apoptosis was originally coined by Kerr *et al*⁵ to describe an active model of cell death, the regulation of which depends on specific genes that take part in this hierarchical process.⁶⁻⁸ The p53 and bcl-2 genes, in particular, may be regarded, respectively, as positive and negative regulators of apoptosis, but other genes are certainly involved. As a consequence, loss of function of the p53 gene and gain of function of the bcl-2 gene could produce tumours resistant to both chemo- and radiotherapy. Recent studies have reported the association of a dysregulated oncogene and expression of a tumour suppressor gene with a poor response to antitumour therapy.⁹⁻¹³

In the present study we investigated the relation between immunohistochemical expression of the p53 and bcl-2 proteins and the rate of tumour response after neoadjuvant chemotherapy and radiotherapy in locally advanced squamous cell carcinoma (SCC) of the oesophagus, and whether antitumour treatment could modify the immunohistochemical expression of these oncoproteins.

Methods

Between January 1993 and June 1994, 22 consecutive patients with SCC of the oesophagus, confirmed by biopsy, were enrolled into the study. To be eligible, disease had to be limited to the oesophagus and regional lymph nodes (stage II-III according to the staging criteria of the American Joint Committee), with no evidence of distant metastases or involvement of the major structures that would preclude removal of tumour. Patients were required to have a Karnofsky performance status of at least 60%. All patients gave informed consent. All patients received preoperative chemotherapy (cis-platinum 20 mg/m²/day and 5-fluorouracil 600 mg/m²/day by continuous intravenous

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Figure 1 Squamous cell carcinoma of the oesophagus: intense p53 immunoreactivity is evident in the majority of nuclei (original magnification $\times 200$).

Figure 2 Immunostaining for p53 in the basal layer of non-neoplastic oesophageal squamous cell epithelium (original magnification $\times 50$).

Figure 3 Malignant cells showing diffuse cytoplasmic immunostaining for bcl-2 protein in a SCC of the oesophagus (original magnification $\times 200$).

infusion, D 1–5, 29–33) and radiotherapy with a cumulative dose of 36 Gy. The average interval between preoperative chemo/radiotherapy and surgery was 52.2 days (range 29–241 days). The patient population comprised 21 men and one woman aged between 41 and 71 years (mean 58.4 years).

After completing preoperative treatment, the patients underwent oesophagectomy and the surgically resected specimens were examined for histological features and for response to chemo/radiotherapy. Quantitation of the change in tumour size was not made because of the difficulty in obtaining accurate serial measurements from radiographic studies. After resection, patients were categorised as either complete responders or as having residual tumour in the resected specimen.

Paraffin wax embedded tissue blocks of formalin fixed biopsy and surgically resected specimens were processed for conventional histopathological analysis (haematoxylin and eosin) and for immunohistochemical staining. Each block was serially sectioned at 5 μ m. Immunohistochemistry for p53 was carried out on the 22 initial diagnostic biopsy specimens and the surgically resected samples (tumour free resection margins included) of the same cases. Twenty four oesophageal biopsy specimens served as controls. The control population comprised 17 men and seven women with a mean age of 56.7 years (range 17–88 years). The control biopsy specimens consisted of normal epithelium (two cases), acanthosis (nine cases), and inflammatory changes (13 cases).

All sections were immunostained for p53 protein using the avidin-biotin peroxidase complex (ABC) method. After rehydration and blocking of endogenous peroxidase activity, the sections were heated in 10 mmol citrate buffer for two minutes in a microwave oven at 300 W and for seven minutes at 100 W. The slides were incubated overnight in primary mouse antibody DO-7, diluted 1 in 300 in phosphate buffered saline (PBS) and washed in PBS. After a second incubation with a biotinylated rabbit anti-mouse antibody, the sections were treated with the avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, California, USA). The primary antibody reacts with wild-type and mutant p53 protein. Sections with intense nuclear staining in at least 10% of neoplastic cells (fig 1) were regarded as positive. Sections in which immunoreactive cells were identified in the basal proliferative layers of the non-neoplastic epithelium only were recorded as basal (fig 2).

Twelve of the 22 cases were randomly chosen and immunostained for bcl-2 protein. Intracytoplasmic staining of more than 10% of the cells was recorded as positive (fig 3). The staining pattern was not specified because distinctive patterns were not observed.

The slides were examined blindly by two pathologists and scored for p53 and bcl-2 expression. Each pathologist looked at all of the slides including controls and, following comparison of individual assessments, an agreed score for each case was reached by dis-

Table 1 Relation between p53 and bcl-2 immunoreactivity and rate of tumour response (Fisher's exact test = not significant)

Pattern of immunostaining	p53 immunoreactivity before treatment (n = 22)	Residual carcinoma at surgery	
		Yes	No
Positive	17	7	10
Negative	5	3	2
	bcl-2 immunoreactivity before treatment (n = 12)		
Positive	6	2*	4
Negative	6	1	5

*bcl-2 expression disappeared in the residual neoplastic cells of a previously bcl-2 positive tumour.

Table 2 p53 staining pattern in oesophageal squamous epithelium of resected specimens

	Tumour-free cases (n=12)	Cases with residual tumour (n=10)		
		Tumour cells	Non-neoplastic cells	Resection margins (n=22)
Positive	0	7	0	0
Basal	4	0	5	13
Negative	8	3	5	9

cussion, if necessary. Both pathologists were unaware of the response of each tumour to chemo/radiotherapy when assessing the preoperative biopsy specimens for oncoprotein expression.

Fisher's exact probability test was used to evaluate associations between oncoprotein expression in the initial diagnostic biopsy specimens and the response of the tumours to chemo/radiotherapy.

Results

On histopathological analysis, residual tumour was detected in the surgically resected specimens of 10 (45%) of the 22 patients. Table 1 shows the correlation between p53 and bcl-2 immunoreactivity in the diagnostic biopsy samples and their response to cytotoxic treatment. Before chemoradiation, 17 (77%) of 22 tumours immunostained positively for the p53 protein, of which seven had residual tumour in the resected oesophagus. Three of the five tumours in which p53 expression was not detected in the biopsy specimens did not respond to preoperative treatment. Furthermore, each of the non-responsive cases showed the same pattern of p53 immunoreactivity both before and after chemo/radiotherapy. Table 2 shows p53 immunostaining in oesophageal squamous epithelium of surgical specimens. Of the 12 cases in which residual tumour was not observed at surgery, non-neoplastic epithelium of four (33%) patients showed a basal immunopattern, whereas in the other eight (67%) p53 protein expression was not detected. The pattern of expression of p53 in the squamous epithelium in tumour-free resection margins was negative in nine (41%) patients and basal in 13 (59%). Finally, the

Table 3 p53 immunostaining in non-neoplastic epithelium of the 24 control sections

Pattern of p53 immunostaining	Normal epithelium (n=2)	Acanthosis (n=9)	Inflammatory changes (n=13)
Positive	0	0	0
Basal	1	8	7
Negative	1	1	6

pattern of p53 protein expression was basal in 16 (67%) of the 24 controls (one normal epithelium, eight acanthosis, seven inflammatory changes) and was not detected in the other eight (table 3).

Prior to preoperative chemo/radiotherapy, cytoplasmic immunostaining of bcl-2 protein was detected in six (50%) of the 12 tumour samples in which protein expression was evaluated. Residual tumour was detected following preoperative treatment in two of the six patients with bcl-2 positive biopsy samples. Only one bcl-2 negative tumour did not respond to treatment (table 1).

No statistically significant association was found between preoperative expression of p53 and bcl-2 proteins and response rates.

Discussion

In the present study p53 positive immunostaining was detected in biopsy specimens of most patients, suggesting the presence of p53 gene mutations. Recent studies have reported a good correlation between p53 overexpression, as detected by immunohistochemistry, and the presence of p53 gene mutations in SCC of the oesophagus.^{14 15} We also evaluated p53 expression after preoperative chemo/radiotherapy and observed no differences before and after treatment. The pattern of immunostaining in all patients with residual carcinoma was the same both before and after preoperative treatment.

As expected, the immunostaining pattern observed in controls was very similar to that of the non-neoplastic oesophageal epithelium in responsive cases. Furthermore, diffuse p53 immunolocalisation was seen in tumour tissue only, whereas immunostaining of the basal layer of the epithelium seemed to be characteristic of non-neoplastic oesophageal epithelium. A similar pattern of immunostaining in tumour-free oesophageal mucosa has been reported by others.¹⁶⁻¹⁸ These data can be explained by considering the mechanisms underlying p53 expression. Initially, it was thought that all immunohistochemically detected p53 protein was mutant. Recent evidence suggests, however, that both wild-type and mutant p53 protein can be detected by immunohistochemistry. In fact, whereas the mutant form is detectable because of its longer half-life, normal protein can be detected when stabilised by interacting with cellular proteins (for example, the product of the mdm2 gene)¹⁹ or in response to some toxic insults.^{20 21} p53 is regarded as a "guardian of the genome" because the wild-type protein protects the genome against mutations and prevents the development of abnormal cell clones.²⁰⁻²³ Therefore, we suggest that p53 accumulation in the basal layer of the oesophageal epithelium represents the expression of a local response to an insult(s) and/or is an early event in oesophageal carcinogenesis, as suggested by other authors.^{16 18}

Recent studies have emphasised that apoptosis occurs after cellular damage is induced by antitumour agents.^{4 11} However, damaged tumour cells harbouring mutant p53 protein

could escape apoptosis and therefore could produce chemotherapy and radiotherapy resistant clones; tumours harbouring p53 mutations are generally insensitive to treatment. Conversely, tumours with few p53 mutations frequently respond well to treatment.³ In the present series, there was no significant difference in response to preoperative treatment by tumours with and without p53 mutations. This observation emphasises that other mechanisms are certainly involved in determining tumour cell sensitivity to treatment. It is also difficult to predict treatment response on the basis of a negative p53 staining pattern. The absence of p53 on immunostaining may suggest the presence of the wild-type protein and these tumours would be expected to respond well to chemo/radiotherapy. Conversely, tumours in which both p53 alleles have been deleted or with a mutation that introduces a stop codon could respond poorly to therapy, although they would have a negative immunostaining pattern.

Apoptosis is regulated by several genes. The bcl-2 gene, in particular, seems to act as a negative regulator of cell death with a possible downstream effect on p53 dependent apoptosis.²⁴ Interestingly, of the three non-responsive cases in which bcl-2 protein expression was evaluated, two had a bcl-2 positive biopsy specimens. This suggests that the tumour cells may be resistant to the action of ionising radiation and cytotoxic drugs, which depends on bcl-2 anti-apoptotic activity that by-passes the induction of apoptosis by p53. However, the number of cases in the present study was too few to permit further testing of this hypothesis.

In conclusion, this study confirms the observation that p53 protein is frequently expressed in SCCs of the oesophagus, probably as a result of a mutation of the TP53 gene. However, no significant association was found between oncoprotein expression and response to chemo/radiotherapy. Anticancer agents do not seem to modify the expression of p53 and bcl-2 proteins.

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