ANTICANCER RESEARCH *24*: 2579-2584 (2004)

# Predictive Effect of p53 and p21 Alteration on Chemotherapy Response and Survival in Locally Advanced Adenocarcinoma of the Esophagus

PIERRE A.M. HEEREN[1], FRANK W.H. KLOPPENBERG[1], HARRY HOLLEMA[2], NANNO H. MULDER[3], RAOUL E. NAP[4] and JOHN TH.M. PLUKKER[1]

*Departments of [1]Surgical Oncology, [2]Pathology, [3]Internal Medicine and [4]Medical Statistics, University Hospital Groningen, Groningen,The Netherlands*

**Abstract.** *Background: Cell cycle regulating proteins (p53/p21) and proliferation index Ki-67 have been associated with prognosis and response to chemotherapy. The aim of this study was to determine the significance of these molecular markers on tumor response and prognostic effect in a group of esophageal cancer patients treated with neoadjuvant chemotherapy. Patients and Methods: Immunohistochemical expression of p53/p21 and Ki-67 was examined in pre-treatment biopsy specimen of 30 patients, in phase II neoadjuvant studies for locally advanced adenocarcinoma of the esophagus, who underwent surgery. Seven patients (23%) had progressive disease. Resection was achieved in all responders (n=23; 77%) and histochemical expression of the above-mentioned proliferating markers was examined in pre-treatment and resection specimens after chemotherapy. Results: Responders had a significantly better survival compared to non-responders (p=0.001). Expression of p53, p21 and high Ki-67 in pre-treatment specimens was 73% (22/30), 63% (19/30) and 30% (10/30), respectively and was not related to response to chemotherapy. However, alteration in expression of p53-positivity in the pre-treatment specimens to p53-negativity in the resection specimens and p21-negativity to p21-positivity in 6 of the 23 (26%) resected tumors was correlated with better response and survival (p=0.011). Conclusion: Data from this study showed that alteration of p53 and p21 expression rather than the initial expression seems to be related to chemotherapy response and overall survival in patients with esophageal adenocarcinoma.*

*Correspondence to:* J.Th.M.Plukker, MD, PhD, Department of Surgery/Surgical Oncology, University Hospital Groningen, 9700 RB Groningen, The Netherlands. Tel: 31- 50-3612317, Fax: 31-50-3614873, e-mail: j.th.plukker@chir.azg.nl

*Key Words:* Esophageal carcinoma, chemotherapy response, apoptotic markers.

Five-year survival in patients with locally advanced carcinoma of the esophagus and gastro-esophageal junction (GEJ) after surgery alone varies between 10-20% (1-2). To improve the outcome multimodality treatment has been proposed with different response and complete regression in 0-25% (3-9). However, considerable side-effects and resistance to chemotherapy remain a major problem. Response is associated with apoptosis controlled by genes involving the cell cycle regulation, including p53 and its downstream regulator p21/WAF (Wild-type Activating Fragment) (10-13). In response to DNA damage p53 is activated and turns on p21. Transcription of p21 stops DNA replication *via* the cyclin-dependent kinase system and PCNA, allowing DNA repair. Loss of functional wild-type p53 and p21 due to mutation or allelic deletion alters suppressor activity and enhances genomic instability and may be associated with resistance to chemotherapy. High expression of Ki-67 as an index for growth fraction and potential aggressiveness is associated with decreased survival and resistance to cytotoxic drugs (14-16). Measuring the effectiveness of multimodality treatment with Computed Tomography (CT) and endoscopic ultrasonography is difficult, due to tumor necrosis and fibrosis during chemotherapy. Currently, Positron Emission Tomography and the use of molecular markers seem to predict response or resistance to neoadjuvant treatment (17,18). Increased expression of apoptotic-related wild-type p53 and p21 and decreased cell proliferation estimated by Ki-67 expression might indicate a favorable response.

We investigated the correlation between immuno-histochemical expression of these molecular markers in pre-treatment and resection specimens with clinicopathological response and overall survival in esophageal cancer patients after neoadjuvant treatment.

## Patients and Methods

*Patients.* Sufficient archival formalin-fixed paraffin-embedded tissue samples from 30 patients, all participants in phase II studies of neoadjuvant chemotherapy in locally advanced adenocarcinoma

of the esophagus and GEJ at the University Hospital of Groningen, The Netherlands, were available for this study (19,20). All patients presented with clinical and radiological response after completion of neoadjuvant chemotherapy and underwent relaparotomy between 1990 and 2001. Histology revealed adenocarcinoma of the intestinal type in 22 (73%) and of the diffuse type in 8 patients (27%). Mean age at diagnosis was 61 (21-78) years. Surgical resection was not performed because of progressive disease in 7 patients (23%). The remaining 23 patients underwent resection with curative intent after chemotherapy was completed. Twenty patients (20/23; 87%) had a microscopic complete (R0) resection and 3 (13%) patients had a microscopically-positive resection margin (R1). Immunohistochemical expression of p53, p21 and Ki-67 was determined in pre-treatment and resection specimens. In patients with progressive disease the tumor was not resected and expression was only determined in pre-treatment specimens.

*Chemotherapy.* Three previously described regimens were given: 5-Fluoro-Uracil (5FU) 750mg/m$^2$, Leucovorin 12.5 mg (LV) and Alpha Interferon 18 microU; a combination of high-dose 5FU; 1.5 g/m$^2$ and Methotrexate; 1.5g/m$^2$ and a scheme consisting of Carboplatin 300 mg/m$^2$, 4-Epiadriamycin 80 mg/m$^2$ and Tenoposide 100 mg/m$^2$. Surgery was performed within 4 weeks after the last chemotherapy cycle. Ten of the 30 patients (33%) received 5FU/MTX, 50% (15/30) CET and 16% (5/30) the 5FU/LV/interferon schedule (19,20).

*Assessment of response.* Assessment of response was determined as clinical response measured by clinical examination and CT, and pathological response after completion of chemotherapy. In absence of progressive disease relaparotomy was performed and the tumor was resected. Response was defined as complete response (CR) in case of total regression on CT and during surgery. Tumor regression of more than 50% but with residual mass was termed partial response (PR) and stable disease (SD) when 50% or less reduction was noted. Tumor enlargement and new locations during treatment were defined as progressive disease (PD), in which case the tumor was not resected. Pathological complete response (pCR) included absence of disease or complete tumor necrosis on pathological examination, while partial responding (pPR) tumors presented with partial necrosis or fibrosis in the resected specimen.

*Surgery.* In all these patients resection with curative intent was not possible at initial surgery. Tumors were considered primarily incurable in case of T4 disease and/or involvement of second echelon para-aortic or celiac lymph nodes at frozen section examination (M1a). Within 4-6 weeks after completion of chemotherapy, relaparotomy was performed if progression was excluded on CT and physical examination. Esophagectomy with curative intent was performed if progressive disease was excluded during surgical exploration. This was accomplished by a right thoraco-abdominal approach with cervical anastomoses in esophageal cancer or a left thoraco-abdominal approach in GEJ tumors, with dissection of mediastinal and abdominal lymph nodes (two-field dissection).

*Immunohistochemistry.* Sections of 5 mm were cut from retrieved paraffin blocks of pre- and post-treatment specimens. For assessment of pathological response with respect to p53, p21 and Ki-67, immunohistochemistry (IHC) was performed using the standard avidin-biotin complex method. Sections were incubated with monoclonal mouse anti-p53-DO7, anti-p21 and polyclonal anti-Ki-67 overnight at 4˚C. Biotinylated swine-anti-rabbit and rabbit-anti-mouse (1:300 dilution-30 min) were applied as a secondary antibody. The color reaction product was obtained with diaminobenzidine hydroxychloride (DAB) and counterstaining of the nuclei with haematoxylin. Omission of primary antibodies provided negative controls. For p53, a breast carcinoma specimen was taken as a positive control (2+) and for p21, a normal colon specimen was used as positive control (1+). Two blinded observers evaluated the intensity and extension of staining, which was graded qualitatively as: -, not detectable; +, weak; ++, moderate and +++, strong. The intensity was expressed relative to corresponding positive controls. Expression of p53 (neg. <5%; weak +, 5-25%; moderate ++, 25-50%; strong +++, >50%. The results of IHC of p53 performed on biopsies were scored positive when a strong staining intensity was found. Expression of p21 (neg.

Table I. *Expression of p53, p21 and Ki-67 in relation to chemotherapy response.*

| Pat no | p53 pre-CTx | p53 after CTx | p21 pre-CTx | p21 after CTx | Ki-67 pre-CTx | Ki-67 after CTx | Response |
|---|---|---|---|---|---|---|---|
| 1 | + | - | - | - | 40% | 40% | PR |
| 2 | + | + | - | + | 40% | 50% | PR |
| 3 | + | + | + | + | 70% | 90% | PR |
| 4 | + | + | + | + | 15% | 5% | PR |
| 5 | + | + | + | + | 80% | 30% | PR |
| 6 | + | - | - | + | 15% | 30% | PR |
| 7 | + | + | - | + | 20% | 80% | PR |
| 8 | + | - | - | - | 50% | 10% | PR |
| 9 | + | + | + | - | 80% | 20% | SD |
| 10 | + | + | + | - | 80% | 35% | SD |
| 11 | - | - | + | - | 80% | 20% | SD |
| 12 | + | + | + | - | 10% | 15% | SD |
| 13 | + | + | + | + | 80% | 30% | SD |
| 14 | + | + | - | + | 50% | 30% | SD |
| 15 | - | - | + | - | 80% | 80% | SD |
| 16 | + | + | + | - | 10% | 80% | SD |
| 17 | + | + | + | + | 50% | 30% | SD |
| 18 | + | + | + | + | 40% | 30% | SD |
| 19 | - | + | + | + | 80% | 30% | SD |
| 20 | - | - | + | + | 80% | 30% | SD |
| 21 | - | - | + | + | 10% | 20% | SD |
| 22 | - | - | + | + | 80% | 60% | SD |
| 23 | + | + | + | + | 70% | 30% | SD |
| 24 | + | N.A. | - | N.A. | 40% | N.A. | PD |
| 25 | + | N.A. | - | N.A. | 50% | N.A. | PD |
| 26 | - | N.A. | + | N.A. | 40% | N.A. | PD |
| 27 | + | N.A. | - | N.A. | 70% | N.A. | PD |
| 28 | + | N.A. | + | N.A. | 60% | N.A. | PD |
| 29 | + | N.A. | - | N.A. | 40% | N.A. | PD |
| 30 | - | N.A. | - | N.A. | 50% | N.A. | PD |

CTx, Chemotherapy;
PR, Partial Response;
SD, Stable Disease;
NA, Not Applicable;
PD, Progressive disease

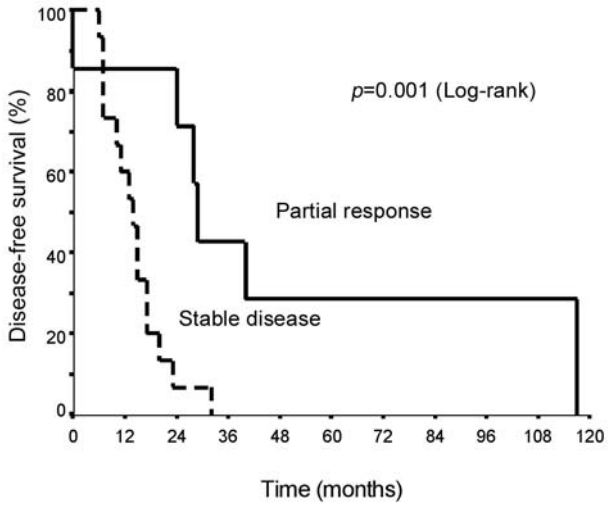


Figure 1. *Correlation of staining intensity with disease-free survival in relation to response to chemotherapy.*

<10%; weak +, 10-25%; moderate ++, 25-50%; strong +++, >50%) were considered positive when 25% or more nuclear staining was found. The Ki-67 index was defined as the total number of Ki-67-positive cells per total number of nuclei counted. To define high and low proliferation indices, cut-off of Ki-67 was set at 30% and 70%, respectively.

*Statistical analysis.* Expression of the parameters and response to chemotherapy were correlated by Fisher's exact and Chi-square analysis. Log-rank testing for dichotomous variables was used to compare the overall survival (OS) in different groups. Survival data were prospectively collected and analysed by the Statistical Package for Social Sciences (SPSS, version 11.0, Chicago, Illinois, USA) software. *P*-values <0.05 were considered statistically significant.

## Results

*Expression before and after chemotherapy.* Pre-treatment group: Expressions of p53 and p21 were found in 22 (73%) and 19 (63%) of the pre-treatment tumors, respectively. Expression of p21 was observed in 12 of the 22 (55%) p53-positive tumors. Ki-67 index was high in 7 of the 22 p53-positive tumors (32%). In 7 of the p53-negative tumors (7/8; 88%) p21 was expressed and 5 p53-negative tumors (5/8; 63%) showed a high Ki-67 expression (Table I).

Resected group: After completion of chemotherapy, p53 expression changed in 17% (4/23) of the resected tumors [p53-positivity into p53-negativity (3/22) and p53-negativity into p53-positivity (1/6)]. Four of 5 p53-positive tumors without p21 expression before neoadjuvant treatment changed to be p21-positive. The expression of either p53 or p21 altered in 12 tumors (52%) of which 5 became positive. Eleven out of 23 tumors (48%) showed a decrease in Ki-67 expression, 10 (43%) remained the same and 2 (9%) had an increased expression.

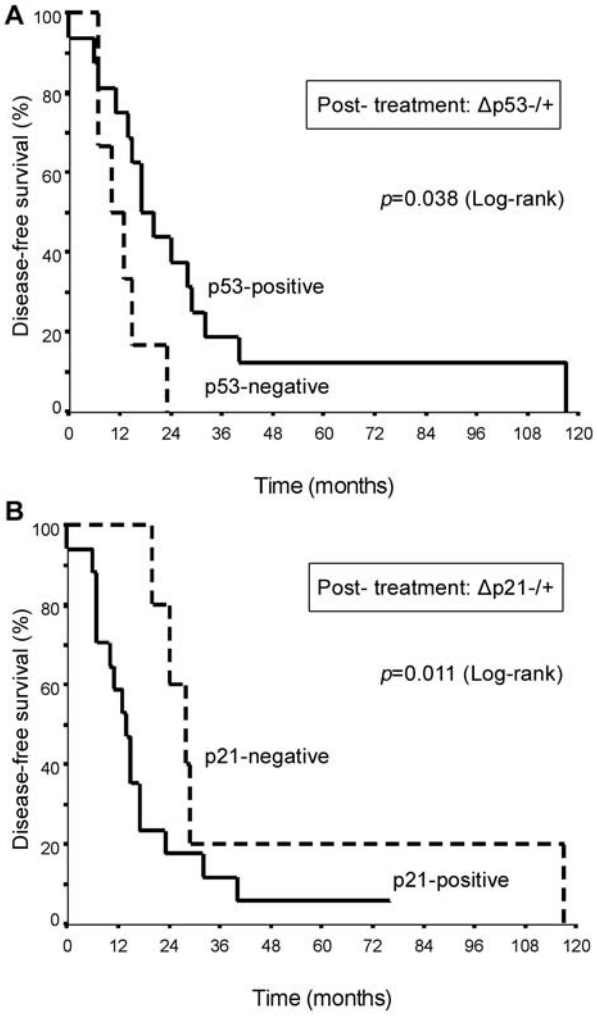


Figure 2. *Survival of patients in relation to the expression of p53 (A) and p21 (B) in specimens after neoadjuvant treatment.*

*Response to chemotherapy and survival.* Clinically, 27% (8/30) of the patients showed partial response (PR) and 50% (15/30) had stable disease (SD). Clinical complete responses were not observed. Disease-free survival (DFS) and OS were compared between patients with PR and SD or PD. The difference in survival between responders and non-responders was significant ($p=0.001$; Figure 1). Median survival was 35 (CI, 16-54) months for patients with PR, compared with 19 months (CI, 15-23) and 11 months (CI, 6-16) for patients with SD or PD. pPR was observed in 5 patients (5/23: 22%), while the other 18 tumors showed only minimal or no signs of pathological response and were classified as stable disease. None of the patients had pCR. Pathological response was not associated with clinical response or with survival. Responders and non-responders were equally divided between different regimens and survival was not different between the treatment arms.

*Expression of p53/p21 and Ki-67 in response to chemotherapy and survival.* To examine whether the expression of p53, p21 or Ki-67 in pre-treatment specimens was able to predict the clinical response to neoadjuvant treatment, a Fisher's exact test was performed. None of these immunohistochemical parameters were able to predict clinical/radiological response, although p53-positive and p21-negative (p53+/p21–) tumors were more likely to response than p53-negative and p21-positive (p53–/p21+) tumors. However, this difference was not significant. Moreover, no significant difference in response was seen between patients with high, medium or low Ki-67 expression levels in pre-treatment specimens. Furthermore, no correlation was found between the expression of the different parameters and the occurrence of pathological response. In 6 out of 23 resected tumors (26%), an alteration in expression from p53-positive to p53-negative and/or p21-negative to p21-positive between pre-treatment and resection specimens was noted. In 5 of these 6 patients (83%) pPR was noted, compared with 3 out of 17 patients (18%) without such an alteration or with a reversed change ($p=0.003$). Moreover, comparison of survival between both groups revealed a significantly better OS for patients with such a change in expression ($p=0.036$). Median survival was 32 months (CI 26-38 months) for the "changed" group compared to 19 months (CI 15-23 months). This was also reflected in the different survival curves for p53-positive and p53-negative and p21-positive and p21-negative tumors before and after chemotherapy (Figure 2 A,B). In the group of patients in whom resection was performed, expression of p53 in endoscopical biopsy specimens was correlated with a significantly increased DFS and OS compared with p53-negative tumors ($p=0.039$ and $p=0.035$, respectively). Although there was a trend for better DFS and OS for p21-negative tumors compared with p21-positive tumors before chemotherapy, a statistically significant difference was not reached ($p=0.09$). For p21-negative and p21-positive tumors, in analogy with p53-expression the Kaplan Meier survival curves after chemotherapy was equal. Change in expression of Ki-67 was not related with response to chemotherapy or survival.

## Discussion

Increasing interest has been focused on multimodality treatment for esophageal carcinoma. Though several phase II /III trials suggested some survival benefit over surgical treatment alone, most studies reported only proportional gains in responders (3-9,19-21). Besides, neoadjuvant chemo- (radio) therapy is frequently complicated by serious side-effects and evaluation of complete response may be a problem. Early prediction of response by evaluating potential molecular markers such as apoptosis and reduction of proliferation indices, *e.g.* Ki-67, in pre-treatment specimens seems to be attractive (14-16). DFS and OS were significantly better in responders compared to patients with stable disease (Figure 1). Pathological response rate in this study was 22% (5/23), presenting only partial necrosis or fibrosis at best. Complete pathological response was not seen (19,20), in contrast to other reports (6-8). Surprisingly, pathological response did not correlate with radiological response as only 2 out of 8 patients (25%) with radiological response showed pathological response. Moreover, pathological response did not predict DFS and OS better than radiological response. Based on these results, assessment of tumor viability in post-treatment endoscopic biopsies may not be a reliable indicator of treatment effect. Expression of p53 (73%; 22/30) and p21 (63%; 19/30) in pre-treatment specimens was comparable with previous studies, noting p53 expression in 42-87% and p21 expression in 65-91% of tumors, respectively (22-24). The impact of p53 and p21 expression in pre-treatment biopsies on response to chemotherapy and survival differs considerably. Literature results of application are conflicting. Some authors described a higher rate of response in p53-positive tumors (25), while others found no significant discrepancies in response between p53-positive tumors and p53-negative tumors (26). Shimada *et al.* showed improved sensitivity for cisplatin in p53-negative tumors compared with p53-positive tumors in a study of 59 tumors of the esophagus (27). We did not find p53, p21 or Ki-67 expression in biopsy specimens to be predictors of identified response. Based on these results, we could not identify p53, p21 or Ki-67 as determined in biopsy specimens to be predictors of treatment efficiency. All responding patients were p53-positive (8/8), but there was no difference with non-responders (5/7; 71%). Shimoyama *et al.* described the alteration in the expression of p53 and p21 proteins before and after chemotherapy in their study of 13 patients with squamous cell carcinomas receiving 5FU and cisplatin (28). Change of expression from p21-negativity to p21-positivity and/or p53 in the opposite direction occurred in 6 of our patients compared to 4 of the 13 patients in their study (26% *vs* 31%, respectively). Change of p53 expression after chemotherapy was already described in patients with squamous cell carcinomas of the cervix without influencing prognosis (29). In 5 of the 6 patients with altered p53/p21 expression (83%) clinical/radiological partial response was noted, compared with 3 out of 17 resected tumors (18%) without this change ($p=0.003$). In addition, comparison of survival between these two groups of patients revealed a significantly better OS for the group of patients in whom this change was noticed ($p=0.036$). There is increased evidence that p53 and p21 are crucial proteins in the effect of chemotherapy for these tumors. We found no association between pretreatment marker expression and treatment response, but noted that a change in p53 expression from positive to negative or in p21 status from negative to positive accompanied improved response and survival. Prospective evaluation of these genetic markers may be valuable in a multimodality approach for the treatment of these tumors.

## References


1 Muller JM, Erasmi H, Stelzner M *et al*: Surgical therapy of oesophageal carcinoma. Br J Surg *77*: 845-57, 1990.
2 Swanson SJ, Batirel HF, Bueno R *et al*: Transthoracic esophagectomy with radical mediastinal and abominal lymph node dissection and cervical esophagogastrpstomy for esophageal carcinoma. Ann Thorac Surg *72*: 1918-25, 2001.
3 Walsh TN, Noonan N, Hollywood D *et al*: A comparison of multimodality therapy and surgery for esophageal carcinoma. N Engl J Med *335*: 462-510, 1996.
4 Forastiere AA, Heitmiller RF, Lee DJ *et al*: Intensive chemoradiation followed by esophagectomy for squamous cell and adenocarcinoma of the esophagus. Cancer J Sci Am *3*: 144-52, 1997.
5 Kelsen DP, Ginsberg R, Pajak TF *et al*: Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. N Engl J Med *339*: 1979-84, 1998.
6 Medical Research Council Oesophageal Cancer Working Party. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. Lancet *359*: 1727-33, 2002.
7 Bosset JF, Gignoux M, Triboulet JP *et al*: Chemoradiotherapy followed by surgery compared with surgery alone in squamous cell cancer of the esophagus. N Engl J Med *337*: 161-7, 1977.
8 Urba SG, Orringer MB, Turrisi A *et al*: Randomized trial of preoperative chemoradiation *versus* surgery alone in patients with locoregional esophageal carcinoma. J Clin Oncol *19*: 305-13, 2001.
9 Heath EI, Burtness BA, Heitmiller RF *et al*: Phase II evaluation of preoperative chemoradiation and postoperative adjuvant chemotherapy for squamous cell and adenocarcinoma of the esophagus. J Clin Oncol *18*: 868-76, 2000.
10 Kerr JFR, Wyllie AH and Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implication in tissue kinetics. Br J Cancer *26*: 239-57, 1972.
11 Koshiji M, Adachi Y, Taketani S *et al*: Mechanism underlying apoptosis induced by combination of 5-fluorouracil and interferon-gamma. Biochem Biophys Res Commun *52*: 376-81, 1997.
12 Kastan MB, Onyekwere O, Sidransky D *et al*: Participation of p53 protein in the cellular response to DNA damage. Cancer Res *51*: 6304-11, 1991.
13 Namba H, Hara T, Tukazaki T, Migita K *et al*: Radiation-induced G1 arrest is selectively mediated by the p53-WAF1/Cip1 pathway in human thyroid cells. Cancer Res *55*: 2075-80, 1995.
14 Youssef EM, Matsuda T, Takada N *et al*: Prognostic significance of the MIB-1 proliferation index for patients with squamous cell carcinoma of the esophagus. Cancer *76*: 358-366, 1995.
15 Okuno Y, Nishimura Y, Kashu I *et al*: Prognostic values of proliferating cell nuclear antigen (PCNA) and Ki-67 for radiotherapy of esophageal squamous cell carcinomas. Br J Cancer *80*: 387-395, 1999.
16 Horii N, Nishimura Y, Okuno Y *et al*: Impact of neoadjuvant chemotherapy on Ki-67 and PCNA labeling indices for esophageal squamous cell carcinomas. Int J Radiation Oncol Biol Phys *49*: 527-532, 2001.
17 Brucher BL, Weber W, Bauer M *et al*: Neoadjuvant therapy of esophageal squamous cell carcinoma: response evaluation by positron emission tomography. Ann Surg *233*: 320-1, 2001.
18 Flamen P, Van Cutsem E, Lerut A *et al*: Positron emission tomography for assessment of the response to induction radiochemotherapy in locally advanced oesophageal cancer. Ann Oncol *13*: 361-8, 2002.
19 Plukker JT, Sleijfer DT, Verschueren RC *et al*: Neoadjuvant chemotherapy with carboplatin, 4-epiadriamycin and teniposide (CET) in locally advanced cancer of the cardia and the lower esophagus: a phase II study. Anticancer Res *15*: 2357-61, 1995.
20 Plukker JT, Mulder NH, Sleijfer DT *et al*: Chemotherapy and surgery for locally advanced cancer of the cardia and fundus: a phase II study with methotrexate and 5-fluorouracil. Br J Surg *78*: 955-8, 1991.
21 Kok TC, Lanschot JV, Siersema PD *et al*: Neoadjuvant chemotherapy in operable esophageal squamous cell cancer: final report of a phase III multicenter randomized controlled trial. Proc Asco *16*: 277, 1997.
22 Hirai T, Kuwahara M, Yoshida K *et al*: The prognostic significance of p53, p21 (Waf1/Cip1), and cyclin D1 protein expression in esophageal cancer patients. Anticancer Res *19*: 4587-92, 1999.
23 Parenti AR, Rugge M, Frizzera E *et al*: P53 overexpression in the multistep process of esophageal carcinogenesis. Am J Surg Pathol *19*: 1418-22, 1995.
24 Wagata T, Shibagaki I, Imamura M *et al*: Loss of 17p, mutation of the p53 gene, and overexpression of p53 protein in esophageal squamous cell carcinoma. Cancer Res *3*: 1195-1200, 1997.
25 Muro K, Ohtsu A, Boku N *et al*: Association of p53 protein expression with responses and survival of patients with locally advanced esophageal carcinoma treated with chemoradiotherapy. Jpn J Clin Oncol *26*: 65-9, 1996.
26 Lam KY, Law S, Ma LT, Ong SK *et al*: Preoperative chemotherapy for squamous cell carcinoma of the esophagus: do histological assessment and p53 overexpression predict chemoresponsiveness? Eur J Cancer *33*: 1221-5, 1997.
27 Shimada Y, Watanabe G, Yamasaki *et al*: Histological response of cisplatin predicts patients' survival in esophageal cancer and p53 protein accumulation in pretreatment biopsy is associated with cisplatin sensitivity. Eur J Cancer *36*: 987-93, 2000.
28 Shimoyama S, Konishi T, Kawahara M *et al*: Expression and alteration of p53 and p21 influence the sensitivity of chemoradiation therapy for esophageal cancer. Hepatogastroenterology *45*: 1497-1504, 1998.
29 Garzetti GG, Ciavattini A, Luccarini G *et al*: Modulation of expression of p53 and cell proliferation in locally advanced cervical carcinoma after neoadjuvant combination chemotherapy. Eur J Obstet Gynecol Reprod Biol *63*: 31-6, 1995.


*Received February 27, 2004*
*Revised June 9, 2004*
*Accepted June 11, 2004*