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Original Paper

Pre-operative Chemotherapy for Squamous Cell Carcinoma of the Oesophagus: Do Histological Assessment and p53 Over-expression Predict Chemo-responsiveness?

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Pre-operative chemotherapy is increasingly used in the treatment of oesophageal carcinoma. However, no features have been identified which can reliably predict a positive response to chemotherapy. The aim of this study was to examine whether histological features and p53 overexpression could predict such response. Prechemotherapy endoscopic biopsies from 55 patients, who subsequently completed two courses of chemotherapy followed by surgical resection, were studied. Patients were classified into responders and non-responders according to clinical and pathological findings. Pathological features of the endoscopic biopsies examined included adequacy of the tumour tissue, histological grade, degree of keratinisation, histologic patterns, mitotic rates and nuclear pleomorphism. Biopsy specimens were also tested for p53 overexpression using p53 protein specific mouse monoclonal antibody DO-7 on paraffin sections. Histologic features and p53 expression were correlated to chemoresponsiveness. 76% (42 of 55) of patients had sufficient biopsy tissue for assessment. Response to chemotherapy was evident in 64% ($n = 27$) of patients. None of the non-responders had tumours with high-grade nuclear pleomorphism compared with 37% (10 of 27) of responders ($P = 0.01$). All patients with high-grade nuclear pleomorphism responded to chemotherapy. No significant differences were found between the responders and non-responders with respect to tumour differentiation ($P = 0.7$), degree of keratinisation ($P = 0.3$) and mitotic rates ($P = 0.8$). Overall, p53 overexpression was noted in 67% (28 of 42) of patients. This was more prevalent in non-responders (12/15) compared to responders (16/27), but this was not statistically significant ($P = 0.08$). The degree of p53 overexpression had no significant relationship with responsiveness to chemotherapy. High-grade nuclear pleomorphism, identified on pretreatment biopsy specimens, correlated with response to chemotherapy, whereas p53 overexpression did not correlate with response. Improved tissue sampling and further investigations should be done so that the assessment of prechemotherapeutic endoscopic biopsies can have significant impact on clinical decision making. © **1997 Published by Elsevier Science Ltd.**

Key words: oesophageal carcinoma, chemotherapy, p53, histology

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INTRODUCTION

SQUAMOUS CELL carcinoma of the oesophagus is an aggressive malignancy [1]. Advances in anaesthesia, risk analysis and operative techniques have led to only modest improvement in the overall prognosis of oesophageal cancer over the last two decades [2, 3]. Although surgery remains the standard treatment for oesophageal cancer, a variety of combined modality treatments have been investigated in order to induce down-staging of the tumour, improve local control, treat micrometastases, and ultimately improve long-term survival [1, 4]. Induction chemotherapy given before surgery has been shown to improve outcome in subgroups of patients [5]. However, no indicators exist which can reliably predict response to chemotherapy. It is important to

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identify such patients so that unnecessary, potentially harmful treatment is not given to those who will not benefit.

Histological grading of oesophageal tumours has been used as a prognostic indicator in patients treated with combined radiotherapy and surgery [6, 7]. In other cancers, *P53* mutations are associated with resistance to chemotherapeutic drugs since p53-dependent apoptosis modulates the cytotoxic effects of common antineoplastic agents [8]. Cells without wild type p53 are resistant to chemotherapy, while cells with wild type p53 are sensitive to drugs and will undergo apoptotic cell death. Also, chemotherapy resistance, in particular 5-fluorouracil (5-FU) resistance, may be closely involved with cellular expression of the 5-FU target enzyme, thymidylate synthase, as increased expression of thymidylate synthase may predict 5-FU resistance [9].

The relationship of *P53* mutations to chemoresponsiveness has not been studied in patients with oesophageal cancer treated by pre-operative chemotherapy. The aim of this study was to assess the feasibility of using histopathological features and p53 protein overexpression in pretreatment endoscopic biopsy specimens to predict response to chemotherapy in patients with oesophageal squamous cell carcinoma.

PATIENTS AND METHODS

Between December 1989 and January 1995, patients with primary oesophageal squamous cell carcinoma managed at the Department of Surgery, Queen Mary Hospital, The University of Hong Kong, were recruited in a prospective randomised controlled trial comparing pre-operative chemotherapy and surgery alone. Patients randomised in the pre-operative chemotherapy arm were analysed in this study. A total of 55 patients completed two courses of chemotherapy and prechemotherapy endoscopic biopsies and resected specimens were available for assessment.

Chemotherapy regime

The treatment protocol consisted of two courses of cisplatin and 5-FU being given to the patients 22–26 days apart. The patients were hydrated prior to commencement of the chemotherapy. For forced diuresis, 20% mannitol was given intravenously for the first day. Cisplatin (100 mg/m^2) was given as an intravenous infusion over 4 h on day 1, followed by 5-FU (500 mg/day^2/day) infusion over days 1–5. Appropriate anti-emetogenic medications were prescribed.

Management of response

The response to pre-operative therapy was assessed by both clinical (endoscopic and radiological examination) and pathological means. The patients were divided into responders and non-responders. Responders were graded as: (1) MR—minimal responder: less than 50% reduction in the size of tumours; (2) PR—partial responder: ≥50% reduction in the size but tumours still evident; (3) CR—clinical complete responders: clinical disappearance of the tumours and (4) pCR—pathological complete responders: absence of any tumour on pathological examination of the surgical specimens. The non-responders (NR) were defined as patients with disease progression or the tumour had no objective reduction in size.

Histological assessment

Histological sections from the endoscopic biopsies of oesophageal cancer before pre-operative chemotherapy were retrieved and reviewed. The size of the biopsies ranged from 0.1 to 0.3 cm at the greatest dimension and the number of pieces from each patient ranged from 1 to 3. These were first examined to determine whether the amount of cancer tissue identified was adequate for histological and immunohistochemical study. The selected cases were graded into well, moderately or poorly differentiated squamous cell carcinoma according to the World Health Organization (WHO) recommendations [10]. The other histological features analysed were simplified from those proposed by Hambraeus and associates [6]. The parameters examined were histological patterns, tendency to keratinisation, nuclear polymorphism and mitotic rates. Each of these morphological parameters was divided into two grades, high and low. High-grade features were infiltrative pattern, minimal or no keratinisation, marked nuclear pleomorphism (ratio of the largest: smallest nuclei was eight or more) and ≥5 mitotic figures/section. The low-grade features were cohesive pattern, moderately to highly keratinised, mild to moderate nuclear pleomorphism and <5 mitotic figures/section.

Immunohistochemical study

From the chosen paraffin blocks, 5 μm sections were cut. They were deparaffinised with xylene and rehydrated through graded concentration of alcohol. They were then treated in a microwave at 95°C for 14 min in 10 mM citrate buffer at pH 6. The sections were washed in water, rinsed with Tris-buffered saline (TBS) and treated with 3% hydrogen peroxide in methanol for 10 min at room temperature to block the intrinsic peroxidase activity. They were again washed with water rinsed with TBS. 10% normal rabbit serum was then added at room temperature for 10 min. Primary mouse monoclonal anti-p53 antibody (NCL-p53-DO7 from Novocastra Laboratories Ltd, Newcastle upon Tyne, U.K.) was then added at a dilution of 1:100 and incubated in a moist chamber overnight at 4°C. The antibody recognises both wild type and mutant p53. The slides were again washed three times in TBS for 3 min. Rabbit anti- mouse IgG-biotinylated (diluted in 10% normal rabbit serum; E354 from Dako, Gloustrup, Denmark) and preincubated (30 min at room temperature) avidin–biotin complex (1:100; Amersham, Buckinghamshire, U.K.) were added for 30 min at 37°C. The slides were washed in TBS as before and were then developed in freshly prepared DAB/H_2O_2 solution for 10 min at room temperature. Afterwards, the sections were washed in water, counterstained with Mayer's haematoxylin for 1 min at room temperature, dehydrated, cleared and mounted. Negative controls were sections treated the same as above but with omission of the primary antibody. A block of squamous cell carcinoma known to be strongly positive for p53 protein (obtained in our previous study) was used as the positive control [11]. The degree of p53 accumulation was graded semiquantitatively into four categories (0, +, + +, + + +) as described in our previous studies [11].

Statistical analysis

The data were computerised and statistical tests were performed with the program Statistical Package for Social Sciences (SPSS-X version 3.1, Chicago, Illinois, U.S.A.). Other tests used were the chi-square test, Fisher's test and Student's *t*-test. The tests were considered significant when the *P* value was less than 0.05.

Table 1. The relationship between histological features and responsiveness to pre-operative chemotherapy

Pathological features	Clinical groups					Significant test
	NR	MR	PR	CR	pCR	
Differentiation						
well	1	0	3	1	1	$P = 0.7$ (chi-square test)
moderately	12	2	13	1	2	
poorly	2	1	2	1	0	
Keratinisation						
prominent	4	0	7	2	2	$P = 0.3$ (chi-square test)
scanty	11	3	11	1	1	
Mitotic count						
low	5	1	4	0	1	$P = 0.8$ (chi-square test)
high	10	2	14	3	2	
Histological pattern						
cohesive	12	1	14	2	2	$P = 0.5$ (chi-square test)
infiltrative	3	2	4	1	1	
Nuclear pleomorphism						
low	15	1	12	3	1	$P = 0.01$ (chi-square test)
high	0	2	6	0	2	

NR, non-responder; MR, minimal response; PR, partial response; CR, complete clinical response; pCR, complete pathological response.

RESULTS

In 13 of the 55 patients, the endoscopic biopsies were not suitable for further histological grading and immunohistochemistry because the quantity of these specimens, though adequate for the diagnosis of carcinoma, was not enough for further histological assessment and immunohistochemical studies. Therefore, 42 patients (35 males, 7 females) were studied in detail.

The mean age of the 42 patients was 65 years (range 47–79). 14% ($n = 6$) of the squamous cell carcinoma were well-differentiated, 71% ($n = 30$) were moderately differentiated and 14% ($n = 6$) poorly differentiated. 36% (15) of patients were non-responders. Response was seen in 64% (27) of patients: pCR was seen in 3 patients, CR in 3, PR in 18 and MR in 3.

The relationship between histologic features before chemotherapy and responsiveness to chemotherapy are listed in Table 1. It was noted that none of the non-responders had tumours with high-grade nuclear pleomorphism compared with 37% (10 of 27) of responders, $P = 0.01$ (Figures 1 and 2). The sensitivity, specificity and positive predictive value of high-grade nuclear pleomorphism in relation to a positive response to chemotherapy were 37%, 100% and 100%, respectively. Other pathological features such as tumour differentiation, degree of keratinisation, mitotic figures and pattern of infiltration had no relationship with responsiveness to chemotherapy.

p53 overexpression was noted in 67% (28 out of 42) of patients (Figure 3). More intense p53 staining was seen in the poorly differentiated tumour cells, while the better differentiated foci had weaker staining. The relationship of p53 overexpression with various clinicopathologic parameters are shown in Table 2. The features examined had no relationship with either the presence of p53 overexpression or the degree of p53 staining. Although p53 overexpression was more prevalent in non-responders compared to responders, at 80% and 59%, respectively, the difference was not statistically significant, $P = 0.08$.

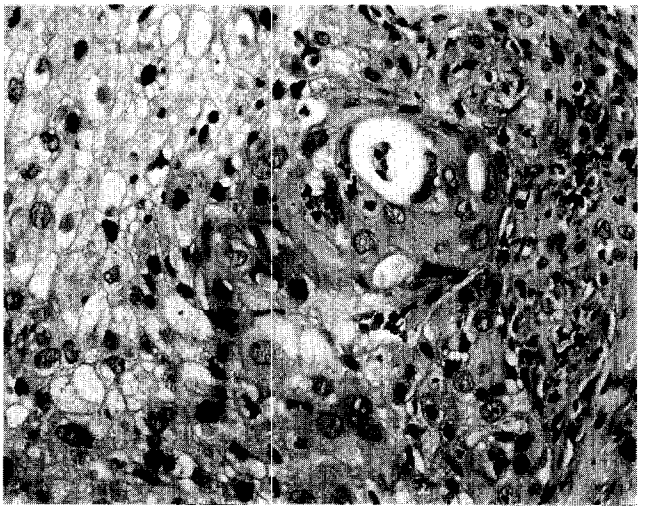

Figure 1. Squamous cell carcinoma of low-grade nuclear pleomorphism and cohesive pattern in a non-responder (haematoxylin & eosin × 360).

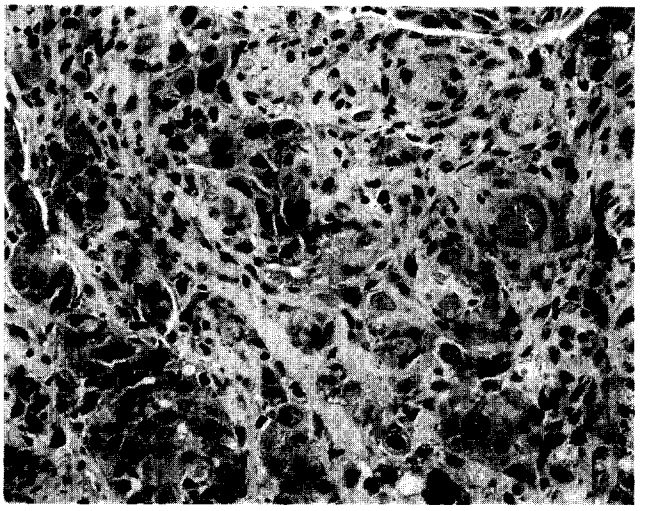

Figure 2. Squamous cell carcinoma of high-grade nuclear pleomorphism and infiltrative pattern in a responder (haematoxylin & eosin × 360).

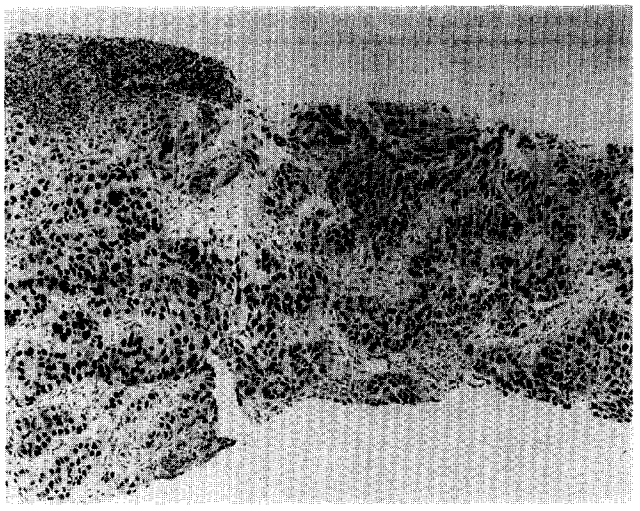

Figure 3. Squamous cell carcinoma that shows positive nuclear staining to p53 in a non-responder (×150).

DISCUSSION

The pathological effects of chemotherapy in oesophageal carcinoma have been studied as investigations on neoadjuvant chemotherapy have gained importance in recent years [12–14]. In subgroups of patients, response to chemotherapy pre-operatively has been associated with better long-term survival. It is important to identify such patients so that unnecessary, potentially harmful treatment is not given to those who are unlikely to benefit.

The survival of patients with oesophageal carcinoma depends on various factors [1]. Thus, we use responsiveness to chemotherapy rather than survival in order to study precisely the effect of chemotherapy. Also, only pretreatment biopsies were studied as the morphology of the post-treatment biopsies are affected by the therapy.

The value of pathological features in pretreatment biopsies in predicting the responsiveness to chemotherapy have never been studied. Hambraeus and associates used a complicated histological grading system to assess the survival of patients with oesophageal squamous cell carcinoma treated by combined radiotherapy and surgery [6]. They suggested that histological parameters were useful prognostic factors. The results, however, were not supported by the findings of Lewinski and associates, who studied 9 patients managed in a similar manner [7]. In the present study, it was demonstrated that only nuclear pleomorphism correlated with the responsiveness of oesophageal squamous cell carcinoma to pre-operative chemotherapy, with high-grade pleomorphism reliably predicting responsiveness to chemotherapy.

For p53 expression, it has been postulated that wild type p53 may induce tumour cells to undergo programmed cell death [8]. Tumour cells lacking wild type p53 are often resistant to chemotherapeutic agents and radiotherapy, whereas tumour cells expressing wild type p53 are sensitive to these antitumour agents. The value of this information on clinical practice remains undetermined. Clinical correlation is seen in carcinomas of the testis, Wilms' tumour, and lymphoid tumour. These tumours often respond well to chemotherapy and rarely manifest *P53* mutations [15].

There has been no previous study which analysed the role of *P53* mutations in predicting the responsiveness of oesophageal squamous cell carcinoma to chemotherapy. In this study, p53 overexpression occurred more frequently in non-responders than in responders, although the difference was not statistically significant. This non-significant relationship

Table 2. Relationship between p53 overexpression and clinicopathological parameters

	p53 positive					Statistical test	
Parameters	+	++	+++	(total)	p53 negative	positive versus negative	+ versus ++/+++
Differentiation							
well	1	2	1	4	2	$P = 1$ (chi-square test)	$P = 0.3$ (Fisher's exact test)
moderately	1	6	13	20	10		
poor	0	0	4	4	2		
Keratinisation							
prominent	1	4	4	9	6	$P = 0.5$ (chi-square test)	$P = 1$ (Fisher's exact test)
scanty	1	4	14	19	8		
Mitotic count							
high	1	2	5	8	3	$P = 0.5$ (Fisher's exact test)	$P = 0.5$ (Fisher's exact test)
low	1	6	13	20	11		
Histological pattern							
cohesive	2	5	12	19	12	$P = 0.3$ (Fisher's exact test)	$P = 1$ (Fisher's exact test)
infiltrative	0	3	6	9	2		
Nuclear pleomorphism							
high	1	7	12	20	12	$P = 0.5$ (Fisher's exact test)	$P = 0.5$ (Fisher's exact test)
low	1	1	6	8	2		
Clinical response							
NR	0	5	7	12	3	$P = 0.08$ (chi-square test)	$P = 0.3$ (chi-square test)
MR	0	0	2	2	1		
PR	2	0	6	8	10		
CR	0	1	2	3	0		
pCP	0	2	1	3	0		

NR, non-responder; MR, minimal response; PR, partial response; CR, complete clinical response; pCR complete pathological response.
+, 10–30% of the tumour cells were positive.
++, 30–50% of the tumour cells were positive.
+++, more than 50% of the tumour cells were positive.

may be due to a type II statistical error as the size of the sample was small. In addition, neither the prevalence of p53 overexpression nor its staining intensity correlated with chemoresponsiveness. It could also be argued that p53 overexpression is not synonymous with *P53* mutations [16]. Sometimes, overestimation of *P53* mutations by immunohistochemistry could be related to mechanisms such as inactivation of an enzymatic pathway responsible for p53 degradation. However, in practice, there was often insufficient tissue taken by endoscopic biopsy to perform studies on p53.

In the current study, 67% of cases were found to be positive for p53 immunohistochemistry. It is worth noting that we have reviewed the studies regarding p53 overexpression in squamous cell carcinomas in the literature in our previous study (ranging from 30 to 85% of cases) [11]. The wide range of positivity may be related to the number of cases studied, the type of antibodies and the methods employed.

The application of histological grading and p53 overexpression studies of prechemotherapeutic biopsies has limitations. The routine biopsies taken were adequate for the diagnosis of carcinoma, but not sufficient for histological grading in 24% of patients. In addition, it was difficult to ascertain if the histology of the biopsies was representative as regional variation of pathological features existed in many tumours. Thus, it is important to obtain large size biopsies from different sites of the tumour in order to reduce sampling error and improve the predictive value. Furthermore, other studies of chemosensitivity, ploidy, and markers such as epidermal growth factor receptor and proliferating cell nuclear antigen, may be potentially useful for predicting chemoresponsiveness [17–19].

In conclusion, high-grade nuclear pleomorphism, identified on pretreatment biopsy specimens, correlated with response to chemotherapy, although because of the limited sample size, this result needs to be confirmed in further investigations. Markers of chemoresponsiveness need to be identified which could have significant impact on clinical decision making.

1. Lam KY, Ma LT, Wong J. Measurement of extent of spread of oesophageal squamous carcinoma by serial sectioning. *J Clin Pathol* 1996, **49**, 124–129.
2. Earlam R, Cunha-Melo JR. Oesophageal squamous cell carcinoma: I A critical review of surgery. *Br J Surg* 1980, **67**, 381–390.
3. Muller JM, Erasmi H, Stelzner M, Zieren U, Pichlmaier H. Surgical therapy of oesophageal carcinoma. *Br J Surg* 1990, 77, 845–857.
4. Kelen D. Neoadjuvant therapy of esophageal cancer. *Can J Surg* 1989, **32**, 410–414.
5. Fink U, Stein HJ, Bochtler H, Roder JD, Wilke HJ, Siewert JR. Neoadjuvant therapy for squamous cell esophageal carcinoma. *Ann Oncol* 1994, **5**, 517.
6. Hambraeus GM, Mercke CE, Willen R, *et al.* Prognostic factors influencing survival in combined radiotherapy and surgery of squamous cell carcinoma of the esophagus with special reference to a histopathologic grading system. *Cancer* 1988, **62**, 895–904.
7. Lewinski T, Morysiński T, Pietrow D, Sikora K. Preoperative radiotherapy combined with resection of squamous cell carcinoma of the mid-thoracic oesophagus. Does a histopathologic malignancy grading system assess actual survival? *Eur J Surg Oncol* 1991, **17**, 571–574.
8. Lowe SW, Ruley HE, Jacks T, Houseman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993, **74**, 957–967.
9. Harwood FG, Frazier MW, Krajewski S, Reed JC, Houghton JA. Acute and delayed apoptosis induced by thymidine deprivation correlates with expression of p53 and p53-regulated genes in colon carcinoma cells. *Oncogene* 1996, **12**, 2057–2067.
10. Watanabe H, Jass JR, Sobin LH, in collaboration with pathologists in eight countries. Definition and explanatory notes. In *World Health Organization International Histological Classification of Tumors: Histological Typing of Oesphagael and Gastric Tumours*, 2nd edn. Berlin, Springer, 1990, 1–8.
11. Lam KY, Loke SL, Chen WZ, Cheung KN, Ma L. Expression of p53 in oesophageal squamous cell carcinoma in Hong Kong Chinese. *Eur J Surg Oncol* 1995, **21**, 242–247.
12. Darnton SJ, Allen SM, Edwards CW, Matthews HR. Histopathologic findings in oesophageal carcinoma with and without preoperative chemotherapy. *J Clin Pathol* 1993, **46**, 51–55.
13. Antonakopoulos GN, Darnton SJ, Newman J, Duffy JP, Matthews HR. Effect of chemotherapy on ultrastructure of oseophageal cell carcinoma. *Histopathology* 1994, **25**, 447–454.
14. Mandard AM, Dalibard F, Mandard JC, *et al.* Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma: clinicopathologic correlations. *Cancer* 1994, **73**, 2680–2686.
15. Batsakis JG, El-Naggar AK. p53: Fifteen years after discovery. *Adv Anat Pathol* 1995, **2**, 71–88.
16. Wynford-Thomas D. p53 In tumour pathology: can we trust immunocytometry? *J Pathol* 1992, **166**, 329–330.
17. Hirai T, Kawano K, Hirabayashi N, *et al.* A novel *in vitro* chemosensitivity test using materials collected by endoscopic biopsy. *Anticancer Drugs* 1991, **2**, 269–274.
18. Segalin A, Ruol A, Panozzo M, Bonavina L, Bianchi LC, Peracchia A. Flow cytometric DNA analysis does not predict the radiochemoresponsiveness of esophageal cancer. *J Surg Oncol* 1993, **54**, 87–90.
19. Hickey K, Grehan D, Reid IM, O'Brian S, Walsh TN, Hennessy TP. Expression of epidermal growth factor receptor and proliferating cell nuclear antigen predicts response of esophageal squamous cell carcinoma to chemoradiotherapy. *Cancer* 1994, **74**, 1693–1698.

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