Expression of DNA Topoisomerase IIα and Topoisomerase IIβ Genes Predicts Survival and Response to Chemotherapy in Patients with Small Cell Lung Cancer[1]

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ABSTRACT

Drug resistance is a major problem in patients with small cell lung cancer; in fact, most die of resistant disease, despite an initial response. Several markers of drug resistance have been described in preclinical models, but the mechanism of drug resistance in lung cancer patients remains unknown. The objective of this study was to evaluate the role of the expression of a number of markers of drug resistance, proliferation, and apoptosis in relation to response to chemotherapy and survival in patients with small cell lung cancer. Tumor samples were derived from 93 previously untreated patients who were randomized in a Phase III study to receive cyclophosphamide, epirubicine, and etoposide or cyclophosphamide, epirubicine and vincristine alternating with carboplatin and etoposide. Paraffin-embedded samples, derived from the primary tumor site prior to chemotherapy, were analyzed by immunohistochemistry for expression of markers implicated in drug resistance [topoisomerase (topo) IIα, topo IIβ, and multidrug resistance-associated protein], apoptosis (p53, p21, and bcl-2), or proliferation (Ki67). Response prediction was analyzed by χ^2 test and logistic regression analysis; overall and disease-free survival curves were compared by log-rank test and Cox regression analysis. Shorter survival was observed in patients with extensive disease ($P = 0.037$) and poorer performance status ($P = 0.028$) and in patients whose tumors expressed high topo IIα levels ($P = 0.01$) and high Ki67 ($P = 0.024$). By multivariate analysis, the following factors were found to be predictive for worse survival: high expression levels of topo IIα, Ki67, and bcl-2; male sex; and extensive disease. High topo IIβ expression was found to be predictive for lower overall and complete response rate. No relationship between apoptotic pathway markers or MRP and response to chemotherapy was observed. In conclusion, high expression of topo IIα was predictive of worse survival, and high expression of topo IIβ was predictive of lower response rates. Furthermore, lower survival probability was observed in patients with bcl-2-positive tumors. Immunohistochemical assessment of these markers in diagnostic biopsies may give important prognostic information and may help selecting patients in the worse prognostic categories for new therapeutic strategies.

INTRODUCTION

Lung cancer is the major cause of cancer-related death in Western countries. The incidence of lung cancer in the United States is 70 per 100,000 men (1). SCLC[4] accounts for 20–25% of all cases. Whereas radical surgery is feasible in 30% of NSCLC cases, the mainstay treatment of SCLC is combination chemotherapy, and surgery is rarely indicated. Although initial high response rates (50–90%) are seen in SCLC, in most cases, highly resistant relapses occur within a year of treatment, and the 5-year survival rate is <5% (2). The drug resistance mechanisms involved in the development of resistant SCLC are unknown.

From the many mechanisms involved in drug resistance, Pgp does not seem to play an important role in lung cancer because expression levels are low or undetectable in lung tumors (3), although in a small study of seven SCLC patients, Pgp expression was reported exclusively in all four samples derived from nonresponding patients by reverse transcriptase-PCR (4).

We investigated two other targets that are of potential interest because they are implicated in resistance to drugs of common use in the treatment of SCLC (*e.g.*, doxorubicin and etoposide). The first targets are the type II DNA topos: topo IIα is mainly expressed in proliferating cells, and a good correlation was observed with the expression of known proliferation markers in solid tumors (5, 6) and in leukemia (7, 8), whereas the expression of topo IIβ appears rather constant during the cell cycle (9). *In vitro* studies have suggested a relationship between topo IIα expression and response to chemotherapeutic drugs in selected and unselected SCLC cell lines (10–12), with low expression levels predicting relative resistance to drugs. In some recent studies, alterations of topo IIβ were also shown to be

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[4] The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; Pgp, P-glycoprotein; topo, topoisomerase; MRP, multidrug resistance-associated protein.

associated with drug resistance (12, 13). The second target is the recently discovered MRP, that has been shown to be overexpressed in SCLC cell lines selected for resistance to chemotherapy (14). Natural drugs as doxorubicin are transported by MRP. Although high expression levels of MRP have been seen in both normal lung tissue and lung cancer samples (15, 16), little is known about MRP expression in SCLC and its relationship with chemosensitivity.

The effector pathway of many of the frequently used chemotherapeutics is induction of apoptosis through the DNA damage that these drugs cause. Recent evidence strongly suggests that downstream events are important determinants of the response to treatment. Wild-type p53 induces either the *WAF1/CIP1* growth suppressor gene, which codes for p21 or the bcl-2/bax pathway. Expression of $p21^{WAF1}$ leads to cell cycle arrest, which may allow the cell to repair DNA damage. However, *WAF1* can also be induced by a p53-independent pathway (17). The interaction between the proto-oncogenes *bcl-2* and *bax* and other members of the bcl-2 family may shift the equilibrium toward apoptotic cell death or survival. Wild-type p53 regulates bcl-2 and bax expression by inhibition and up-regulation, respectively (18); however, the tumor suppressor gene *p53* is mutated in ~70% of SCLCs (19, 20). Both bcl-2 and bax expression in SCLC cell lines and tumors are high (21–23), and no difference in expression was observed between drug-sensitive and drug-resistant cell lines (23). Transfection studies have shown that bcl-2 can confer drug resistance to etoposide and doxorubicin (24, 25).

Through aberrant expression, either increased or decreased, of the above-mentioned molecules, drug resistance can occur in SCLC. The aim of this study was to find which markers from the above-mentioned group were involved and could be used to predict response to chemotherapy and survival in SCLC patients. This study represents one of the largest investigation of several clinical and biological markers ever conducted in SCLC patients treated in a homogeneous fashion.

MATERIALS AND METHODS

The patients analyzed in this study were included in a Phase III trial of the Noord-Hollandse Oncology Group.[5] Previously untreated patients with SCLC were randomly assigned to receive three courses of cyclophosphamide, epirubicine, and vincristine alternating with three courses of carboplatin and etoposide or six courses of cyclophosphamide, epirubicine, and etoposide; chemotherapy cycles were given every 3 weeks. Inclusion criteria were: age $<$ 75 years; no second primary tumor; no prior chemotherapy; adequate liver, kidney, and heart function; no symptomatic brain metastasis; and WHO performance status $\leq$ 3. Response was evaluated according to standard criteria (26). Radiological responses were revised by two independent observers. All patients achieving a complete remission and those with limited disease who achieved a partial remission were given locoregional radiotherapy. Prophylactic cranial irradiation was given only to patients with complete response, which was confirmed by rebronchoscopy and negative histology. Patients were selected for this study based on the availability of adequate paraffin-embedded material from the primary tumor prior to chemotherapy, consisting of $>$10% tumor cells. From eight patients, we had also tumor biopsies taken at restaging, and these could be compared with the prechemotherapy biopsies.

Immunohistochemistry. The following antibodies were used, along with isotype antibodies as negative controls: Ki-S1 for topo IIα (Boehringer-Mannheim, Mannheim, Germany), diluted 1:100 (27); 3H10 for topo IIβ, kindly provided by Dr. Kikuchi (28), diluted 1:100; MIB1 for Ki67 (DAKO, Glostrup, Denmark), diluted 1:40; DO7 for p53 (DAKO), diluted 1:500; clone EA10 for p21 (Oncogene, Manahasset, NY), diluted 1:100; clone 100 for bcl-2 was kindly provided by Dr. J. L. Cordell (29), diluted 1:50; and MRPr1 for MRP, kindly provided by Dr. R. J. Scheper (30), diluted 1:100. 3H10, clone EA10, and clone 100 were processed by signal intensification with biotinylated tyramine.

Four-μm sections were cut from the paraffin-embedded tumor samples and collected on poly-L-lysine-coated glass slides. The slides were allowed to dry overnight in a 37°C incubator and stored at room temperature until use. The samples were evaluated for the percentage of tumor cells by H&E staining. All staining procedures were according to the avidin-biotin complex method (31): briefly, samples were deparaffinized in xylol, rehydrated, and incubated with methanol-0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. When appropriate, antigen retrieval was performed by microwave heating of the slides in a 10 mmol/liter citrate buffer at pH 6.0; subsequently, slides were allowed to cool down at room temperature for at least 20 min. Slides were preincubated with normal rabbit serum (DAKO; 1:50) or normal goat serum (DAKO; 1:50; MRPr1) for 10 min followed by overnight incubation at 4°C with the primary antibody or with an irrelevant isotype-matched antibody as negative control. The next day, the slides were rinsed in PBS and the biotinylated secondary antibody rabbit-antimouse (DAKO; 1:500) or, in the case of MRPr1 staining, goat-antirat (Jackson Immunoresearch Laboratories, West Grove, PA; 1:200) was applied for 30 min. After rinsing, the slides were incubated with the avidin-biotin complex (Strept ABComplex, 1:200; DAKO) for 1 h. When the use of biotinylated-tyramine intensification step was used, the avidin-biotin complex was applied for 30 min in 1:1000 dilution and washed in PBS, followed by a 10-min incubation with biotinylated tyramine (1:1000) supplemented with 0.01% H_2O_2, and after careful rinsing, the avidin-biotin complex (1:200) was applied for 30 min. As substrate, chromogen 3′-diaminobenzidine tetrahydrochloride (DAKO, Carpinteria, CA) was applied for 3 min. The slides were counterstained with hematoxylin-NH_3, dehydrated, and coverslipped with Depex (BDH Laboratory Supplies, Poole, Dorset, England). Percentage of staining tumor cells was assessed independently by two investigators who were blinded to knowledge of clinical outcome of patients.

Statistics. Cutoff points were decided before assessing the percentage of staining tumor cells in the samples. No established cutoff points for the topos and Ki67 could be found in the literature. On the basis of our previous experience with these markers, three groups of topo IIα-, topo IIβ-, and Ki67-express-

[5] Manuscript in preparation.

ing tumors of similar dimensions were identified: low (<30% positive tumor cells), intermediate (30–60% positive tumor cells), and high (>60% positive tumor cells) expression levels, because these values could be easily discriminated due to the distribution pattern. For p53, p21, bcl-2, and MRP, samples were divided in two groups, positive or negative, with a cutoff value of 10% positive tumor cells, which was based on findings of previous reports (32–35).

Statistics was performed by using the SPSS software program (SPSS Inc., Chicago, IL). To evaluate concordance between the two observers in estimating the percentage of tumor cells, κ values were calculated. Groups were compared by using the Pearson χ^2 test. Kaplan-Meier analysis was used to analyze survival rates, and the log-rank test was used to compare survival curves. The logistic regression model was used to estimate prognostic factors for response rate and 1- and 2-year survival rates. Prognostic factors for survival were evaluated by Cox regression analysis. For both logistic and Cox regression analysis, the backward stepwise regression strategy was adopted and all variables that had a P of <0.2 in univariate analysis were included in the model (36). Differences were regarded as significant when the P was <0.05. All Ps were two-sided, and the data used were consistent with the assumptions of Cox proportional hazards analysis.

Table 1 Patient characteristics

Characteristics	No. of patients
Total no. of patients	93
Age, yr (mean ± SD)	63 ± 8.4
Sex (male/female)	73/20
Disease extent	
Limited	46
Extensive	47
Chemotherapy[a]	
CEE	45
CEV/PE	48
Response	
Complete response	33
Partial response	44
Stable disease	4
Progressive disease	11
Not evaluable	1
WHO performance score	
0	56
1	33
2	3
3	1
Median follow-up, weeks	291
Median survival, weeks	47

[a] CEE, cyclophosphamide-epirubicine-etoposide; CEV/PE, cyclophosphamide-epirubicine-vincristine alternated with carboplatin-etoposide.

RESULTS

Of the 179 patients included in the Phase III trial, for this study, adequate material of the primary lung tumor prior to treatment was available for 93 patients. In some of the patients, there was no primary tumor material because the diagnosis was made by needle aspirate or biopsy of a metastatic tumor site. In other cases, the biopsies were (nearly) completely used for diagnostic purposes. Patient characteristics of the 93 patients are displayed in Table 1, and they are similar to the characteristics of the total group (data not shown). At the time of this analysis, 6 of the 93 patients were still alive, 3 with no evidence of disease. Median follow-up was 291 weeks (first patient censored after a follow-up of 113 weeks).

Immunohistochemistry Results. κ values for concordance between the two observers varied between 0.40 and 0.73, with the lowest κ value for the evaluation of MRP staining. The majority of samples contained enough tumor material to perform staining with all antibodies. When three groups of nuclear topo IIα expression were distinguished (n = 92), 40 patients displayed low, 26 displayed intermediate, and 26 displayed high expression levels. In 26 cases, cytoplasmic staining was also observed; in 13 of these cases, cytoplasmic staining was observed in the group with low nuclear topo IIα expression. topo IIβ (n = 84) stained in a discrete granular nuclear staining pattern in most cases; 28 patients had low levels of topo IIβ expression, 21 patients had intermediate expression levels, and 35 had high expression levels. Most tumors were in the intermediate expression level group for Ki67 (n = 91: 58 in the intermediate, 19 in the low, and 14 in the high expression group). p53 nuclear staining was positive in half of the patients (48 of 93 cases). p21 staining (n = 85) was nuclear, with scattered positive cells and low expression levels, and in addition, immunopositivity was observed in normal bronchial epithelium covering the tumor: 63 patients had p21-negative tumors. Staining with bcl-2 was cytoplasmic in all cases, and bcl-2 expression was present in the majority of tumors (71 of 91 evaluable cases). MRP displayed a granular cytoplasmic staining pattern with the typical membranous staining in sporadic cases, and MRP was positive in 69 of 89 evaluable cases.

Table 2 shows the relationships among all of the markers tested. A significant positive correlation was observed between topo IIα and topo IIβ. Significant positive correlations were also observed between p21 and topo IIβ and both p53 and bcl-2, but no correlation was observed between p53 and bcl-2 expression. Furthermore, p53 was correlated significantly with both topo IIα and topo IIβ. A weak but not statistically significant correlation was observed between topo IIα and the proliferation marker Ki67. No difference in expression of the markers was seen between limited and extensive disease.

From eight patients from whom we had multiple samples of the primary tumor, before and after chemotherapy, no significant change was observed of any of the markers analyzed (data not shown), with the exception of topo IIβ expression, which significantly increased in the postchemotherapy samples (P = 0.018; Wilcoxon signed rank test).

Survival Analysis. The median overall survival was 47 weeks (95% confidence interval, 41–53 weeks), and the median progression-free survival was 32 weeks (95% confidence interval, 30–35 weeks). No difference in overall or progression-free survival was observed between the two treatment arms [median overall survival was 42 *versus* 48 weeks for the alternating and sequential regimens, respectively (P = 0.42)]. Overall and progression-free survival rates were longer in patients with limited disease than in patients with extensive disease [58 *versus* 40 weeks (P = 0.037) and 34 *versus* 27 weeks (P = 0.009), respectively]. In addition, longer survival rates were observed in

Table 2 Relationships between expression of biological markers[a]

	topo IIα			Ki67			topo IIβ			p53		p21		bcl-2	
Marker	−	+	++	−	+	++	−	+	++	−	+	−	+	−	+
MRP															
−	8	7	5	3	13	4	10	4	5	10	10	17	2	7	12
+	32	18	18	16	41	10	18	16	27	37	32	43	19	12	57
bcl-2															
−	7	7	6	4	11	5	6	5	7	12	8	18	1		
+	32	19	19	15	45	9	21	16	27	35	36	43	21[b]		
p21															
−	29	15	18	11	42	10	26	13	19	37	26				
+	6	9	7	5	13	4	0	7	15[c]	5	17[d]				
p53															
−	27	11	9	13	29	5	21	9	13						
+	13	15	17[e]	6	29	9	7	12	22[f]						
topo IIβ															
−	17	7	3	7	20	1									
+	5	10	6	6	9	6									
++	12	6	17[g]	6	24	5									
Ki67															
−	10	6	3												
+	27	12	18												
++	2	7	5												

[a] For topo IIα, topo IIβ, and Ki67: −, <30%; +, 30–60%; ++, >60% staining tumor cells. p53, p21, and bcl-2: −, ≤10%; +, >10% staining tumor cells. Only significant *P*s (Pearson χ^2 analysis) are indicated. All *P*s are two-sided and were considered statistically significant at <0.05.
[b] $P = 0.018$.
[c] $P = 0.001$.
[d] $P = 0.004$.
[e] $P = 0.019$.
[f] $P = 0.008$.
[g] $P = 0.003$.

patients with performance score 0 than in patients with performance score 1, 2, or 3 at diagnosis (52 *versus* 36 weeks, $P = 0.028$). Women had a longer overall survival than men (median, 62 *versus* 45 weeks), but this difference did not reach statistical significance ($P = 0.17$). Table 3 reports 1- and 2-year survival rates, and the *P*s reported were calculated by χ^2 analysis because all patients had a follow-up of at least 2 years.

Because similar survival was observed between patients in the low and intermediate expression level groups for topo IIα (Table 3 and data not shown), these two groups were combined in further analyses. Patients with high topo IIα levels had worse overall ($P = 0.01$; Fig. 1) and progression-free ($P = 0.006$) survival rates than did patients in the combined low and intermediate expression level group. When stratified by disease extent, high topo IIα levels predicted worse survival in the patients with limited disease ($P = 0.0006$) but not in those with extensive disease ($P = 0.60$; Fig. 2). The same result was observed in the patients who obtained a response to chemotherapy. Similar to the results obtained with topo IIα were those observed with Ki67: when all three expression groups were considered, the difference did not reach statistical significance ($P = 0.07$), but the difference between the group with low expression and the combined group with intermediate and high Ki67 was significant ($P = 0.024$; Fig. 3).

No difference in overall or progression-free survival was observed between patients depending on p53 and p21 expression, in the total group or in groups stratified by response or disease extent. Shorter survival rates were observed in patients with bcl-2-positive tumors, but this did not reach statistical significance ($P = 0.09$). When patients were stratified according to response to chemotherapy (responders *versus* nonresponders), in the responders group significant worse survival rates were observed in the patients with bcl-2-expressing tumors ($P = 0.02$). No difference was observed between patients based on disease extent. In addition, when four subgroups were created based on p53 and bcl-2 expression, in the p53-negative group, worse survival rates were observed in the patients with bcl-2-positive tumors than in patients with bcl-2-negative tumors ($P = 0.07$), whereas the survival rates were not different in the p53-positive group. No difference was observed in survival rates between patients with MRP-positive and -negative tumors.

Table 4 displays the Cox regression analysis results, for which only those variables with *P*s of <0.2 by univariate analysis were included. For overall survival, disease extent, sex, performance score, topo IIα, Ki67, and bcl-2 were entered in the regression analysis, of which disease extent, sex, bcl-2, Ki67, and topo IIα were found to be of prognostic value. Female sex, limited disease, and low expression of bcl-2, Ki67, and topo IIα were related to longer survival. In patients with limited-stage disease, topo IIα and performance score were prognostic factors for overall survival, and Ki67, topo IIβ, and bcl-2 were deleted from the model. In patients with extensive-stage disease, only Ki67 was of prognostic value for survival, and topo IIβ and performance score were dropped from the regression equation.

Response. Most patients obtained a complete or partial response to the treatment (overall response rate of 83%). Response rates were similar between the two treatment arms, but patients with limited disease achieved a complete response to

Table 3 One- and two-year survival rates[a]

	1-yr survival (%)	χ^2	*P*	2-yr survival (%)	χ^2	*P*
Disease extent						
Limited	52			20		
Extensive	34	3.12	0.08	9	2.36	0.12
Sex						
Male	40			12		
Female	55	1.49	0.22	20	0.88	0.38
Performance score						
0	52			16		
1–3	30	4.42	0.04	11	0.51	0.47
topo IIα						
<30%	53			18		
30–60%	50			15		
>60%	23	6.18	0.046	8	1.30	0.52
topo IIβ						
<30%	46			14		
30–60%	43			19		
>60%	40	0.26	0.88	11	0.62	0.73
Ki67						
<30%	68			32		
30–60%	38			12		
>30%	36	5.86	0.053	0	7.21	0.027
p53						
≤10%	42			15		
>10%	44	0.07	0.79	13	0.03	0.86
p21						
≤10%	43			14		
>10%	41	0.03	0.87	9	0.39	0.53
bcl-2						
≤10%	45			30		
>10%	42	0.05	0.83	10	5.17	0.023
MRP						
≤10%	35			10		
>10%	46	0.82	0.37	16	0.44	0.51

[a] All patients were followed for at least 2 years. All *P*s are two-sided and were considered statistically significant at <0.05.

treatment more frequently than did patients with extensive disease. Table 5 displays the relationships between response rates and the investigated markers as well as other known prognostic markers for this disease. When three groups were available, statistics were also performed on the combination of two groups *versus* the third. Lower overall response rates were observed in the patients with high topo IIβ-expressing tumors, and when only two groups of topo IIβ-expressing tumors were distinguished (low plus intermediate *versus* high expression), this difference reached significance ($\chi^2 = 5.0$, $P = 0.025$). Furthermore, higher complete response rates were seen in the patients with low and intermediate topo IIβ-expressing tumors ($\chi^2 = 7.58$, $P = 0.006$). None of the other investigated markers was predictive for response to treatment; however, when two groups were made for topo IIα expression, the high expression group had less complete responses than the combined group of low plus intermediate expression, and the difference approached significance ($P = 0.062$). Only the parameters that generated $P < 0.2$ by univariate analysis were included into the multivariate analysis: topo IIβ was the only predictor for overall response rate by multivariate analysis (regression coefficient, 1.30; $P = 0.03$), whereas disease extent (regression coefficient, 2.21; $P = 0.0003$), topo IIβ (regression coefficient, 1.31; $P = 0.031$), and topo IIα (regression coefficient, 1.39; $P = 0.045$) were found to be predictive for complete response rate.

DISCUSSION

In this study, we assessed a number of biological markers related to cell survival and drug resistance in patients with SCLC. To our knowledge, this is the largest study in which several biological prognostic markers were studied in a homogeneous group of SCLC patients. Due to the difficulty in obtaining sufficient and adequate tumor material, only a few small studies have thus far been performed in SCLC, in contrast to studies in other malignancies. Furthermore, in our study, we exclusively analyzed samples obtained from the primary tumor site because expression of some markers, for example, p53, may be different in metastatic sites (37).

Response to Chemotherapy. Although most SCLC patients achieve a good response to chemotherapy, the majority of patients relapse and die of the disease. Only patients who attain a complete response may experience long-term survival or be cured of the disease (2). There have not been substantial improvements in the treatment of SCLC in extensive-disease patients in the last 20 years, and probably only the addition of radiation therapy to combination chemotherapy has had an impact in limited disease (2).

Disease extent is a known prognostic marker with regard to response to chemotherapy and survival (2). Also, in our study, patients with limited disease had higher complete response rates and longer survival than patients with extensive disease. However, performance status, often reported as an important prognostic factor for survival and response, was not retained in the multivariate analysis in our study. Because most patients with SCLC obtain a response to chemotherapy, response prediction is not easy to assess. Furthermore, the clinical assessment of complete responses is rather imprecise because the discrimination between residual tumor and scar tissue is very difficult by routine radiological tools. An interesting finding in our study was the relationship between topo IIβ expression and the response to treatment, with higher response rates in patients with low or intermediate topo IIβ-expressing tumors. This observation contrasts with *in vitro* studies in which decreases of topo IIβ are associated with increased drug resistance in cell lines selected for resistance to topo II inhibitors (12, 13).

In our study, multivariate analysis showed that disease extent and low expression of both topo IIβ and topo IIα were of prognostic value for the complete response rate. Although topo IIα and IIβ isoforms have been reported to have different functions, we observed a good correlation between them, and both were of prognostic value for complete remission rate. A good correlation between topo IIα and topo IIβ expression has been reported in other malignancies (7, 38) but not in NSCLC (5). Most *in vitro* studies have reported a direct correlation between expression of topo IIα and sensitivity to drugs (39); furthermore, the higher expression of this enzyme in SCLC compared to NSCLC has been reported as a possible reason for the higher chemosensitivity of SCLC (40). Our results are in contrast with the general idea that a higher expression of topo enzymes correlates with a greater chemosensitivity. However, thus far, no clinical study has convincingly correlated the ex-

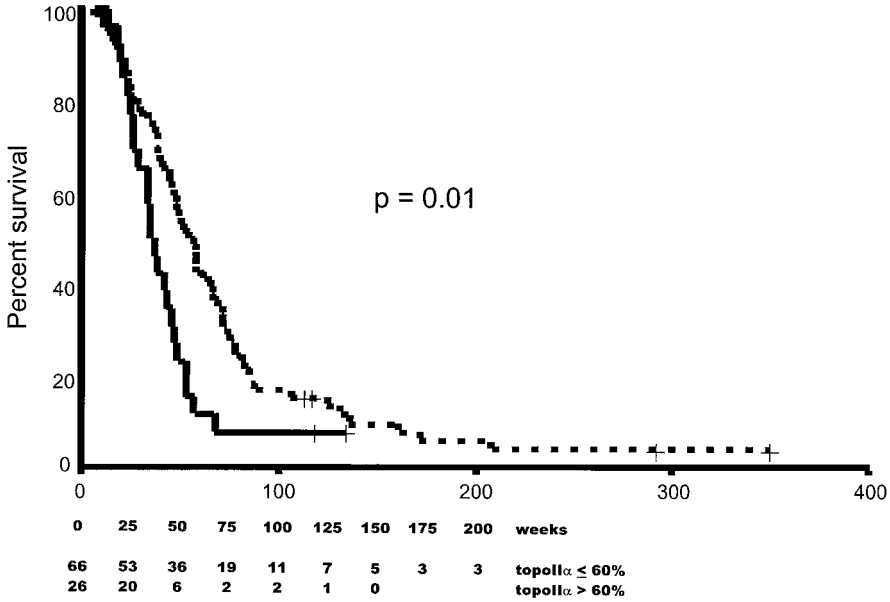

Fig. 1 Kaplan-Meier curves of the overall survival for all series depending on topo IIα expression. Two groups of topo IIα-expressing tumors were distinguished: topo IIα ≤ 60% (*dotted line*, $n = 66$) and topo IIα > 60% (*solid line*, $n = 26$). Significantly worse overall survival rates were observed in the patients with high topo IIα levels ($P = 0.01$). +, censored values. *Numbers*, numbers of patients at risk.

pression of topo genes to chemosensitivity (41). Moreover, although both isoenzymes have been reported to be target of topo II poisons, it is unclear which of these enzymes is more important in determining drug action and resistance (39). In our study, topo II inhibitors were actually administered together with other agents which have different targets, and this might partially explain the contrast between the *in vitro* results and these findings. The relatively high rate of p53 mutations in SCLC (19–20), compared to other solid tumors and hematological malignancies, may, in part, be responsible for this discrepancy. Wild-type p53, in fact, acts as a transcriptional repressor of *topo IIα* gene expression (42, 43), and mutations of p53 may lead to reduced regulatory suppression of this enzyme, accelerated cell proliferation, and dysregulation of cell growth. The resulting higher expression of topo IIα may, therefore, have little influence compared to the lower sensitivity to drugs induced by mutant p53 (44). This hypothesis is further suggested by the correlation between the expression of p53 and both topo II enzymes (see Table 2). Although not known, these data suggest that also the transcription of topo IIβ is under the control of p53.

A cytoplasmic staining has been reported in cell lines with a mutated *topo IIα* gene (45, 46) and selected for resistance to chemotherapy. The reported mutations belong to clusters constituting Nuclear Localization Signals, essential for transportation of the mature protein into the nucleus. In our study, however, no relation was observed between cytoplasmic localization of topo IIα, which we observed in part of the samples, and reduced response to chemotherapy.

From eight patients, multiple samples were available, before and after chemotherapy, at restaging of bronchoscopy; the remaining tumor material in the second biopsy may be regarded as resistant tumor. Of all of the markers analyzed, no significant change was observed between the two samples, with the exception of topo IIβ expression, which significantly increased in the postchemotherapy samples. This finding further supports the lack of correlation between chemosensitivity and high topo II levels of expression and actually strengthens the opposite hypothesis, that there is a correlation between chemosensitivity and low levels of topo II expression.

Interestingly, expression of MRP, a transmembrane transporter of drugs, originally identified in a doxorubicin-resistant SCLC cell line (14), was of no predictive value for response or survival in our study. Another transporter, MDR1, has been shown not to be expressed or expressed at very low levels in lung cancer (3). In two small studies, MDR1 expression was reported to be predictive of lack of response to chemotherapy in SCLC (4, 47), and in one (47), the presence of MDR1 expression was also correlated with a significantly poorer survival, but these results were based on a very small sample size. Both studies applied reverse transcriptase-PCR, which is a bulk method and does not take into consideration the expression levels in surrounding or infiltrating cells and is not well correlated with other methods (48). Despite several attempts, we were unable to obtain reproducible results of Pgp expression by immunohistochemistry, in the small biopsies we had available.

Survival. Among the clinical factors, disease extent and sex were important prognosticators for survival, retained in multivariate analysis for the whole series, and performance status had prognostic value in limited-disease patients. Interestingly, survival was also correlated with markers of cell proliferation: by multivariate analysis, those with high topo IIα, high Ki67, and high bcl-2 expression had worse survival in the whole series, and topo IIα and Ki67 retained their prognostic value in limited and extensive disease, respectively (see Table 4). topo IIα had prognostic value in patients with limited disease but not in patients with extensive disease (see Fig. 2). In a recent study in breast cancer patients, Ki-S1 expression was identified as an independent prognostic marker, with worse survival in patients with >30% Ki-S1-positive tumor cells (27). Ki-S1 has been

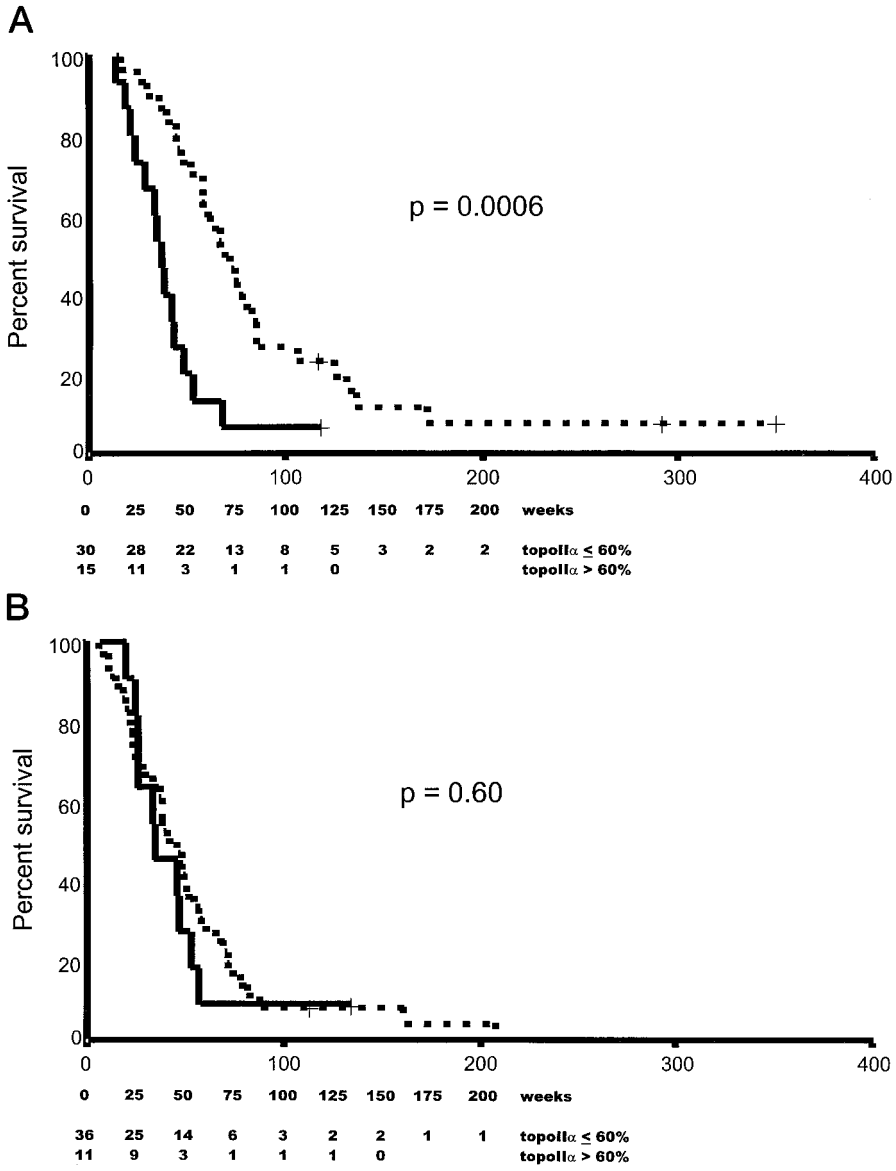

Fig. 2 Kaplan-Meier curves of overall survival stratified by disease extent, according to topo IIα expression. Two groups of topo IIα-expressing tumors were distinguished: topo IIα ≤ 60% (*dotted line*, $n = 30$ and $n = 36$ for limited and extensive disease, respectively) and topo IIα > 60% (*solid line*, $n = 15$ and $n = 11$ for limited and extensive disease, respectively). +, censored values. *A*, results for patients with limited-stage disease ($P = 0.0006$). *B*, results for patients with extensive disease ($P = 0.60$). *Numbers*, numbers of patients at risk.

recognized as a topo IIα-specific antigen (49). Interestingly, although we found a correlation between the expression of the two topo II isoforms, only topo IIα had prognostic value for survival. topo IIα in our study was weakly associated with the proliferation marker Ki67 expression. In the survival analysis, it appears that these markers are correlated because, in fact, one or the other or both had prognostic value for survival. The expression of topo IIα and proliferation by Ki67 or PCNA has been described in several solid tumors and hematological malignancies (41). In breast cancer, topo IIβ and lymph node status were found to be independent prognostic factors for relapse-free survival rates, with high topo IIβ mRNA levels predicting for higher risk of relapse (50). In this study, however, a bulk method (RNase protection assay) was used, by which the estimated expression level is derived from an admixture of stromal and tumor cells, which does not accurately reflect tumor cell expression; furthermore, no relationship between topo IIβ RNA levels and the immunohistochemical data was observed.

With the use of the monoclonal antibody DO7 and the cutoff value of 10%, we observed p53 accumulation in ~50% of cases. The incidence of *p53* gene mutations observed by sequencing appears to be in excess of 70% (20). Because DO7 detects both wild-type and mutant p53, in the attempt to attain more information of the p53 functional status, we also analyzed the expression of p21. Because p21 is induced by wild type p53, p21 expression may indicate the presence of wild-type p53, although p53-independent pathways for p21 induction have recently been described (51). In agreement with another small study in SCLC, a low percentage of p21-positive tumors was observed (52). Interestingly, the tumors with p21 positivity were more frequently observed in correlation with p53 and bcl-2 positivity. In a recent study, p21 expression was found to be a

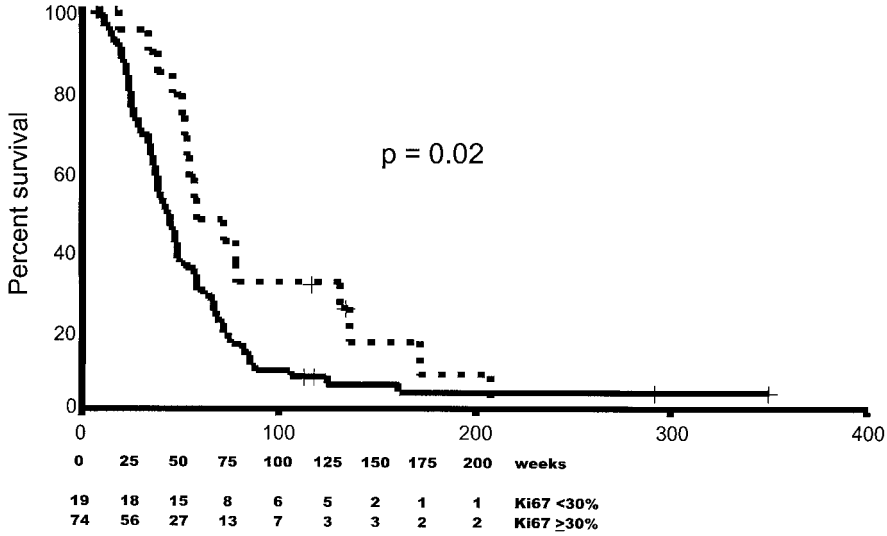

Fig. 3 Kaplan-Meier curves of overall survival for all series depending on Ki67 expression ($P = 0.024$). Two groups of Ki67-expressing tumors were distinguished: Ki67 $< 30\%$ (*dotted line*, $n = 19$) and Ki67 $\geq 30\%$ (solid line, $n = 74$). +, censored values. *Numbers*, numbers of patients at risk.

Table 4 Cox regression analysis for survival

	Regression coefficient	*P*
Total group		
Disease extent	0.71	0.002
Sex	0.71	0.01
topo IIα[a]	0.53	0.04
Ki67[a]	0.85	0.003
bcl-2	0.74	0.02
Limited disease		
topo IIα[a]	1.63	0.0001
Performance score	0.79	0.049
Extended disease		
Ki67[a]	0.63	0.07

[a] For both topo IIα and Ki67, two groups were distinguished: topo IIα low-intermediate *versus* high expression and Ki67 low *versus* intermediate-high expression. All *P*s are two-sided and were considered statistically significant at <0.05.

favorable prognostic marker for survival in patients with radically resected early-stage squamous carcinomas of the lung (53) and in patients with early bladder cancer (34). Maintenance of p21 expression has been suggested to abrogate the deleterious effects of p53 alterations (34). In our study, a correlation was observed between p21 expression and expression of topo IIβ but not topo IIα; the reason for this correlation remains unclear.

bcl-2 expression in SCLC has been reported to be high (54), and in our study, most patients displayed bcl-2 expression. Expression in SCLC is definitely higher than that reported in NSCLC (54, 55). In a large series of 164 SCLC patients, higher complete remission rates, although not statistically significant, were observed in patients with bcl-2-positive tumors (56). We did not observe such a difference, even when we analyzed our data with the cutoff point of 50%, which was used in the study by Kaiser *et al.* (56). In addition, Kaiser *et al.* (56) observed a trend, again not significant, for longer survival rates in patients with bcl-2-positive tumors. This finding was also in general observed in early-stage (in particular, stage I) NSCLC (29, 55, 57). In contrast, in our study, bcl-2 negativity was an independent prognostic factor for longer overall survival, and this was particularly significant in responding patients. The reason for this discrepancy is unclear but does not appear to be related to the choice of the cutoff point. Findings similar to those in this study have been observed in another smaller study of 38 SCLC patients (35) and in a study of patients with head and neck cancer treated by radiotherapy (58); furthermore, bcl-2 expression increased in postchemotherapy biopsies of the head and neck cancer patients (58) and also in neuroblastoma patients after chemotherapy (59). Given the involvement of bcl-2 in the apoptotic pathway induced by chemotherapy, together with bax, bcl-x, and several other members of the bcl-2 family and the delicate balance between these molecules (60), it cannot be excluded that there are different equilibria in different series.

Because p53 and bcl-2 expressions were reported to be inversely correlated in a number of studies, the combination of these two markers was investigated. In a previous study of patients with operable NSCLC, the combination of bcl-2 negative and p53 positive had the worst survival (55). In contrast, here, the combination bcl-2-positive/p53-negative had the worst survival. This finding is likely to reflect a major difference in prognostic value of bcl-2 between NSCLC (55) and SCLC.

In conclusion, in addition to clinical prognostic factors such as disease extent, performance status, sex, and response to chemotherapy, in this study, new biological prognostic markers were observed to have an impact on response to chemotherapy and survival of patients with SCLC. topo IIβ expression was predictive for response to chemotherapy, with higher response rates in patients with low topo IIβ levels. High topo IIα, Ki67, and bcl-2 expression predicted for shorter survival, indicating that the proliferation rate has a major impact on survival in SCLC. Although suggestive, our study must be considered exploratory, and the expression of the topo II enzymes topo IIα and topo IIβ together with Ki67 should be investigated prospectively in a larger patient pop-

Table 5 Impact of clinical and biological markers on response to chemotherapy[a]

	Overall			Complete		
	ORR	χ^2	*P*	CRR	χ^2	*P*
Disease extent						
Limited	89			54		
Extensive	78	1.99	0.16	17	13.66	0.000
Sex						
Male	81			36		
Female	95	2.39	0.12	35	0.01	0.93
Performance score						
0	86			42		
1–23	81	0.31	0.58	27	2.10	0.15
Treatment arm						
CEE	87			40		
CEV/PE	81	0.57	0.45	32	0.56	0.42
topo IIα						
<30%	85			38		
30–60%	85			46		
>60%	80	0.31	0.86	20	4.00	0.14
topo IIβ						
<30%	89			46		
30–60%	91			48		
>60%	71	5.01	0.08	18	7.58	0.02
Ki67						
<30%	84			21		
30–60%	83			38		
>30%	85	0.04	0.98	46	2.52	0.28
p53						
≤10%	85			34		
>10%	82	0.14	0.71	38	0.14	0.71
p21						
≤10%	86			36		
>10%	77	0.79	0.38	41	0.21	0.65
bcl-2						
≤10%	79			32		
>10%	85	0.33	0.56	37	0.17	0.68
MRP						
≤10%	90			32		
>10%	83	0.53	0.47	38	0.24	0.62

[a] ORR, overall (complete plus partial) response rate (%); CRR, complete response rate (%); CEE, cyclophosphamide-epirubicine-etoposide; CEV/PE, cyclophosphamide-epirubicine-vincristine alternated with carboplatin-etoposide. All *P*s are two-sided and were considered statistically significant at <0.05.

ulation. Patients with low chance to enter a complete remission or a long-term survival based on these biological markers may be offered intensified follow-up and experimental treatment.

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