

## BIOLOGICAL MARKERS AS INDICATORS OF PATHOLOGICAL RESPONSE TO PRIMARY CHEMOTHERAPY IN ORAL-CAVITY CANCERS

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**The predictive role in terms of pathological response and prognostic role of biomarkers such as GST- $\pi$ , p53, bcl-2 and bax expression, immuno-histochemically detected, and of the S-phase cell fraction, autoradiographically determined as thymidine labeling index (TLI), were investigated within a prospective randomized phase III clinical trial on squamous-cell carcinoma of the oral cavity, including surgery or primary chemotherapy (PCT), which foresaw the prospective determination of biological markers. Pathological response was defined as the achievement after PCT of a pathological complete remission or the presence of microresidual disease. The study was performed on tumors obtained from a series of 100 previously untreated patients with resectable T2–4N0–2M0 carcinoma. All biomarkers were unrelated, except for an inverse relation between TLI and GST- $\pi$  and a direct relation between bcl-2 and bax expression. In patients treated with surgery alone, 3-year disease-free survival (DFS) appeared to be weakly, but not significantly, related only to GST- $\pi$  and p53 expression. In patients treated with PCT, pathological response and DFS were independent of p53 expression and cell proliferation. Conversely, low GST- $\pi$  and bax expression were indicative of pathological response but lost relevance as predictors of DFS, whereas absence of bcl-2 was associated with high probability of 3-year DFS in the overall series as well as in non-responding patients. Within this latter sub-set, all patients with bcl-2-positive tumors relapsed within 1 year of surgery, whereas a 60% probability of 3-year DFS was observed for patients with bcl-2-negative tumors ( $p = 0.02$ ). This interim analysis appears to indicate that some biofunctional markers can provide information on pathological response to PCT and could help in understanding treatment efficacy at a cellular level. *Int. J. Cancer (Pred. Oncol.)* 79:619–623, 1998.**

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Primary chemotherapy (PCT) for advanced head-and-neck cancer has been studied in clinical trials for more than a decade without a clear demonstration of benefit for local-regional tumor control or overall patient survival (Browman, 1994). The benefit derived from PCT's demonstrated ability to reduce distant failure is not matched by a clear benefit in terms of survival since it fails to improve local-regional control. PCT provides a high percentage of tumor response and, in some cases, ranging from 10% to 30%, pathologically complete remission. Some patients may achieve a very good result, with the presence in the specimens of only microresidual disease. This means that a substantial fraction of tumors is highly chemosensitive to treatment and that patients may benefit from PCT. These important aspects for the cure of advanced head-and-neck cancer patients should stimulate basic research to identify and evaluate biological variables in order to define a "biological profile" of a chemosensitive patient. Such variables could help clinicians to indicate the optimal treatment for an individual patient, thus maximizing treatment efficacy in terms of cure and organ preservation.

In the past, *in vitro* chemosensitivity assays have been proposed and used to define drug sensitivity on individual tumors at a pre-clinical level. However, the limited amount of tumor material before PCT and the moderate feasibility of *in vitro* tests represented, for this tumor type, important constraints on wide utilization of the approach. Conversely, the determination of biological

markers involved or associated with different cellular aspects appears feasible on small tumor samples and has already provided predictive information on treatment response for several tumor types (Harris and Hollstein, 1993; Corvò *et al.*, 1997; Silvestrini *et al.*, 1998).

Molecular markers involved as determinants or targets of the cellular response to cytotoxic agents, including P-glycoprotein, glutathione S-transferase- $\pi$  (GST- $\pi$ ) and topoisomerases, were found to correlate with the efficacy of different pharmacological agents on clinical tumors. In particular, GST- $\pi$  has been reported to be indicative of cisplatin resistance (Okuyama *et al.*, 1994), and GST- $\pi$  plasma levels have been proposed as useful to predict treatment efficacy or recurrence after surgery in oral-cavity cancer. Moreover, alterations of tumor-suppressor genes and/or oncogenes, which proved to be related to tumor aggressiveness in several tumor types, have been reported to affect sensitivity to drugs (Gottesman, 1994). Alterations of the TP53 tumor-suppressor gene (the most common genetic alterations found in human malignancies) were found to be a negative prognostic factor in different tumor types (Harris and Hollstein, 1993) and appeared to provide information on resistance or sensitivity to different chemical and physical agents (Righetti *et al.*, 1996).

Resistance to chemotherapy has also been associated with decreased susceptibility to apoptosis (Reed, 1995), raising the possibility that cell-death determinants may influence treatment outcome. However, control of the apoptotic pathway is highly complex since it involves a multiplicity of growth-control factors, suggesting the combined consideration of its positive and negative regulators. In particular, among the different controlling genes, bcl-2 is known to be a negative regulator of cell death, whereas bax appears as a promoting factor with similar structure, able to form heterodimers with bcl-2 and thus counteract its death-repressor activity in several experimental models. The de-regulated expression of these genes in neoplastic tissues may confer resistance to chemotherapy by enabling cells to avoid apoptosis. Another tumor feature influencing clinical response to treatment is cell-proliferation rate, defined by quantification of S-phase cells (thymidine- or bromodeoxyuridine-labeling index, flow-cytometric S fraction) or of the whole cell-cycling fraction (Ki67, AgNORs) (Quinn and Wright, 1990). In fact, *in vitro* and *in vivo* experimental data indicate a direct relation between cell proliferation and response to chemotherapy or radiotherapy, and evidence in this regard has consistently emerged from retrospective and prospective studies in human tumors (Corvò *et al.*, 1997; Silvestrini *et al.*, 1998).

In the present study, we proposed to define the predictive role in terms of pathological response and the prognostic role in terms of

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disease-free survival (DFS) of GST- $\pi$ , p53, bcl-2 and bax, immuno-histochemically detected, and of S-phase cell fraction, as defined by thymidine labeling index (TLI) (Silvestrini *et al.*, 1991), within a randomized phase III clinical trial on oral-cavity cancer, including surgery or primary chemotherapy.

#### MATERIAL AND METHODS

##### Patient population

Biological determinations were performed on tumors obtained from a series of 100 previously untreated patients, with resectable T2(>3 cm)-4N0-2M0 squamous-cell carcinoma of the oral cavity. Cases were among the series of 197 patients who entered a still open multicenter randomized trial started in 1989 in order to establish the role of PCT in patients with advanced resectable oral-cavity cancer (Grandi *et al.*, 1994). In particular, the biological characterization was prospectively planned and carried out on tumors from all patients enrolled at the Istituto Nazionale Tumori of Milan from November 1989 to December 1997. Patients were less than 70 years old and randomly assigned to receive surgery (group A) or PCT followed by surgery (group B). PCT consisted of 3 cycles of cisplatin (100 mg/m<sup>2</sup>) plus 5-fluorouracil (1,000 mg/m<sup>2</sup>) on days 1 to 5 as a 120-hr infusion repeated every 21 days. Surgery was planned within 3 weeks from the last chemotherapy cycle. Only high-risk patients (defined as those with positive surgical margins, extracapsular tumor spread and/or more than 3 node metastases) of both groups received 50 Gy of post-operative radiotherapy.

In group B, patients progressive after the first cycle were immediately operated on. Surgery was performed in patients with stable disease after 2 cycles. Responsive patients, as defined by the WHO criteria, were operated on after having completed 3 cycles of PCT. Resected specimens were analyzed, and chemotherapy-induced necrosis was evaluated according to a 3-grade scale. Grade I was classified as the presence of gross residual tumor, grade II as the presence of isolated microscopic residual tumor foci and grade III as no detectable viable tumor, this last being defined as pathological complete remission. Pathological response was defined as the achievement of grade II regression (microresidual disease) and grade III (pathological complete remission).

Biological determinations were performed on biopsy specimens before PCT for patients in group B and on surgical specimens for patients in group A. For the determination of bax expression, tumor samples were available for 92 of the 100 patients. For the series of tumors for which biological information was available, clinical features, according to the TNM classification system (UICC, 1987), were similar to those of the overall series of patients entered in the randomized study (Table I).

##### In vitro determinations

Immediately after removal, biopsy or surgical tumor specimens were incubated with <sup>3</sup>H-thymidine (Silvestrini kit; Euroframe, Asti,

Italy) and then processed for conventional histological procedures for determination of TLI, p53, bcl-2, bax and GST- $\pi$  expression. Investigators from Oncologia Sperimentale C of the National Cancer Institute of Milan have actively participated in national quality control programs activated in 1989 for TLI (Silvestrini *et al.*, 1991) and for the other investigated biological markers. For all of the biomarkers under investigation, 2 investigators independently evaluated the specimens and inter-observer variability was always less than 10%.

##### TLI determination

TLI was determined by autoradiography as described (Silvestrini *et al.*, 1991) and evaluated independently by 2 observers by scoring a total of more than 3,000 tumor cells on different areas from the same tumor specimen. TLI was defined as the percentage ratio between labeled cells and total number of tumor cells.

##### Immuno-histochemical determinations

**p53 expression.** Histological sections (4  $\mu$ m) were incubated for 1 hr with the PAb1801 monoclonal antibody (1:50 dilution; Oncogene Science, Uniondale, NY), which was raised against human p53 protein and recognizes wild-type and mutant forms of p53 protein. Specimens were then incubated with a goat anti-mouse immunoglobulin and processed with an avidin-biotin complex peroxidase method (Vectastain ABC kit; Vector, Burlingame, CA).

**bcl-2 expression.** Histological sections (4  $\mu$ m) were incubated for 1 hr at room temperature with a mouse monoclonal anti-human bcl-2 oncoprotein (1:40 dilution; clone 124; Dakopatts, Copenhagen, Denmark). Specimens were then incubated with a goat anti-mouse immunoglobulin and treated with an avidin-biotin complex peroxidase method (Vectastain ABC kit, Vector).

For the short-fixation time (6 hr), incubation of slides in a microwave oven was not necessary to retrieve antigen expression (Silvestrini *et al.*, 1995). The fraction of p53- and bcl-2-positive tumor cells was evaluated independently by 2 observers by scoring a total of 1,000 to 3,000 tumor cells. Immunostaining for p53 and bcl-2 (at nuclear level for the former and cytoplasmic for the latter) was defined as the percentage ratio between positive and total number of tumor cells. Samples were considered positive if unequivocal brown staining was seen.

**bax expression.** Histological sections (4  $\mu$ m) were incubated for 2 hr at 4°C with a rabbit polyclonal antibody (1:400 dilution, clone N-20; Santa Cruz Biotechnology, Santa Cruz, CA). Specimens were then processed by using the Dako quick-staining labeled alkaline-phosphatase kit (Dako Chemate, Dakopatts).

**GST- $\pi$  expression.** Histological sections (4  $\mu$ m) were incubated with a rabbit polyclonal antibody (1:50 dilution; Ylem, Avezzano, Italy) for 2 hr at 4°C. Specimens were then incubated with a goat anti-rabbit immunoglobulin (Vector) and processed by using the Dako quick-staining labeled alkaline-phosphatase kit (Dako Chemate, Dakopatts).

The bax and GST- $\pi$  immunostaining was defined according to a qualitative scale. Tumor samples were considered negative in the absence of any cytoplasmic immunoreactivity or when weakly stained and positive when unequivocally stained.

Head-and-neck squamous-cell carcinomas with high p53, bcl-2, bax or GST- $\pi$  immunoreactivity were used as positive controls, while negative controls were obtained by omission of the specific primary antibody.

##### Statistical analysis

Spearman's rank-correlation coefficient was used to assess the association between TLI, p53 and bcl-2 expression considered as continuous variables. Wilcoxon's rank-sum test and Fisher's exact test were used to assess the relation between bax or GST- $\pi$ , qualitatively graded, and TLI, p53 and bcl-2 expression.

In group B, the relation between pre-treatment values of biological variables and pathological complete remission and grade II pathological response was assessed by the  $\chi^2$  test. DFS was

TABLE I - CLINICAL STAGE (TNM) OF TUMORS FROM PATIENTS ENTERED IN PHASE III STUDY AND FROM THOSE WITH BIOLOGICALLY CHARACTERIZED TUMORS

	Group A <sup>1</sup>		Group B <sup>2</sup>	
	Overall series (n = 99)	Biologically characterized (n = 51)	Overall series (n = 98)	Biologically characterized (n = 49)
Initial tumor size				
T2 (>3 cm)	43%	45%	51%	43%
T3	38%	40%	35%	47%
T4	19%	15%	14%	10%
Initial nodal status				
N0	56%	55%	58%	57%
N1	27%	31%	27%	26%
N2a	6%	4%	5%	6%
N2b	9%	8%	8%	11%
N2c	2%	2%	2%	—

<sup>1</sup>Surgery  $\pm$  RT. <sup>2</sup>Chemotherapy + surgery  $\pm$  RT.

computed in both groups, starting from the date of surgery, by the Kaplan-Meier product-limit method. The Cox model was used to assess differences among sub-groups. Since patient accrual is ongoing, the present is an interim analysis based on available data.

For clinical correlations, biomarkers were used as categorical variables. For TLI, the cut-off value of 14% (which represents the median value for the present series) was used. p53, bcl-2, bax and GST- $\pi$  expression were categorized as negative vs. positive.

## RESULTS

Immunoreactivity to p53 protein, characterized by nuclear staining, was detected in 52% of cases. The number of p53-positive cells in individual tumors ranged from 1% to 86%, with a median value of 29%. Positivity to bcl-2, bax and GST- $\pi$  proteins was localized in the cytoplasm of tumor cells, and the frequency of positive tumors was 15%, 55% and 57%, respectively. TLI values showed a log-normal distribution, ranging from 0.01% to 32.6% for the different tumors, with a median value of 14%. Tumor biological characteristics were similar for patients entered in the 2 groups of the clinical study (Table II).

The only relation found among the biomarkers was an inverse one between TLI and GST- $\pi$  expression and a trend in favor of a direct relation between bcl-2 and bax expression ( $p = 0.03$ ). In particular, the median TLI value was significantly higher in GST- $\pi$ -negative than in GST- $\pi$ -positive tumors (16.6% vs. 12.8%, respectively;  $p = 0.01$ ), whereas 9 of 11 bcl-2-positive tumors also expressed bax.

### Biomarkers as prognostic or predictive variables

In light of the clinical protocol, we analyzed the relevance of biological variables as prognostic indicators in terms of DFS in patients treated with surgery alone (group A) and as predictors of pathological response and DFS in patients treated with PCT and surgery (group B). The median follow-up was 37 months (range, 1 to 90 months). At this writing, 31 patients have relapsed: 16 at local-regional sites, 3 at distant sites and 12 at a regional level. The overall clinical outcome of patients with available biological information was similar to that of all enrolled patients (data not shown).

In patients treated with surgery alone, neither bcl-2/bax expression nor TLI provided information on long-term clinical outcome (Table III), and moderate/strong GST- $\pi$  and lack of p53 protein expression were favorable, though not statistically significant, indicators of DFS.

Pathological response was obtained in 18 patients treated with PCT: 9 grade III pathologically complete remissions and 9 grade II pathological regressions. Pathological response rates and DFS (Table IV) were independent of TLI and p53 expression. Conversely, the lack of GST- $\pi$  and bax expression was related to pathological response rates 3- and 2-fold higher, respectively, than

TABLE II – BIOLOGICAL CHARACTERISTICS IN TUMORS FROM RANDOMIZED PATIENTS

	Group A <sup>1</sup>	Group B <sup>2</sup>
p53 expression		
Positive cases	55%	49%
p53 <sup>+</sup> cells (median, %)	34.5	24.6
bcl-2 expression		
Positive cases	13%	16%
bcl-2 <sup>+</sup> cells (median, %)	5.0	12.5
bax expression		
Positive cases	55%	56%
GST- $\pi$ expression		
Positive cases	62%	53%
TLI		
Median value	11.9%	14.5%

<sup>1</sup>Surgery  $\pm$  RT. <sup>2</sup>Chemotherapy + surgery  $\pm$  RT.

TABLE III – BIOMARKERS AND CLINICAL OUTCOME IN PATIENTS SUBMITTED TO SURGERY AS FIRST-LINE TREATMENT (GROUP A)

	Disease-free survival at 3 years, % <sup>1</sup>	HR (95% CI) <sup>2</sup>
GST- $\pi$ expression		
Negative/weak	51 (32)	1.5 (0.5–4.2)
Moderate/strong	71 (19)	
p53 expression		
Negative	76 (24)	2.0 (0.7–5.8)
Positive	52 (27)	
bcl-2 expression		
Negative	61 (44)	1.2 (0.3–5.3)
Positive	71 (7)	
bax expression		
Negative	67 (21)	1.2 (0.4–3.4)
Positive	58 (26)	
TLI		
$\leq 14\%$	62 (31)	1.5 (0.6–4.2)
$> 14\%$	64 (20)	

<sup>1</sup>In parentheses, number of cases. <sup>2</sup>HR, hazard ratio; CI, confidence interval.

TABLE IV – BIOMARKERS AND CLINICAL OUTCOME IN PATIENTS SUBMITTED TO PRIMARY CHEMOTHERAPY (GROUP B)

	Pathological response <sup>1, 2</sup>	Disease-free survival at 3 years, % <sup>2</sup>	HR (95% CI) <sup>3</sup>
GST- $\pi$ expression			
Negative/weak	50 (15/30)	72 (30)	1.2 (0.4–3.3)
Moderate/strong	16 (3/19)	67 (19)	
p53 expression			
Negative	40 (10/25)	67 (25)	1.3 (0.5–3.8)
Positive	33 (8/24)	74 (24)	
bcl-2 expression			
Negative	37 (15/41)	75 (41)	2.8 (0.9–8.9)
Positive	38 (3/8)	47 (8)	
bax expression			
Negative	50 (10/20)	68 (20)	1.1 (0.4–3.4)
Positive	28 (7/25)	71 (25)	
TLI			
$\leq 14\%$	33 (7/21)	70 (21)	1.0 (0.4–3.0)
$> 14\%$	39 (11/28)	70 (28)	

<sup>1</sup>Grade-II and Grade-III regression. <sup>2</sup>In parentheses, number of cases. <sup>3</sup>HR, hazard ratio; CI, confidence interval.

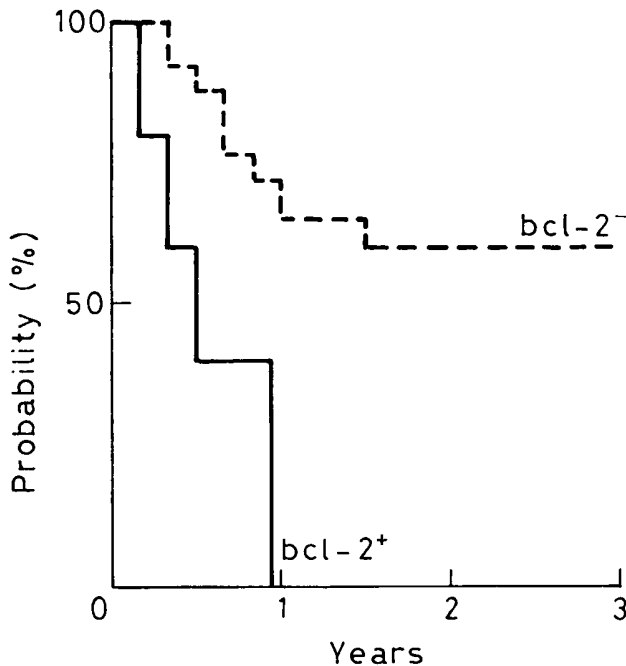
those observed for patients with tumors over-expressing GST- $\pi$  and bax. However, both variables lost relevance as predictors of DFS. Patients with bcl-2-negative tumors had a 75% probability of DFS compared with only 47% observed for patients with bcl-2-expressing tumors ( $p = 0.07$ ).

Since all responder patients were alive and disease-free at 3 years, we analyzed DFS as a function of biological variables within the sub-set of non-responder patients. Again, bcl-2 expression was the only discriminant of DFS (Fig. 1). In particular, all patients with bcl-2-positive tumors (5 cases) relapsed within 1 year, whereas a 60% probability of DFS was observed for the 26 patients with bcl-2-negative tumors ( $p = 0.02$ ).

## DISCUSSION

Correlative studies, in which the determination of biological markers is foreseen and prospectively performed within the context of clinical treatment protocols, are of remarkable interest to validate preliminary evidence of some relation between biomarkers and clinical outcome and to verify the usefulness of biological characterization in clinical practice. In fact, for head-and-neck cancers, the role of biomarkers on clinical outcome has been generally derived from retrospective analyses on different sub-sets of patients and rarely prospectively validated, with possible selection bias for case series under investigation, as well as





**FIGURE 1** – Disease-free survival as a function of bcl-2 expression in non-responder patients. ---, bcl-2-negative tumors; —, bcl-2-positive tumors. Hazard ratio = 4.3 (95% confidence interval = 1.3–14.3),  $p = 0.02$ .

possible different predictivity of biomarkers in different tumor sites, even in consideration of clinical and biological heterogeneity of this neoplasm. Moreover, the different functional aspects of tumor cells have been occasionally investigated within a multifactorial frame, to assess their relative role in predicting treatment response. The activation of randomized clinical protocols on neo-adjuvant chemotherapy, in which pathological response to chemical agents is assessed after a few cycles, and the high feasibility of immuno-cytochemical determination of multiple markers on small tumor specimens prompted correlative studies on consecutive series of patients.

In the present study, we investigated the prognostic and predictive role of biomarkers involved in different cell functions, such as proliferation (TLI), repair of DNA damage (p53 expression), control of programmed cell death (bcl-2 and bax expression) and detoxification of electrophilic compounds (GST- $\pi$  expression), on clinical outcome of patients with resectable oral-cavity cancer and entered in a randomized trial comparing surgery alone with primary chemotherapy plus surgery. A main purpose of the study was to evaluate the predictive role of pre-treatment biomarkers in terms of obtaining a pathological response, with a view to the clinical need to look at biomarkers able to indicate highly chemosensitive patients who may really benefit from PCT. Although the biological characterization was prospectively carried out on tumor specimens obtained only at the Istituto Nazionale Tumori of Milan (accounting for about 50% of the whole enrolled series), results could be considered as representative of those achievable from a multicenter study. In fact, the main clinico-pathological features of the patient series biologically characterized were superimposable to those observed for the overall series.

In the present series of resectable T2–4N0–2 squamous-cell carcinomas of the oral cavity, the fraction of tumors rapidly proliferating and over-expressing p53, bcl-2, bax or GST- $\pi$  was in keeping with published results on this tumor type.

As a whole, in the group subjected to surgery alone with or without radiotherapy, none of the investigated markers significantly influenced clinical outcome. The findings regarding expres-

sion of p53, GST- $\pi$  or bcl-2 were in agreement with results on series of patients similar in terms of site and stage of disease (Veneroni *et al.*, 1997). The lack of prognostic relevance of cell proliferation, as reported earlier, could in part be explained by the impact of radiation therapy, which was administered in about 40% of cases. In fact, a radiation benefit for sub-sets of patients with tumors characterized by specific biological features or similar molecular pathways has been reported (Silvestrini *et al.*, 1997).

Conversely, the investigated markers variously predicted chemotherapeutic efficacy and outcome. In particular, patients with GST- $\pi$ -negative tumors more frequently achieved pathological response to cisplatin and 5-fluorouracil treatment than patients with GST- $\pi$ -positive tumors. Similar findings have been observed in patients with non-small-cell lung cancer submitted to a similar chemotherapeutic regimen (Bai *et al.*, 1996) as well as in patients with pharyngeal and laryngeal carcinoma treated with radiotherapy (Tanita *et al.*, 1993). bcl-2 expression was not indicative of pathological response, in agreement with results reported for patients with locally advanced squamous-cell carcinoma of the esophagus (Puglisi *et al.*, 1996) or with epithelial ovarian tumors (van der Zee *et al.*, 1995; Herod *et al.*, 1996) treated with chemotherapeutic regimens including cisplatin. However, bcl-2 expression appeared to be a significant predictor of 3-year DFS. Such a finding indicates that absence of bcl-2 expression, by itself an unfavorable prognostic marker in several neoplasms submitted to local-regional treatment, becomes a favorable indicator of long-term outcome in non-responder patients, *i.e.*, in a situation of natural history. A similar observation was reported by Gallo *et al.* (1996) and Friedman *et al.* (1997) in patients with early-stage head-and-neck cancer treated with curative radiotherapy or surgery. The mechanisms by which disordered bcl-2 expression could lead to shorter survival are at present speculative. In fact, in addition to the possibility that absent or weak bcl-2 expression favors apoptosis induced by drugs, the maximum influence of bcl-2 in non-responder patients could be explained by the hypothesis that bcl-2 over-expression prevents natural apoptosis in such tumors, thereby inducing more rapid accumulation of malignant cells. Although in other neoplasms the determination of other apoptosis-related markers allowed more accurate detection of patients with an unfavorable clinical outcome, in the present study, the combined analysis of bcl-2 and bax only marginally improved bcl-2 predictivity. In fact, lack of bax expression provided prognostic information only in non-responder patients with bcl-2-negative tumors (data not shown).

No relation was observed between p53 expression and chemotherapy response, in agreement with some data reported in esophageal (Puglisi *et al.*, 1996) and ovarian (van der Zee *et al.*, 1995; Herod *et al.*, 1996) carcinomas but not with other findings on the latter tumor type (Righetti *et al.*, 1996). Such an observation emphasizes that resistance to regimens including cisplatin is a complex process, involving defects in cell-membrane transport, detoxification and alternative metabolic pathways or changes in DNA repair. Cell proliferation did not appear as a predictive factor in patients submitted to primary chemotherapy. However, the presence of an S phase-specific drug, *i.e.*, 5-fluorouracil, in the chemotherapeutic regimen could be responsible for a possible advantage derived from primary chemotherapy for rapidly proliferating tumors, whose clinical behavior paralleled that observed for more indolent, slowly proliferating tumors.

In conclusion, this interim analysis indicates that some biofunctional markers, which appear as prognostic indicators in several tumor types, can provide information on pathological response to primary chemotherapy and may help us to understand treatment efficacy at a cellular level. Obviously, suggestions emerging from this correlative study should be interpreted with caution and need to be confirmed within this ongoing clinical trial, as well as validated in other and similar clinical settings.

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