ERCC1 and topoisomerase I expression in small cell lung cancer: Prognostic and predictive implications

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Abstract. Small cell lung cancer is the most aggressive lung cancer subtype. The standard treatment approach is based on cisplatin regimens. Although response rates to treatment are approximately 60-80%, the median survival is still very poor. Excision repair cross complementation group 1 (ERCC1) is an enzyme that removes cisplatin-induced DNA adducts and has been related with prognosis and cisplatin response. Topotecan is the standard treatment as second-line therapy and it is an inhibitor of topoisomerase I (TOP I). We selected 76 patients with small cell lung (SCLC) to analyze the ERCC1 and TOP I mRNA expression. ERCC1 was studied both by quantitative PCR and immunohistochemistry. A significant association was found between the inmunohistochemistry expression of ERCC1 and the lack of platinum response (p=0.001). Moreover, low levels of TOP I RNA were shown to be linked to cisplatin response (p=0.002). In the survival analysis, a significant correlation between a better PFS with a low TOP I RNA expression as well as a negative ERCC1 inmunostaining were found, in both cases with a significant p-value (p=0.02 and 0.009, respectively). In summary, our results suggest the use of ERCC1 immunohistochemistry and TOP I mRNA analysis to predict cisplatin response and prognosis in SCLC patients.

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Introduction

Small cell lung cancer (SCLC) is the most aggressive lung cancer subtype, with a strong tendency for early dissemination as well as high frequency of metastasis. SCLC accounts for 14% of new lung cancer cases diagnosed in USA and Europe (1) and staging determines prognosis and treatment. The standard treatment approach to patients with limited stage (SCLC) and good performance status is a combination of chest radiation and chemotherapy based on cisplatin (P) and etoposide (E), which results in complete response rate of 50-80% and a 5-year survival probability of 12-25% (2). In case of extensive disease (ED), the main treatment is a combination of cisplatin with either etoposide or irinotecan. Reponses rates are approximately 60-80% and the median survival reaches 7-12 months.

Cisplatin causes monoadducts and intrastrand or interstrand cross-links in DNA (3,4). Nucleotide excision repair plays a central role among DNA repair pathways and has been associated with resistance to cisplatin-based chemotherapy. The excision repair cross complementation group 1 (ERCC1) enzyme, plays a rate-limiting role in the nucleotide excision repair pathway which recognizes and removes cisplatin-induced DNA adducts (5). The role of ERCC1 in resolving DNA interstrand cross-link-induced double-strand breaks has been clearly shown (6-8). Various studies have reported the relationship between ERCC1 expression and the effect of cisplatin-induced DNA adducts in human ovarian cancer cells *in vitro* (9), in primary gastric adenocarcinomas (10), colorectal cancer (11) and, more recently, in esophageal cancer (12). Pivotal data from primary non-small cell lung cancer (NSCLC) specimens have suggested a link between ERCC1 immunoexpression and cisplatin resistance. However, for NSCLC untreated patients, a high ERCC1 expression in selected cases was associated with a better survival (13,14). Taken together, all these studies show that ERCC1 expression level could be inversely associated with cisplatin response. However in terms of prognosis, high levels of ERCC1 expression are a favourable prognostic factor of survival in some tumors like NSCLC. Nevertheless, the value of ERCC1 as cisplatin predictor and as prognosis marker in SCLC has scarcely been explored with only two previous studies showing contradictory results (15,16).

Topotecan and irinotecan are topoisomerase I (TOP I) inhibitors that are active in the treatment of chemotherapy-naive and chemotherapy-sensitive patients with recurrent SCLC. Topoisomerases are enzymes capable of altering DNA topography for the purposes of relieving torsional strain during processes like replication, transcription, and recombination. These enzymes facilitate passing one strand of DNA through a break in the opposing strand (type I subfamily) or passing a duplex from the same or different molecule through a double stranded gap (type II subfamily). Topoisomerases can relax either negative supercoils, or both positive and negative supercoils of the DNA. These unique features are needed because of the double helical structure of DNA, in which TOPOs help access stored information for transcription, recombination and replication purposes (17).

Topotecan (T) is the standard treatment as second-line setting for patients with chemotherapy-sensitive disease (durable response to first line therapy of at least 3 months), with response rate of 19-37%. In addition, topotecan has been studied as doublet and triplet combination regimens to help assess its potential in first-line treatment of SCLC. Topotecan has shown preliminary results of 75% of objective responses in combination with paclitaxel (18), 90% with paclitaxel and carboplatin, (19) 81% with etoposide and carboplatin, (20) and 67% with ifosfamide (21). Although TOP I inhibitors have attracted extensive interest in the treatment of SCLC, there are no studies analyzing the role of changes in TOP I expression in this tumor.

Resistance to chemotherapy is the main cause of poor outcome in patients with SCLC (22). Therefore, the identification of markers that may identify those patients who would benefit from the current chemotherapy or chemoradiotherapy (CRT) approaches has strong clinical implications. To this aim, we retrospectively analyzed the ERCC1 and TOPO I expression as predictors of response and survival in a SCLC patients cohort treated with platinum-based combination chemotherapy.

Patients and methods

Patients and samples. Between January 2000 and December 2007, 228 patients were diagnosed with SCLC. Of these clinico-pathological data and adequate tissue were available in 76 (33.3%) patients; therefore, this group was selected for the study. All patients were treatment naive. Tissue samples were taken from their primary tumor by bronchoscopic or thoracoscopic biopsy in 73 cases and from the metastatic sites (bone marrow) in three patients. The Veterans Administration Lung Study Group system was used for staging patients (22).

The most commonly administered chemotherapy regimen was EP in 53 cases (etoposide 100 mg/m^2, cisplatin 25 mg/m^2 on days 1-3; 50 patients), followed by carboplatin-etoposide in 22 patients (carboplatin AUC 5 y 6 etoposide on day 1 and etoposide 100 mg/m^2, on days 1-3). Chemotherapy was repeated every 3 weeks. Among all the patients who underwent chemotherapy, 19 received 4 cycles and 54 patients received 6 cycles of an etoposide-platinum combination. In patients with ED SCLC, two to four additional cycles of chemotherapy were administered if a response was achieved after two cycles of chemotherapy. Thoracic concurrent irradiation (45 Gy) was performed in patients with limited disease and it was started with the second-third courses of chemotherapy on the same day. Among second line chemotherapy regimens, the most common treatment used was CAV (cisplatin, adriamycin and vincristine) in 31.5% followed by paclitaxel (21%) and topotecan (10%). Fifteen patients received chemotherapy and the most common regimen administered was topotecan (11.8% of all the patients included) (Table I).

Response to treatment was evaluated with chest CT after the third and sixth course of chemotherapy in ED SCLC, and after the concurrent chemo-radiation treatment in limited stage (LD), according to the World Health Organization criteria (23). Patients were defined platinum-resistant when time to progression after EP was less than 3 months. After the completion of treatment, patients were evaluated with chest CT every 3 months for the first and second year, every 6 months for the next 3 years, and yearly thereafter.

ERCC1 immunohistochemistry. Bronchoscopic lung biopsies were fixed in 4% buffered formaldehyde and embedded in paraffin (FFPE). Four-micrometer-thick sections were cut and stained with hematoxylin and eosin for pathological review. The study was approved by the institutional review board of the hospital. Gene expression analysis was performed blinded to the clinico-pathological data.

Sections of 4 μm were cut from formalin-fixed, paraffin-embedded tissue blocks from the afore-mentioned patients. Slides were de-paraffined and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 10 min at room temperature (RT). Antigens were retrieved by incubation in EDTA for 45 min at 155ºC. The primary mouse anti-human monoclonal antibodies against ERCC1 (ERCC1 Ab-2, Neomarkers, Fremont, CA, USA) was diluted at 1:100 in 1% BSA in TBS. Tissue slides were incubated with the antibody for 30 min at RT. Slides were then rinsed in TBS and incubated with the peroxidase-based EnVision™ kit (Dako Corp., Carpinteria, CA, USA) for 30 min at RT. Afterwards, specimens were incubated with diaminobenzidine chromogenic substrate (Dako Corp.) for 5 min at RT. Sections were counterstained with hematoxylin, stepwise dehydrated through graded alcohols and cleared in xylene.

Immunohistochemical score. The negative controls for these proteins were made by omission of the primary antibody during the process of immunohistochemical staining. The positive controls for ERCC1 were the presence of staining in the non-tumor bronchial mucosa. The slides were examined independently by two expert surgical pathologists blinded to both clinical and pathologic data. Discordant results were discussed and agreed on by both pathologists. Immunohistochemical staining was quantified using a visual grading system based on the extent of staining (percentage of positive tumor cells) according to the grading system of Soria *et al*. It was considered positive when more than 10% of the cells showed nuclear staining (24).

ERCC1 and TOP I quantitative PCR. RNA isolation was performed from the formalin-fixed paraffin-embedded bronchoscopic lung biopsies. Ten (5 μm) sections from each endoscopic specimen block were placed into a microcentrifuge tubes. One additional 4-μm section was stained with hematoxylin and eosin (H&E). Paraffin was removed by xylene extraction, and RNA was isolated using a commercially available kit:

Table I. Base characteristics of the patients.

Characteristics of the patients	Number (%)
Age (years)	62
Range	43-81
Gender	
Male	66 (86)
Female	10 (14)
Stage	
Limited	27 (35)
Disseminated	49 (65)
First line chemotherapy	76 (100)
Platinum/etoposide	76 (100)
Second line chemotherapy	49 (64.7)
Cisplatin/adriamycin/vincristine	25 (31.5)
Paclitaxel	16 (21)
Topotecan	8 (10)
Third line chemotherapy	15 (19.7)
Paclitaxel	5 (6.5)
Topotecan	9 (11.8)
Gemcitabine-vinorelbine	1 (1.3)
Treatment modality in limited stage	27 (35)
Concomitant chemo-radiation	22 (28.9)
Sequential chemo-radiation	1 (1.3)
Chemotherapy alone	4 (5.2)

Epicentre Technologies Inc. (Madison, WI), MasterPure RNA Purification kit according to the manufacturer's instructions. Total RNA was measured spectrophotometrically, measurements were performed in UltraPure destillated water DNAse, RNAse free (Gibco, Invitrogen). Reverse transcription of RNA derived from FFPE material was performed from 250 ng of total RNA of each case and 125 U Multiscribe reverse transcriptase (High-Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA, USA). Random primers were used to primer cDNA synthesis. Relative cDNA quantitation for both ERCC1 and TOPO 1 against the reference gene (β-actin) was done using a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (TaqMan); Applied Biosystems]. We profiled expression of the three genes ERCC1 (Hs Hs01012158_m1 assay), TOP I (Hs01052825_m1) and the internal reference ACTH (Hs99999903m1 assay) (in triplicates). These TaqMan primers and probes are designed to produce amplicons of around 100 nucleotides. ERCC1 and TOP I Gene expression levels are expressed as ratios (differences between the C_t values) between two absolute measurements (genes of interest/internal reference gene).

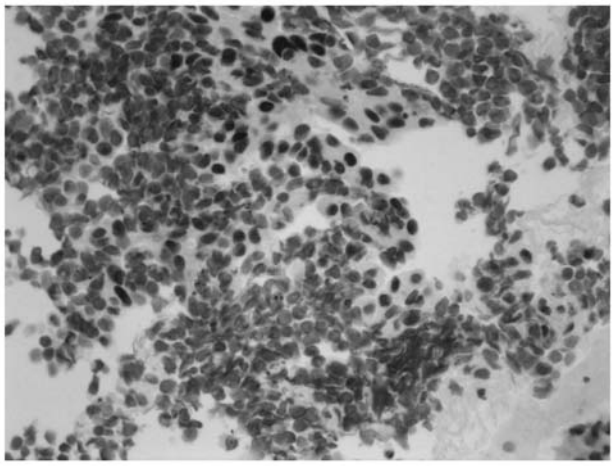

Figure 1. Positive inmunohistochemical expression of ERCC1 in a small cell lung cancer sample.

Statistical analysis. Overall survival (OS) and progression-free survival (PFS) was calculated using the Kaplan-Meier method. The differences between the survival curves were tested by using the log-rank test.

The Cox proportional-hazards regression model was used to determine the joint effects of several variables on survival. A comparison of clinico-pathological characteristics was evaluated with Fisher's exact test. Cox's proportional hazards multivariate analysis was used to evaluate which of the significant factors at the univariate analysis had a significant influence on survival. Statistical significance was set at p=0.05. All analyses were performed with SPSS for Windows 13.0 software.

Results

Patient characteristics. The clinico-pathological characteristics of the 76 patients are listed in Table I. Of the patients 49 (65%) had an ED whereas 27 (35%) had LD. All received chemotherapy based on PE with concurrent radiation in LD. Among the 76 patients selected, 43 (51.3%) were considered platinum responders and 33 (48.7%) platinum resistant, according to the definition previously given.

ERCC1 expression and cisplatin response. Positive immunoexpression of ERCC1 was observed in 29 (52%) whereas in 24 patients the ERCC1 immunostaining was negative (45.3%) (positive immunostaining of ERCC1; Fig. 1). Twenty-six cases could not be evaluated for the immunohistochemistry and PCR analysis. A significant association was found between negative immunohistochemistry expression of ERCC1 and the platinum response (7 patients in the cisplatin-resistant group showed negative staining vs 31 patients in the cisplatin responders group, 12.5 vs 87.5 % respectively, p=0.001) (Fig. 2).

The analysis of ERCC1 mRNA by qPCR showed low ERCC1 expression in 24 cases (50%) and 17 cases showed a high ERCC1 expression. Among low expression cases, there were 27 patients considered cisplatin responders (70.8%). In contrast, among the 30 non-cisplatin responder patients, 10 patients (41%) had high ERCC1 expression. There was a correlation between low expression of ERCC1 and cisplatin response that did not reach statistical significance (p=0.54) (data not shown).

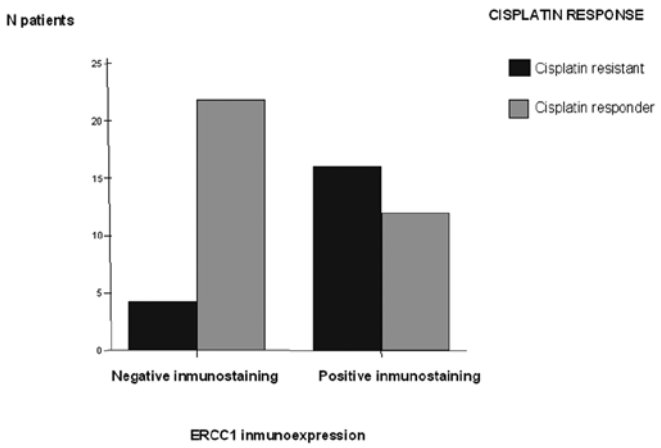

Figure 2. Correlation between ERCC1 inmunoexpression and cisplatin response.

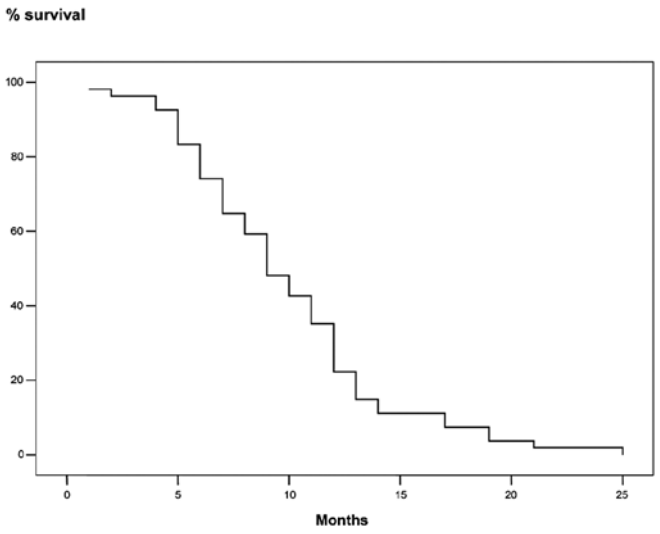

Figure 4. Kaplan Meyer overall survival curve.

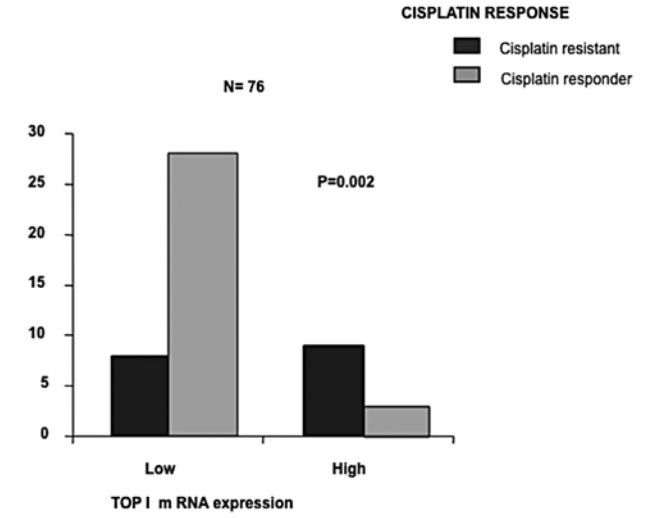

Figure 3. Correlation between cisplatin response and mRNA expression of ERCC1.

TOP I expression and cisplatin response. Only 48 cases could be evaluated for the PCR analysis because of problems of availability of the samples. We observed an opposite relationship between TOP I RNA expression levels and the response to cisplatin: 28/36 (77.8%) patients in the responder group showed a low level expression of TOP I expression, whereas 9 patients (75%) patients in the resistant group showed high expression of TOP I (p=0.02) (Fig. 3).

ERCC1 and TOP I expression and prognosis. After a median follow-up time of 12 months, the median PFS was 6 months (CI 95%, 5-6.9 months) and OS was 9 months (CI 95%, 7.4-10.6) (Fig. 4).

Patients with negative ERCC1 immunostaining showed a lower risk of PFS compared to cases with positive ERCC1 immunostaining HR=0.456 (CI 95% 0.256-0.819; p=0.009) (Fig. 5a). In contrast, the qPCR analysis of ERCC1 did not show the risk of PFS, with only a trend towards a lower risk of progression in patients with low ERCC1 mRNA levels HR=0.641 (CI 95% 0.35-1.15; p=0.14) (Fig. 5b).

Table II. Univariate analysis of progresion-free survival (PFS).

Variable	P-value
Stage	0.09
Limited	
Extensive	
Gender	0.96
Male	
Female	
Response to cisplatin based-chemotherapy	0.0001[a]
Positive	
Negative	
ERCC1 inmunoexpression	0.009[a]
Low	
High	
ERCC1 mRNA expression	0.14
Low	
High	
TOP I mRNA expression	0.002[a]
Low	
High	

Patients with low TOP I mRNA expression showed a significantly lower risk of progression compared to those patients with high TOP I expression HR=0.446 (CI 95% 0.223-0.888; p=0.02) (Fig. 5c).

We also analyzed for a possible correlation between TOP I and ERCC1 expression using Pearson correlation, but we could not demostrate an association (*Sq r lineal* = 0.036). The results of the univariate analysis are shown in Table II. A multivariate analysis was performed including gender, stage, cisplatin response, ERCC1 and TOP I expression. Response to cisplatin

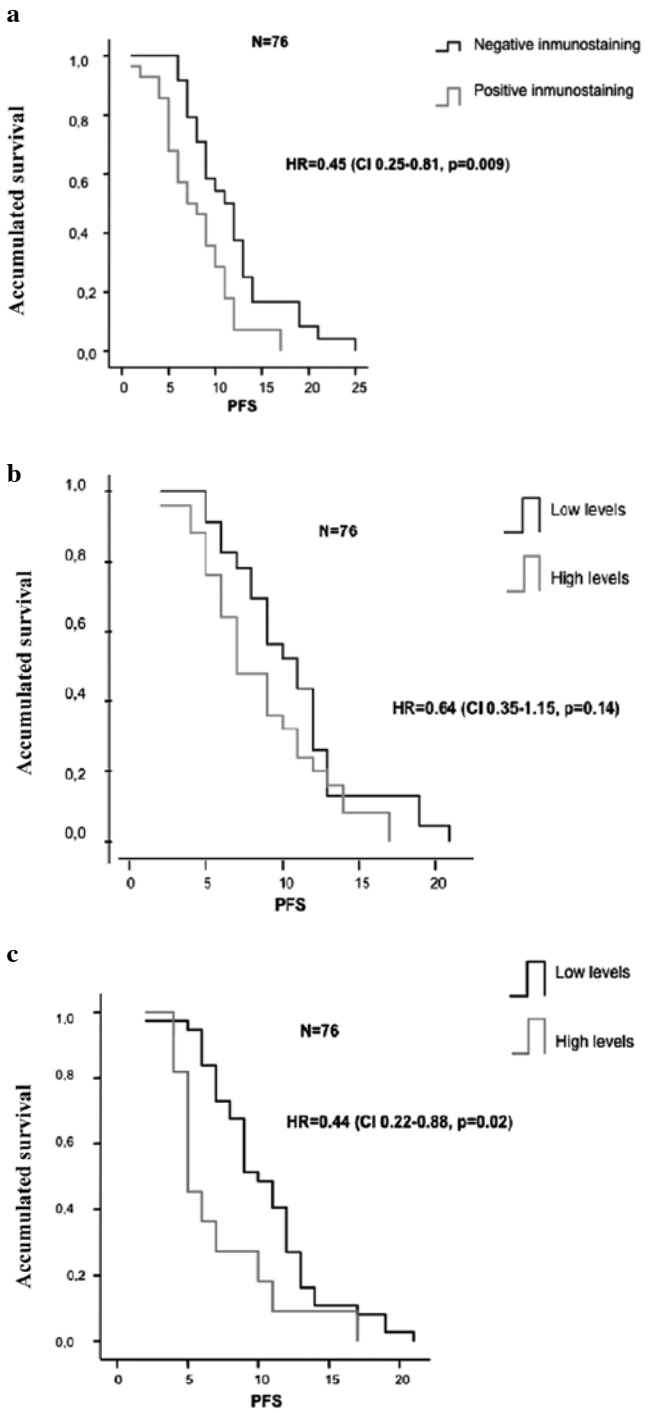

Figure 5. (a) Correlation between ERCC1 inmunostaining and PFS. (b) Association of PFS and mRNA ERCC1 levels. (c) Correlation of mRNA TOP I levels and PFS.

as well as TOP I expression were the parameters with the most relevant impact on survival. Cisplatin response reached statistical significance in the multivariate analysis (p=0.003) but TOP I expression showed only a trend with a statistical significance in the first case I expression (p=0.06) (data not shown).

Discussion

In this study, we explored the relationship between ERCC1 and TOP I expression and cisplatin-response and PFS in patients diagnosed with SCLC. ERCC1 status has been related to cisplatin response in other tumors such as NSCLC (22-24), bladder cancer (25) and head and neck cancer (26,27). However, scarce data have been reported with regard to SCLC, which represents one of the most aggressive cancer types. Moreover, SCLC therapy has not experienced significant improvements despite active research in this field in recent years. Several reports have demonstrated that alterations in DNA damage repair proteins are associated with resistance to cisplatin based chemotherapy or radiotherapy (25-27). Cisplatin resistance is one of the most important causes of treatment failure in SCLC (28). In this study we analyzed ERCC1 expression in relation to cisplatin response. This potential relationship could help us to select those patients with a potential cisplatin response and those with primary resistance. In these resistant cases, cisplatin administration could be avoided; decreasing associated platinum toxicity in patients with a positive ERCC1 expression. Our group showed a highly significant relationship between ERCC1 expression and cisplatin resistance as analyzed by IHC (p=0.001). These findings have a direct influence on survival: patients with ERCC1 positive staining had a significant worse PSF compared to those patients with negative staining (p=0.009). Moreover, when ERCC1 was analyzed by QPCR, it did not reach statistical significance. One possible explanation for this discordance could be the insufficient sample size or the lack of linear correlation between mRNA and protein expression. In this sense, our study is the first to compare ERCC1 expression both by QPCR and immunohistochemistry and the results point to immunohistochemistry as the method of choice for analysis of ERCC1.

There are two published studies that have analyzed ERCC1 expression and PFS in SCLC (15,16). Lee *et al* analyzed ERCC1 immunoexpression in tumor specimens from 77 patients with SCLC who were treated with a platinum regimen. The authors found that in LD patients, high expression of ERCC1 was an independent prognostic factor for poor OS (p= 0.046), along with male gender (p=0.033) (15). Along the same lines, Ceppi *et al* analyzed ERCC1 RNA expression in 85 patients, also showing that ERCC1 was an independent prognostic factor for survival in LD patients; however these results could not be reproduced in ED (16). Consistent with the two previous studies, we have shown that tumor stage and ERCC1 immunoexpression were the only variables with significant impact on PFS, p=0.04 and 0.006, respectively. In contrast to the results of Ceppi *et al*, neither gender nor stage could be analyzed in our study due to the unbalanced sample size. This could also be the reason why the stage did not reach the statistical significance in univariate analysis.

Apart from ERCC1, in this report TOP I expression was also explored. An interesting relationship between low TOP I RNA expression and a better cisplatin response and PFS was found. The multivariate analysis showed that TOP I expression had a significant influence on PFS, as well as cisplatin response, but in the first case the association did not reach statistical significance. There is no previous evidence regarding the role of TOP I in survival nor in cisplatin prediction in SCLC. However, there is controversy regarding the prognosis value of TOP I in other types of tumors (29-33). Tsavaris et al found that levels of expression of TOP I were higher in malignant cells from tumor recurrences compared to primary tumors, suggesting a role of TOP I in tumor recurrence (34), but these

results could not be confirmed by Paradiso et al (35). TOP I is the target of topotecan and irinotecan, which have been explored mainly as second line treatments, and recently, in a first line setting (32). Our study is the first to demonstrate a positive relationship between low levels of TOP I and better cisplatin response as well as a longer PFS in SCLC patients. TOP I plays a crucial function in DNA replication (36-39). Low levels of TOP I indicate inappropriate DNA replication, therefore, a lower rate of proliferation. Perhaps a high level of TOP I is a surrogate marker of proliferation and could be a prognostic factor as well as a predictive marker to response to chemotherapy, in this case, to cisplatin. A recent study by Kohara et al demonstrated in preclinical data, responses to TOP I inhibitors in platinum-resistant cells (40). In this study, we were not able to analyze the relationship between TOP I expression and topotecan or irinotecan treatment because only 10% of the patients received topotecan as second line treatment, and none received topotecan as first line treatment. The reason is that at the beginning of recruitment, topotecan was not yet approved for SCLC treatment in Spain. Further studies will be developed to explore a potential relationship between TOP I expression and response with contradictory results (37). Maden et al found that higher levels of TOP I correlated with sensitivity to TOP I inhibitors (38); however on the other hand, MacLeod et al reported that gene copy number and protein expression are inversely correlated with sensitivity to SN 38, an inroinotecan metabolite, in vitro in several breast and colon tumor cell lines (39).

Based on our results, we suggest that there is a positive impact of the low levels of TOP I mRNA on PFS, but these findings should be confirmed in further studies. We identified a profile of patients with SCLC with negative ERCC1 immunostaining as well as low mRNA levels of TOP I with a good cisplatin response as well as a longer PSF. Mukai *et al* reported similar results in different tumors using immunohistochemistry and PCR analysis in tissue specimens of breast, gastric and non-small cell lung cancer. They found that high levels of ERCC1 and TOP I mRNA are related to recurrence (37).

In addition to this prognostic information, our results could be the first step in designing other studies with a large patient sample sizes to validate these findings. They may suggest a therapeutic algorithm for first line treatment based on ERCC1 and TOP I mRNA expression/levels. Those patients with poor ERCC1 expression and low levels of mRNA of ERCC1 could be treated with a platinum regimen. However, if ERCC1 immunohistochemistry is positive or mRNA levels are high, cisplatin resistance is indicated, and in these cases we could analyze TOP I levels. According to previously reported data, high levels of TOP I may be involved in TOP I inhibitors (topotecan and irinotecan) response, but low levels would be associated with a poor response. In this study, it was not possible to report a correlation with TOP I inhibitor treatment, therefore, we are working on further studies to explore this aspect.

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